ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

The Membrane Bioreactor: A Novel Tool to Grow Anammox Bacteria as Free Cells

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Received 30 December 2007; revision received 4 March 2008; accepted 4 March 2008

Published online 12 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21891

ABSTRACT: In a membrane bioreactor (MBR), fast growth of anammox bacteria was achieved with a sludge residence time (SRT) of 12 days. This relatively short SRT resulted in a—for anammox bacteria—unprecedented purity of the enrichment of 97.6%. The absence of a selective pressure for settling, and dedicated cultivation conditions led to growth in suspension as free cells and the complete absence of flocs or granules. Fast growth, low levels of calcium and magnesium, and possibly the presence of yeast extract and a low shear stress are critical for the obtainment of a completely suspended culture consisting of free anammox cells. During cultivation, a population shift was observed from *Candidatus* "Brocadia" to *Candidatus* "Kuenenia stuttgartiensis." It is hypothesized that the reason for this shift is the higher affinity for nitrite of "Kuenenia." The production of anammox bacteria in suspension with high purity and productivity makes the MBR a promising tool for the cultivation and study of anammox bacteria.

Biotechnol. Bioeng. 2008;101: 286–294.

KEYWORDS: anammox process; anaerobic ammonium oxidation; anoxic ammonium oxidation; "Kuenenia stuttgartiensis"; "Brocadia"; MBR; cultivation methods; selective pressure; planktonic cells

Introduction

The cultivation of slow-growing microorganisms requires efficient retention of biomass and relies mostly on the ability of microorganisms to form biofilms or aggregates such as flocs or granules. Startup of these reactors can be impeded by insufficient biomass build-up: a continuous loss of "small" amounts of biomass via the effluent might lead to significantly longer observed biomass doubling times in the case of slow-growing microorganisms (Strous et al., 1998). Granular sludge-based reactor design (Nicolella et al., 2000) leads to compact reactors, which combine a short hydraulic retention time (HRT) with a long and stable solid retention time (SRT). Processes employing slow-growing organisms like nitrification (Tanaka and Dunn, 1982), anaerobic digestion (Lettinga et al., 1980), ferrous ion oxidation (Ebrahimi et al., 2005), and phosphate removal (De Kreuk and Van Loosdrecht, 2004) all can be implemented successfully, on lab-scale as well as on full-scale, in retention-based reactors with a high volumetric loading rate. Typical examples of such reactors are airlifts (Heijnen et al., 1990), sequencing batch reactors (SBR, Irvine et al., 1977; Wilderer and McSwain, 2004), internal circulation reactors (Pereboom and Vereijken, 1994), and upflow anaerobic sludge bed reactors (UASB, Lettinga et al., 1980; McHugh et al., 2004).

Although particle-based bioreactors are advantageous for the cultivation of slow-growing microorganisms, and thus valuable from a technological point of view, the produced granules are not the most suitable forms for the study of these microorganisms. Biokinetic parameters such as substrate affinities, maximum growth rate or maintenance need cannot be well assessed due to diffusion limitations within the floc or granule itself (Chu et al., 2003; Harremoës, 1977). Disruption procedures performed in order to obtain single cells for several microbial tests—such as the most probable numbers (MPN) method and fluorescence in situ hybridization (FISH)—require large amounts of biomass and often introduce biases in the obtained results. Finally, also the energy required for bacteria to agglomerate (e.g., additional production of extracellular polymers) might

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Contract grant sponsor: Dutch Technology Foundation (STW)

Contract grant number: NPC.5987

reduce the observed maximum specific growth rate (μ_m) and agglomeration therefore potentially also leads to underestimation of μ_m (Characklis, 1990).

In the membrane bioreactor (MBR), biomass retention is not based on settling of biomass. The effluent is withdrawn via a membrane which is impermeable for microbial cells. Unlike the reactors with granular biomass, the MBR enables cultivation of slow-growing microorganisms with full biomass retention but without a selection on settling ability. This reactor type is currently employed for the growth of sensitive cells like plant/animal cells as well as for cell tissue production (Drioli and De Bartolo, 2006). The MBR is also employed in full-scale wastewater treatment (Sutton, 2006; Yang et al., 2006), where the membrane separation reduces the surface area which is normally required for settling of flocculated sludge.

In this research, we demonstrate the possibility of cultivating slow-growing anammox cells in an MBR, with high purity and productivity. The anammox process is the biological conversion of ammonium and nitrite to dinitrogen gas (Van de Graaf et al., 1996) and is performed by slow-growing deep-branching Planctomycetes (Strous et al., 1999a). Anammox bacteria are autotrophic and have a notoriously low growth rate with minimum doubling times of several days (Strous et al., 1998; Tsushima et al., 2007). Despite considerable interest in their cultivation, only enrichments (which typically contain 60–80% anammox bacteria) are available consisting of agglomerates or biofilms. This might even lead to the perception that anammox bacteria are preferentially growing in biofilms or granules. This perception is strengthened by the—valid—observation that selection for biofilm growth *in granular sludge or biofilm reactors* often improves with better biomass retention (Strous et al., 1998). However, the high abundance of free anammox bacteria at the oxic-anoxic interface in several marine systems (Schmid et al., 2007) indicates that growth as free cells is (also) a natural mode of growth.

