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Rapid and Simple Cryopreservation of Anaerobic Ammonium-Oxidizing Bacteria

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A quick and simple protocol for long-term cryopreservation of anaerobic ammonium-oxidizing bacteria (anammox bacteria) was developed. After 29 weeks of preservation at -80°C , activity recovery for all tested cultures under at least one of the applied sets of preservation conditions was observed. Growth recovery was also demonstrated for a single-cell culture of “*Candidatus Kuenenia stuttgartiensis*.”

Anaerobic ammonium oxidation (anammox) (15, 21) in the nitrogen cycle is a recent discovery of global importance (2, 3, 12, 13). Application of the anammox process has already resulted in increases in the energy efficiency of full-scale wastewater treatment plants (1) and could potentially even make wastewater treatment plants capable of generating energy in the near future (10). Due to their long doubling times, anammox cultures are difficult to cultivate *ex situ*, requiring carefully controlled continuous reactor systems. Currently, these microorganisms are kept viable through continuous culturing or storage in cold-rooms. Both of these methods are prone to calamities and community shifts and can result in genetically dissimilar material after a certain time period. In fact, the fastidious nature of the bacteria necessitates a long-term, high-quality storage method. In addition, dissemination of biomass, formulation for commercial purposes and patent applications (4), and deposition in a qualified culture collection (7) all require knowledge of a suitable storage method.

Research on storage of enrichment cultures, consortia, or other nonpure cultures is still in its infancy. Nevertheless, the few available reports have clearly demonstrated the feasibility of preservation of enrichment cultures and its effectiveness for recovery of specific activity (14, 17, 27) and conservation of community composition (14, 17). The only two available studies on anammox storage found freezing in liquid nitrogen and subsequent lyophilization in skim milk medium (17) and storage at 4°C (26) suitable for preservation of viable cultures and activity (not growth) recovery of biofilm aggregates ($\sim 70\%$ enriched) from the genera “*Candidatus Brocadia*” and “*Candidatus Kuenenia*,” respectively. There are no reports on the preservation and resuscitation of anammox bacteria that are cultivated as faster-growing and highly enriched ($\sim 95\%$) planktonic cells (8, 24). Although possible for their aggregated counterparts, storage at 4°C was not successful for single-cell cultures of “*Candidatus Kuenenia stuttgartiensis*” and the marine anammox species “*Candidatus Scalindua*” sp.: the cells lysed within days, potentially due to the lack of the protective polymeric matrix present in aggregated cultures. Highly enriched anammox cultures became available only recently and have already resulted in a breakthrough in research on the physiology and biochemistry of anammox bacteria (11); therefore, it is of utmost importance to have a routine methodology to store and reactivate these cells.

In the present study, we were interested in a quick, simple, and widely applicable method for long-term preservation of both ag-

gregated and single-cell anammox cultures and therefore opted for cryopreservation. Dimethyl sulfoxide (DMSO) was chosen over glycerol as the cryoprotective agent (CPA), as a pairwise comparison in the cryomicrobiology literature showed the latter to be less effective (6). Yeast extract, sugars, or alcohols are almost always included in prepreservation growth medium and freezing medium of heterotrophs, but their cryoprotective role has not been frequently recognized (6, 20). Previous work demonstrated that the combined protective actions of DMSO and carbon compounds and addition of carbon compounds during prepreservation growth significantly increased viability recovery after cryopreservation of aerobic methane oxidizers (data not shown). Therefore, the experimental design of the study presented here incorporated the combination of Trypticase soy broth (TSB) and trehalose, as well as careful execution of preservation and resuscitation manipulations, taking into account CPA penetration time, suitable addition temperature, and toxicity, all previously described as crucial parameters for successful preservation (5).