The anammox process is applied on full-scale in biofilm-based bioreactors (Rosenwinkel and Cornelius, 2004; Van der Star et al., 2007). Contrary to problems associated with these reactors in the *study* of anammox organisms, for the *application* of the anammox process in nitrogen removal, these types of reactors are preferable over MBRs, since anammox bacteria easily form sludge granules or biofilms which form a simple and economic way to obtain high biomass concentrations in the reactor. Moreover, wastewater always contains a certain amount of suspended solids. Since these solids are also (unintentionally but efficiently) retained by membrane filtration—and because the biomass production of anammox bacteria is relatively low due to their autotrophic nature—the sludge activity is also expected to decrease rapidly in a full-scale MBR-based anammox process.

In this study, the successful enrichment of the slow-growing anammox bacteria in a lab-scale MBR to high purity as single cells is reported. The resulting high production of completely suspended anammox bacteria during more than 9 months makes the reactor a promising tool for study of the anammox process.

Materials and Methods

Inoculation of the MBR

The reactor was inoculated with granular anammox sludge from the bottom of the lower compartment of the full-scale anammox reactor (Van der Star et al., 2007) at the Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam (The Netherlands). After removal of the 20% heaviest (i.e., quickest settling) fraction of the solids (with a high precipitates content), the reactor was inoculated with 1.5 L of the remaining granular biomass.

Reactor Operation

A 15 L reactor was used for the cultivation (see Fig. 1). The liquid volume was 8 L and the reactor was fed continuously with 3.9–4.1 L/day medium with different compositions, resulting in a HRT of 2 days. The liquid level was maintained via a liquid level-controlled (peristaltic) effluent pump connected to a membrane microfiltration module type Zeeweed (Zenon Environmental, Ontario, CA) which was placed within the reactor vessel. The membrane fiber (absolute pore size: 0.1 μm) was designed for operation in MBRs for wastewater treatment and is impermeable for microbial cells. The lab-scale module that was used (ZW1) consists of about 100 tubes (diameter ca. 1 mm, length ca. 300 mm). The module was replaced every 10 days to prevent biofilm growth on the membrane surface, and was subsequently cleaned (outside the reactor) with a protease-containing detergent (Tergazyme, Alconox, NY). Great care was taken to remove all detergent after cleaning by prolonged and intensive rinsing with water. Replacement of the membrane took only 1–2 min and mixing was stopped during the replacement to avoid the entrance of large amounts of air into the reactor.

To maintain anoxic conditions and to provide buffering capacity, the reactor was sparged continuously at 25 mL/min with 95%Ar-5%CO_2. pH was not controlled, but was always between 7.1 and 7.5. The temperature was controlled at 38°C, and the stirring speed was 160 rpm. To avoid growth of phototrophic organisms (and the related oxygen production, which would enable growth of other non-anammox microorganisms like nitrifying bacteria), the reactor was covered completely by a PVC cover (1-mm thickness) to prevent penetration of light. The reactor was fed with a concentrated medium according to Van de Graaf et al. (1996) containing 120 mM ammonium and 120 mM nitrite (Table I).

The start-up period began with a first week in which the feed contained 100 mM sodium nitrate to avoid potential sulfate reduction. In this week, the influent ammonium nitrite levels were increased gradually in three steps from

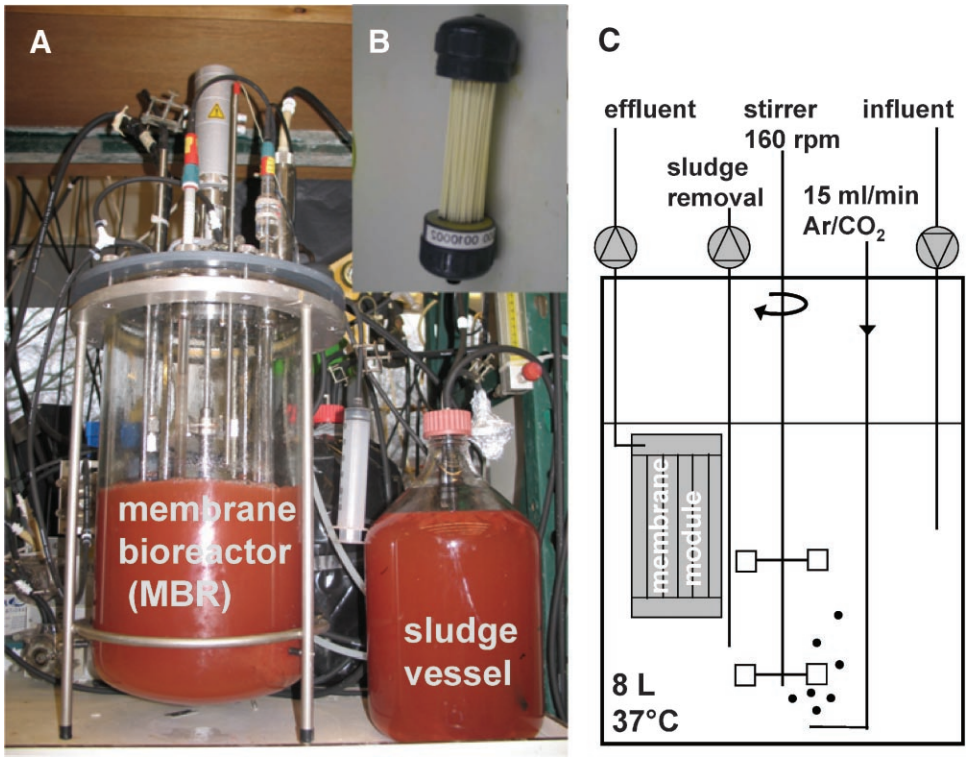

Figure 1. **A**: Photograph of the membrane bioreactor (MBR) for enrichment of anammox bacteria as free cells and the sludge withdrawal vessel; The MBR contains the completely suspended red anammox cells, which stay in suspension also in the (non-mixed!) sludge withdrawal vessel. The membrane (B) is completely immersed in the reactor. (**B**) photograph of the membrane module which was used; (**C**) scheme of the MBR showing the position of the membrane, gas sparger, and influent and effluent lines.

20 to 120 mM. Then, after 30 days of full biomass retention (practically infinite SRT), the SRT was controlled at ca. 16 days by removing once per day (during 30 min) 0.5 L from the reactor using an excess-sludge pump which was not connected to the membrane, but which pumped out the reactor-suspension directly. Operation of this excess-sludge pump was computer-controlled. This day, when the SRT started to be controlled at 16 days, is from here on designated as day 1 of the experiment.

On day 85, after a moment of incomplete conversion of nitrite, the medium composition was adjusted. Calcium and magnesium levels in the medium were lowered by 75%, and addition of 1 mg/L yeast extract was started (adjusted medium composition in Table I). On day 127, the SRT was reduced to 12 days. This SRT was maintained for another 100 days. From day 150 on, ammonium was lowered from 120 to 100 mM to reduce the excess of ammonium in the reactor. The reactor was operated for more than 250 days.

Determination of Nitrite, Nitrate, Ammonium, Nitrous Oxide, and Nitric Oxide

Ammonium (0.14–3.4 mM), nitrite (1.1–43 μM), nitrate (16–964 μM) were analyzed using commercial Dr.Lange test

Table I. Different medium compositions for enrichment of anammox organisms.

Nutrient	Units	Van de Graaf et al. (1996)	Initial period (this study)	Final period (this study)	Trigo et al. (2006)
Ammonium	mg-N/L (mM)	420 (30)	1680 (120)	1680[a] (120)[a]	366 (26)
Nitrite	mg-N/L (mM)	420 (30)	1680 (120)	1680 (120)	370 (26)
Calcium	mg/L (mM)	49 (1.2)	164 (4.1)	41 (1.0)	1.5 (0.038)
Bicarbonate	mg-C/L (mM)	60 (5.0)	179 (15)	179 (15)	120 (10)
Magnesium	mg/L (mM)	30 (1.2)	39 (1.6)	9.9 (0.41)	5.8 (0.24)
EDTA	mg-C/L (mM)	6.5 (0.054)	6.0 (0.050)	6.0 (0.050)	8.1 (0.067)
Phosphate	mg-P/L (mM)	6.2 (0.20)	5.7 (0.18)	5.7 (0.18)	2.3 (0.073)
Iron	mg/L (mM)	1.0 (0.018)	2.5 (0.045)	2.5 (0.045)	2.3 (0.041)
Yeast extract	mg/L	—	—	1.0	—

Values in parenthesis are in mM.
[a]This value was reduced from day 70 on to 1,400 mg-N/L (100 mM).

kits (Hach Lange GmbH, Düsseldorf, Germany) and determined on a designated spectrophotometer (CADAS 50S). The off-gas was collected every 20 min in a 1 L sample bag and nitric oxide (NO) was determined from this bag by a chemiluminescence analyzer (CLD700e, Ecophysics, Dürnten, Switzerland). N_2O was determined occasionally by gas chromatography: 100 μL off-gas samples were directly injected on a Hayesep Q 80/100 Ultimetal micropacked column (0.25 m × 1/16″ × 1 mm) in a Varian 3800 gas chromatograph (Varian, Palo Alto, CA). N_2O was determined in an electron capture detector with a lower limit of ca. 2 ppm.