Biomasses from three enrichment cultures of anammox bacteria (Table 1) were harvested from active continuous reactors after growth at 33°C and pH 7.3 to 7.5 as previously described (8, 9, 22, 24, 25). The identity of the anammox bacteria in the cultures was confirmed with fluorescent *in situ* hybridization (FISH) as previously described (8, 11, 18, 19). Samples were concentrated by centrifugation and washed with and resuspended in 3 ml of fresh medium (supplemented with 2.5% [wt/vol] Red Sea salt for the “*Candidatus Scalindua*” biomass). Samples (in duplicate) were directly frozen in mineral medium without CPA, with 5% (vol/vol) DMSO, or with a combination of 5% DMSO (vol/vol), 1% trehalose (wt/vol), and 0.3% TSB (wt/vol) as CPA or incubated while shaken in mineral medium with 1% trehalose (wt/vol) for 2 h under an anaerobic headspace at 30°C and then frozen with 5% DMSO (vol/vol). DMSO was always added to biomass at 4°C , to decrease toxicity to cells and related adverse effects on survival, and the cells were left to equilibrate for 15 to 30 min to enable

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TABLE 1 Overview of anammox cultures included in this study

Culture	Type	Biomass concn (fold)	Preserved biomass (mg/ml protein)	Reference
Aggregated " <i>Candidatus Kuenenia stuttgartiensis</i> "	Aggregate	4	3.39 ± 0.58	9
Single-cell " <i>Candidatus Kuenenia stuttgartiensis</i> "	Single cell	10	0.39 ± 0.01	8, 24
Single-cell " <i>Candidatus Scalindua</i> " sp.	Single cell	5	1.35 ± 0.04	8

intracellular uptake. All samples were frozen at -80°C in 10-ml glass serum vials. All manipulations, from biomass harvest to freezing, were performed without any precautions to avoid exposure of cultures to air (no inhibitory effects of aerobic manipulations were observed in activity tests with anammox cultures as also previously observed) (8, 9, 11, 25).

After 29 weeks, preserved biomass was thawed at 37°C , CPAs were quickly removed by centrifugation to decrease toxicity to cells, and biomass was washed with and resuscitated in appropriate fresh medium. The success rate of cryopreservation was evaluated by the recovery of specific activity compared to that of fresh biomass, which was considered a proxy for the survival rate. To this end, biomass was incubated while shaken with fresh medium under an anaerobic headspace at 30°C . Specific activity, defined as $^{29}\text{N}_2$ production from ^{15}N -nitrite and unlabeled ammonium, was determined by gas chromatography (Agilent 6890 system equipped with a Porapak Q column at 80°C) combined with an Agilent 5975c quadrupole inert mass spectrometer (MS); ammonium and nitrite levels were determined colorimetrically (9). To-

tal protein content was determined by the bicinchoninic acid assay (Pierce) using a bovine serum albumin standard according to the manufacturer's instructions. All preserved cultures were active after a 29-week cryopreservation period (Fig. 1), but variations of recovery of specific activity were observed. Aggregated "*Candidatus Kuenenia*" culture, in which cells were protected by the surrounding matrix and biomass, recovered well from preservation even without any added CPA (40% recovery), in contrast to single-cell cultures ($<5\%$ recovery). Single-cell cultures more easily take up CPA during the equilibration time prior to freezing, and indeed, single-cell "*Candidatus Kuenenia*" cultures and "*Candidatus Scalindua*" cultures, which both showed only low activity without CPA, recovered better (51% and 30%, respectively) using DMSO, especially combined with carbon compounds (Fig. 1). Nevertheless, use of a CPA did also improve the survival rate of aggregated "*Candidatus Kuenenia*," with no significant differences seen with the use of CPA combined with carbon compounds versus prepreservation incubation with trehalose.