Community Analysis Using Molecular Methods

Fluorescence In Situ Hybridization

Samples were fixed for FISH as described by Pernthaler et al. (2001). Briefly, cells were washed, fixed in paraformaldehyde and spotted onto Teflon-coated multi-well slides. After dehydration, the cells were hybridized with the following fluorescently-labeled oligonucleotide probes: EUB-338, Pla-46, AMX-368, AMX-820, or KST-157. Details on the target organisms and the sequences can be found in Table II. Microscopic observations were performed with a Zeiss Axioplan epifluorescence microscope (Zeiss, Stuttgart, Germany). Fixation took place on days 35, 64, 77, 149, 196, and 267. On day 267, the enrichment level was estimated by counting those cells which were visible under the microscope, but which did not hybridize with the AMX-820 probe (the number of non-anammox cells). This number was compared to the total number of visible (anammox and non-anammox) cells (circa 10,000).

DNA Extraction, PCR Amplification, and Phylogenetic Analysis

Samples (5 mL cell suspension) were taken from the reactor (on days 17 and 118) and directly centrifuged for 5 min at 13,000*g*. The cell pellets were stored at −20°C. Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. The quality of the extracted DNA was analyzed by agarose gel electrophoresis.

Subsequently, the extracted DNA was used to amplify the nearly complete 16S rRNA gene using primers Pla-46F, 907RM, and 1392R (see Table II for details). The PCR products were analyzed with agarose gel electrophoresis, purified using the Qiaquick PCR purification kit (Qiagen, Düsseldorf, Germany) and sequenced by a commercial company (BaseClear, Leiden, The Netherlands). The sequences were first compared to sequences stored in GenBank using blastn. Thereafter, they were imported into the SILVA database (Pruesse et al., 2007) with the ARB software program (Ludwig et al., 2004). The sequences were automatically aligned and alignments were corrected by hand after which a tree was created using the neighbor-joining algorithm with Felsenstein correction. The nearly complete 16S rRNA sequences were deposited in the Genbank database under accession numbers EU361730 (day 17, TUD-1) and EU361731 (day 118, TUD-2).

Results

General Reactor Operation

Within 4 weeks after inoculation (the designated day 1), the reactor could be operated at a conversion rate of 0.8 kg-NO_2^--N/m^3/day. The reactor was operated under nitrite limitation and nitrite consumption was generally complete (≫99%) throughout the study. The inoculum consisted of granules, and this changed to small flocs in the first 60–65 days. Lowering of calcium and magnesium ion levels in the medium, in combination with the addition of yeast extract to the reactor medium resulted in disappearance of the flocs and the culture changed fully to suspended free cells with the red color which is characteristic for anammox cells (Fig. 1). Removed sludge did not settle at all. In the biomass effluent vessel (which was not stirred and had a residence time of 2.5 days) most of the biomass remained in suspension and only a small fraction of the biomass accumulated as a floatation layer after several weeks of operation.

The MBR could be operated stably for more than 250 days with very little maintenance at SRTs of 16 and 12 days. The

Table II. Oligonucleotides used in the study.

Oligonucleotide	Target organisms	Sequence (5′–3′)	Reference
EUB I	Bacteria	GCT GCC TCC CGT AGG AGT	Daims et al. (1999)
EUB II		GCA GCC ACC CGT AGG TGT	
EUB III		GCT GCC ACC CGT AGG TGT	
Pla-46	Planctomycetes	GAC TTG CAT GCC TAA TCC	Neef et al. (1998)
AMX-368	Anammox bacteria	CCT TTC GGG CAT TGC GAA	Schmid et al. (2003)
AMX-820	"Kuenenia"/"Brocadia"	AAA ACC CCT CTA CTT AGT GCC C	Schmid et al. (2000)
KST-157	"Kuenenia"	GTT CCG ATT GCT CGA AAC	Schmid et al. (2001)
Pla46F	Planctomycetes	GGA TTA GGC ATG CAA GTC	Neef et al. (1998)
907RM	Bacteria	CCG TCA ATT CMT TTG AGT TT[a]	Schäfer and Muyzer (2001)
1392R	Universal	ACG GGC GGT GTG TAC	Schäfer and Muyzer (2001)

[a]M is A or C.

correspondingly achieved effective growth rates (where $\mu_m = 1/SRT$) of 0.0026 and 0.0035 h^{-1} represent doubling times of 11 and 8.3 days, respectively. Occasional in-reactor determination of the maximum conversion capacity during operation at an SRT of 12 days was performed by increasing the influent flow for 1–2 h to such an extent, that not all nitrite was converted and thus a slow nitrite accumulation could be observed. By subtraction of this nitrite accumulation from the imposed nitrite loading rate, the nitrite conversion rate under these non-nitrite limiting conditions could be estimated and related to the conversion rate under normal operating conditions. These short experiments indicated that the reactor was operated at 70–90% of the maximum conversion rate. A clear relation between operating conditions and the differences between individual measurements could not be observed. The nitrite:ammonium conversion ratio was 1.1–1.3 and the nitrate:ammonium ratio 0.10–0.25. These numbers are in accordance with values obtained in other reactors (López et al., 2008; Schmid et al., 2000; Strous et al., 1998). Nitric oxide was on-line measured in the off-gas of the MBR, and the levels were generally well-below 1 ppm. The (off-line) N_2O level was below 2 ppm. The fraction of the nitrogen converted to NO and N_2O therefore was below 0.01% of the nitrogen conversion.

Membrane fouling was heavier in the first 60 days of operation, but never led to clogging of the membrane before its scheduled replacement (every 10 days). Membrane replacement did not lead to disturbances in conversion during the first 120 days, but after that, the conversion started again only after a delay of about 1 h. Probably, the absence of heterotrophic organisms and nitrifiers during the 120 days of stable operation, increased the time which was required to remove the oxygen leaking into the reactor during the procedure (Strous et al., 1997), thus inhibiting the anammox process temporarily. The inability of the culture to remove oxygen also became apparent from batch tests with the enrichment in the presence of ammonium under oxic conditions. In these batch tests, the oxygen concentration dropped less than 0.1 mg/L/h, so oxygen consumption could not be detected.

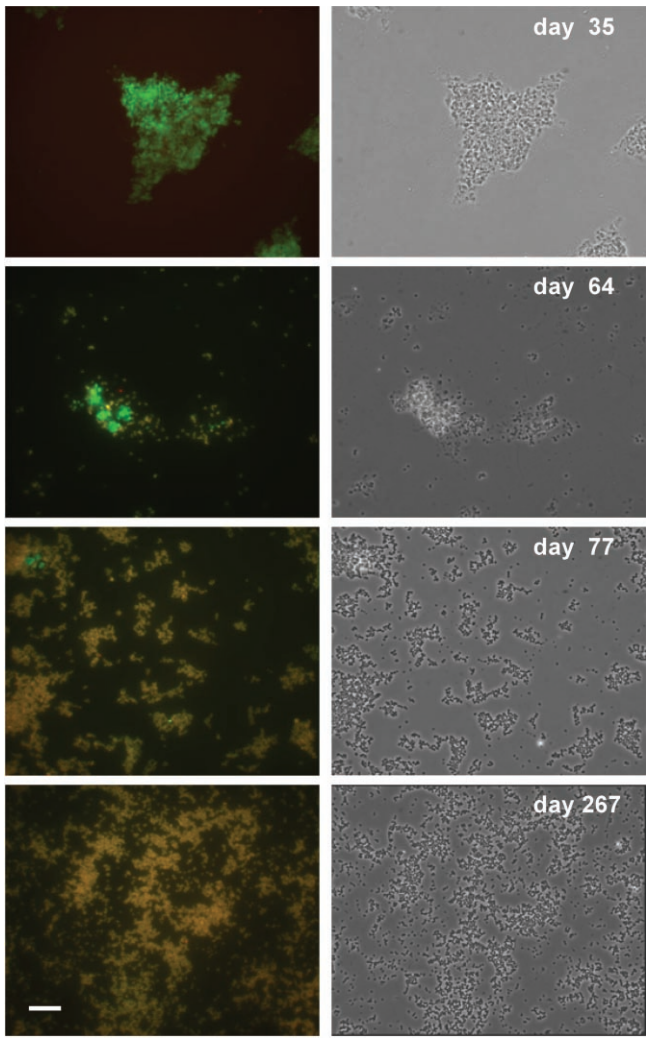

Figure 2. The reactor population in the reactor in time as shown by FISH (**left**) and phase contrast microscopy (**right**). The anammox population shifts from Brocadia (green, hybridization with the "Brocadia"/"Kuenenia"-specific probe AMX-820 in cy3) to "Kuenenia" (orange, hybridization *also* with "Kuenenia"-specific probe KST-157 in cy5). The purity in "Kuenenia" increases to 97.6% on day 267. Scale bar is 10 μm.

Microbial Community Analysis

The anammox bacteria in the inoculum consisted of *Candidatus* "Brocadia" (Van der Star et al., 2007), and on day 35 this was still the main population since hybridization took place with the "Kuenenia"/"Brocadia"-specific probe (AMX-820), but not with the Kuenenia-specific probe (Fig. 2). However, the sample on day 64 shows also a significant number of "Kuenenia" cells, which became the main population after day 149. After this date no change in the main population or level could be seen and "Brocadia" could not be detected anymore (<0.5%). The level of enrichment increased from 60% to 80% in the first days to more than 90% after ± day 100. No cells could be found at any time that did not hybridize with the "Kuenenia"/"Brocadia"-specific probe (AMX-820), but which did hybridize with the *Planctomycetes*-specific PLA-46 probe or with the all-anammox-specific AMX-368 probe in any of the samples. This indicates that other described anammox bacteria were not present (or only present in very low amounts). Since free cells were obtained, quantification using FISH was possible by viewing each individual cell and determining whether this cell had hybridized with the "Kuenenia"/"Brocadia" specific probe (AMX-820). The enrichment level at day 267 was 97.6 ± 0.2% (ca. 10,000 cells counted).

The population change from *Candidatus* "Brocadia" to *Candidatus* "Kuenenia" was confirmed by 16S rRNA sequence analysis. The sequences of the sample on day 17

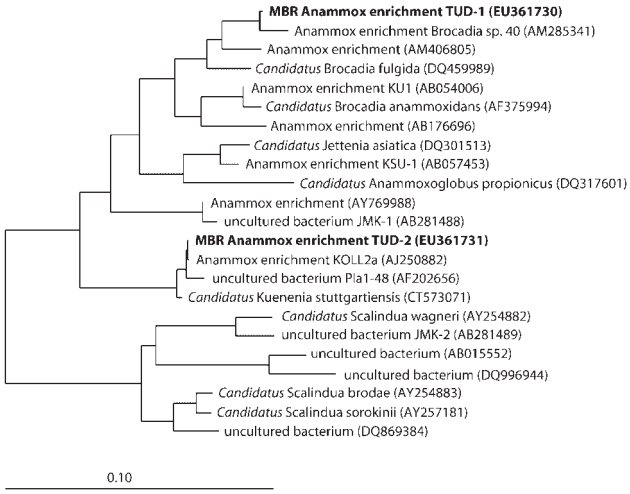

Figure 3. Phylogenetic tree based on nearly complete 16S rRNA sequences obtained from the dominant organisms of the membrane bioreactor (MBR). The sequence obtained from enrichment TUD-1 was related to members around *Candidatus* "Brocadia fulgida." The sequence obtained from enrichment TUD-2 was related to members around *Candidatus* "Kuenenia stuttgartiensis." The sequence of *Thermotoga maritime* was used as an outgroup, but pruned from the tree. Bar indicates 10% sequence difference.

showed the strongest similarity (98.9%) with *Candidatus* "Brocadia" sp. 40 (Kieling et al., 2007). The sample on day 118 showed a sequence similarity of 100% with a *Candidatus* "Kuenenia stuttgartiensis" strain (Kölliken enrichment, Egli et al., 2001), and 99.7% with the original *Candidatus* "Kuenenia stuttgartiensis" (Stuttgart enrichment, Schmid et al., 2000). Figure 3 shows a phylogenetic tree based on the 16S rRNA sequences of the strains.

Affinity for Nitrite

The nitrite level in the reactor fluctuated considerably but was generally between 2 and 6 μM. Since the reactor was operated at 70–90% of the maximum specific conversion rate, the half saturation constant (K_S) must be 10–50% of this range (as $\frac{\mu}{\mu_m} = \frac{C_{NO_2^-}}{C_{NO_2^-}+K_S} \rightarrow K_S = C_{NO_2^-}\frac{\mu_m-\mu}{\mu}$, where $C_{NO_2^-}$ is the nitrite concentration in the reactor), and is thus estimated to be between 0.2 and 3 μM. The low nitrite level is another indication that the bacteria are really present as free cells, since any significant agglomeration would have led to a lower (apparent) substrate affinity due to diffusion limitation (i.e., a higher K_S).

Discussion

Why Was a Suspension Culture Achieved?

Although the obtainment of aggregates is a logic consequence of enrichment reactors which select on settling ability (like SBRs, gaslift reactors, etc.), the reversed statement is not necessarily true. Thus, an enrichment system where no selection on settling ability is present (like the MBR), might still lead to the formation of aggregates. Aggregates were indeed present in the first 70 days of this enrichment, and also in other MBRs where the anammox process (Trigo et al., 2006; Wyffels et al., 2004) or nitrification (Wyffels et al., 2003) was employed.

What was then the trigger for growth as free cells? It appears that the direct cause was the reduction of calcium and magnesium levels, which took place in combination with the addition of small amounts of yeast extract. Bivalent ions (like calcium and magnesium ions) are known flocculation enhancers (Mahoney et al., 1987; Pevere et al., 2007; Sobeck and Higgins, 2002). Moreover, small calcium or magnesium precipitates in the reactor (e.g., hydroxyapatite, calcite) might act as nucleation seeds for the growth of small granules. If the presence of flocs was a result of extracellular polymeric substances (EPS)—produced as a result of stress because of the absence of a micronutrient—addition of yeast extract potentially has led to a lower EPS production and aided suspended growth. This study unfortunately can not differentiate between the effect of addition of yeast extract on one hand, and lowering of calcium and magnesium levels on the other, since addition of yeast extract coincided with lowering of calcium and magnesium levels. Because the effect of bivalent ions on flocculation is a much more established effect, we assume here that this has been the determining parameter in the production of suspended anammox cells. However, a low level of bivalent ions cannot be the only determining factor, since Trigo et al. (2006) obtained granules even after calcium and magnesium levels were lowered (see Table I for an overview of feed conditions).

An important other difference in reactor operation is the applied SRT: in the MBR reactor of this study, the SRT was carefully controlled at 12 or 16 days whereas the retention time was "nearly infinite" in the MBRs of Wyffels et al. (2004) and Trigo et al. (2006) since no biomass (and hence also no precipitate) was actively removed. Besides the fact that competition in systems with an extremely high SRT is much lower (because the growth rate is lower and this enhances the required time for a change in community composition), a low growth rate is a known enhancer of granule formation (De Kreuk and Van Loosdrecht, 2004). A (relatively) short SRT therefore seems to be also important for obtaining suspended cells.

A low shear stress is another parameter which is disadvantageous for aggregation and can therefore aid suspended growth (Beun et al., 2000; Liu and Tay, 2002). With the absence of baffles in the reactor and the low stirring speed (160 rpm) this condition is also met in this reactor.

In conclusion, it seems that the cultivation of anammox bacteria as suspended cells is only possible when several requirements are satisfied: (i) the absence of selective pressure for settling (MBR or chemostat cultivation), (ii) a high growth rate (obtained by short SRT), (iii) low levels of bivalent ions (i.e., calcium and/or magnesium).

Furthermore, addition of yeast extract and low shear stress might play a role in the obtaining of a suspended culture of anammox bacteria.

Growth Rate of Anammox Bacteria

With the continuous cultivation at an SRT of 12 days, this is the first report of a reactor study on anammox bacteria with a doubling time (t_d) of less than 10 days (as $t_d = \ln[2]\,\mathrm{SRT}$). Typical doubling times in anammox reactors are 15–30 days (Fux et al., 2004; Strous et al., 1998). Moreover, the produced biomass could be efficiently harvested because it was obtained separate from the effluent (which was removed via a membrane). In view of the maximum conversion capacity in the reactor, the minimum doubling time for anammox bacteria was estimated to be 5.5–7.5 days. Also from the rate at which the population shift from "Brocadia" to "Kuenenia" took place, a doubling time could be estimated. Assuming from microscopic observations that "Kuenenia" constituted 10% of the population on day 64, and 90% on day 77 (and assuming that the growth of "Kuenenia" was not substrate-limited during this period, and the total amount of anammox ("Brocadia" and "Kuenenia") biomass was constant), the "Kuenenia" biomass had increased by a factor 9 in 13 days at an SRT of 16 days. The growth rate required for this is

$$\mu = \frac{1}{\mathrm{SRT}} + \frac{\ln\left(C_{\text{"Kuenenia",day 77}} / C_{\text{"Kuenenia",day 64}}\right)}{t_{\text{day 77}} - t_{\text{day 64}}} = 0.062 + 0.17 = 0.23\,\mathrm{day}^{-1}$$

where $C_{\text{"Kuenenia",day } i}$ = concentration of "Kuenenia" bacteria on day i, which corresponds to an estimated doubling time of only 3 days. Although this is an indication for a relatively fast growth of anammox bacteria, it should be stressed that it only constitutes a one-time observation based on qualitative population estimations and can therefore only be regarded as an *indication* for this fast growth.

From these observations it can be concluded that the doubling time of anammox bacteria is at most 5.5–7.5 days, but possibly as low as 3 days. Also the growth rate of anammox bacteria during the exponential growth phase (in shake flasks, Tsushima et al., 2007) was estimated to be within this range. By quantitative PCR, the doubling time in those systems was estimated to be 3.6–5.4 days. Isaka et al. (2006) have reported a doubling time of 1.8 days, which is faster than the fastest estimates of the doubling time not only in the present study but also in the study of Tsushima et al. However, the growth rate estimation by Isaka et al. (2006) was based on the comparison of the number of anammox cells of two different reactors inoculated at the same time under similar conditions. From the comparison of the cell numbers at two different points in time—each measurement in one of those two *different* reactors—the growth rate was calculated. The validity of the method used by Isaka et al. (2006) is highly questionable and the obtained results are therefore doubtful.

Affinity Constant for Nitrite of Anammox Bacteria

The K_S of anammox bacteria for nitrite was estimated to be 0.2–3 μM in this study (with "Kuenenia") and <5 μM by Strous et al. (1999b) (with "Brocadia"). The estimated K_S values for nitrite of (aerobic) nitrite oxidizers vary considerably (12–955 μM, Both et al., 1992; Hunik et al., 1993; Schramm et al., 1999) both between different studies and between different species, but the values obtained for anammox bacteria are definitely lower than the lowest reported affinity for nitrite oxidizers.

In (aerated) nitrite-limited systems, nitrite oxidizing bacteria compete with anammox bacteria for nitrite. Nitrite oxidizers maintain themselves in the aerobic part of the biofilm, while anammox bacteria reside in the anoxic regions. Due to the required diffusive transport of nitrite from the aerobic to the anoxic region nitrite oxidizers will observe somewhat higher nitrite concentrations than anammox cells. In the competition this can be compensated by a lower value for the affinity constant for anammox bacteria. It has been predicted by mathematical modeling that an affinity constant ratio between anammox and nitrite oxidizing bacteria of $\left(K_{NO_2^-}^{\text{nitrite oxidizers}} / K_{NO_2^-}^{\text{anammox}}\right) > 3$ is required for anammox bacteria in order to maintain themselves in nitritation-anammox biofilm (CANON/OLAND/deammonification) processes (Hao et al., 2002). The estimated lower affinity constants of anammox bacteria for nitrite in this experimental study indeed suggest that competition in these systems can be solely based on nitrite, and thus the one-reactor nitritation-anammox process does not have to take place under strict oxygen limitation. Operation without this limitation would be advantageous for process control, since a strictly oxygen-limited system is harder to operate because the oxygen load has to be carefully balanced with influent loading rate.

Also in natural systems, a higher affinity for nitrite is a major competitive advantage that might be responsible for the relatively large abundance of anammox bacteria in marine systems (Schmid et al., 2007). However, in natural systems also competition for nitrite with denitrifying microorganisms takes place. The affinity for nitrite of anammox bacteria could be in the same range as that of denitrifiers (4–25 μM, Almeida et al., 1995; Betlach and Tiedje, 1981) and therefore, the outcome of competition between those two groups cannot be assessed.

Niche Differentiation of Different Anammox Bacteria

In lab-scale enrichments with ammonium and nitrite, always either "Brocadia" or "Kuenenia" cells become dominant. Therefore, a clear niche difference exists between the "Brocadia"/"Kuenenia" clade, and other genera, like *Candidatus* "Scalindua" (detected under marine conditions) or *Candidatus* "Anammoxoglobus" (enriched under propionate addition). The niche differentiation between *Candidatus* "Brocadia" and *Candidatus* "Kuenenia" themselves however is still unresolved. It has been suggested that

''Brocadia'' cells are more susceptible to nitrite inhibition, and therefore are not enriched in reactor systems at high nitrite levels (Gaul et al., 2005). The recent finding of several ''Brocadia'' enrichments in full-scale reactors—which are operated at higher nitrite levels (Olburgen NL, Rotterdam NL, Van der Star et al., 2007)—disproves this hypothesis. Also aeration does not seem to play a determining role, as no clear difference could be found between the population of (aerated) one-reactor nitritation-anammox processes [''Brocadia'' in Olburgen NL (Van der Star et al., 2007) and Strass CH (Innerebner et al., 2007); but Kuenenia in Gent BE (Pynaert et al., 2003)] and (non-aerated) anammox reactors [''Brocadia'' in Rotterdam NL; Kuenenia in Lichtenvoorde (NL) (Van der Star et al., 2007)].

In our MBR, the switch from a ''Brocadia''-dominated culture to a ''Kuenenia''-dominated culture took place during cultivation at an SRT of 16 days. The minimum specific growth rate of 0.0026 h^{-1} which was required for cultivation at this SRT is around or below the maximum specific growth rate of both ''Kuenenia'' and ''Brocadia'' organisms. Therefore—and because the switch took place in such a short period of time—the competition has probably not taken place based on growth rate. Selection in MBRs is (like in chemostats, Harder and Kuenen, 1977) likely based on (apparent) affinity for the limiting substrate, in this case nitrite. In lab-scale enrichments (without additions of acids, or extra salts, etc.) in sequencing fed-batch reactors in time the culture always seems to become dominated by ''Kuenenia'' [several unpublished occasions in Nijmegen (NL) and Delft (NL)]. Also in these cases affinity for nitrite might be the determining factor.

Based on these observations we hypothesize that ''Kuenenia'' is an affinity (K) strategist, and ''Brocadia'' is a growth rate (r) strategist. The affinities of both ''Brocadia'' (Strous et al., 1999b) and Kuenenia (this study) could not be assessed with enough accuracy to prove this hypothesis. Therefore, more reports of population switches—preferably in studies where replicate reactors can be operated—as well as determination of K_S and μ_m are necessary to confirm this hypothesis. The hypothesis also entails that operation of the anammox process at a shorter SRT will lead to enrichment of ''Brocadia.''

Conclusion

Ten years ago, the SBR was presented as the ''powerful tool'' to obtain a stable enrichment of anammox bacteria (Strous et al., 1998). The SBR is now widely used for the enrichment of anammox bacteria. With the successful enrichment of anammox bacteria in a suspended culture, the MBR promises to be an even more powerful tool: the availability of ample amounts of almost pure suspended anammox cells offers great opportunities for the research on anammox physiology.

The authors thank Estefanía Muñoz Atienza for experimental work during the initial phase of the project and Herman Evenblij for providing the first membrane module. Boran Kartal, Marc Strous, and Mike Jetten are thanked for their discussions.

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