As a proof of principle for linking recovery of activity to

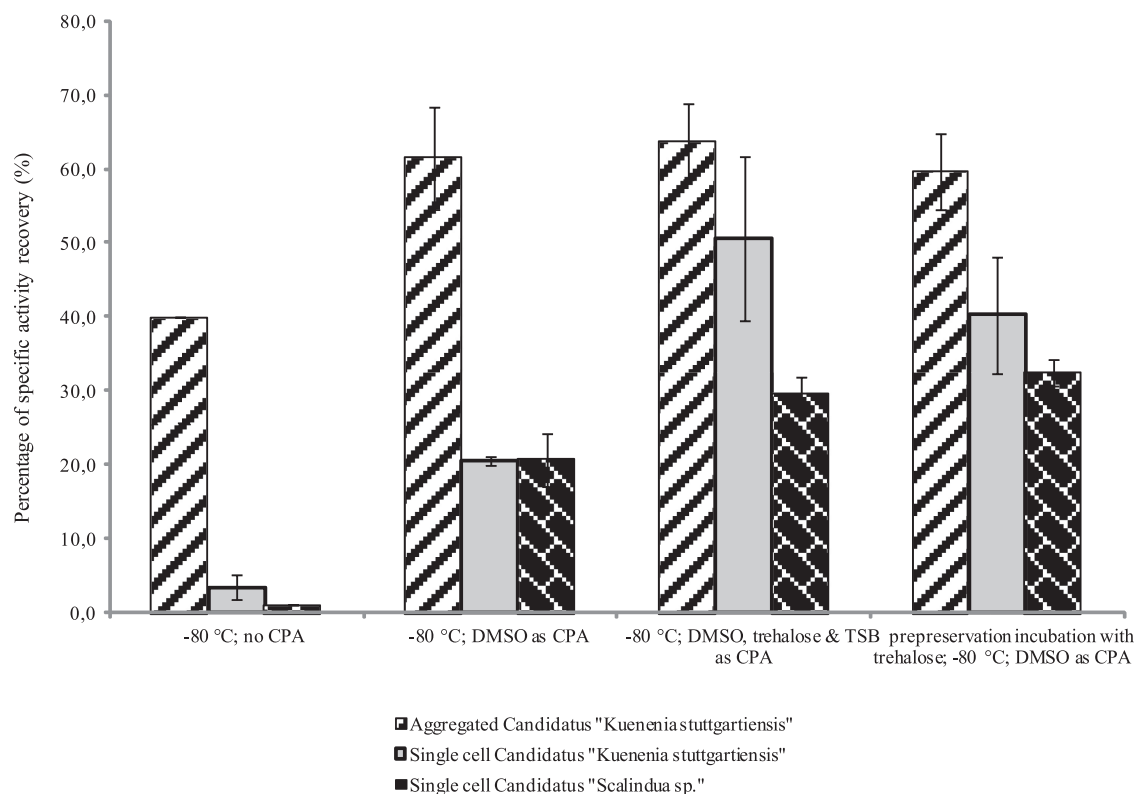


FIG 1 Percent recovery of specific activity of anammox biomass in batch tests after preservation for 29 weeks at -80°C under the indicated storage conditions. Specific anammox activity was based on ^{29}N - N_2 production from ^{15}N -nitrite and unlabeled ammonium. Preservation experiments and batch tests were performed in duplicate (error bars represent variations in activity between individual cryopreserved tubes). No variation in activity was observed for aggregated "*Candidatus Kuenenia stuttgartiensis*" and single-cell "*Candidatus Scalindua*" sp. without CPA.

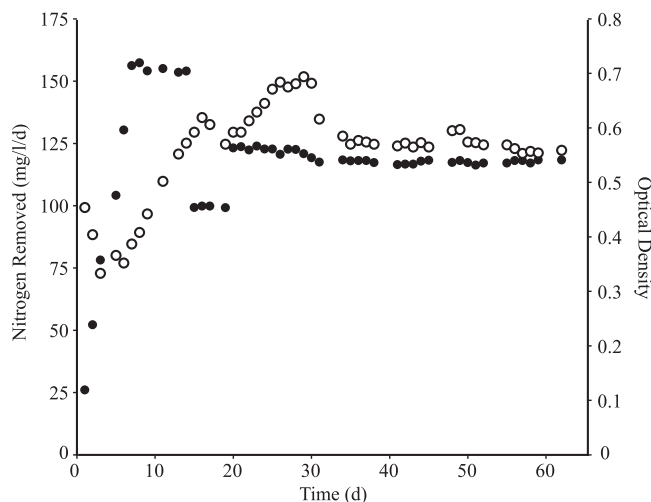


FIG 2 Resuscitation of preserved planktonic cells of “*Candidatus Kuenenia stuttgartiensis*.” The two-liter bioreactor was operated as a membrane bioreactor (MBR) as described elsewhere (8, 24). Open circles, optical density; closed circles, nitrogen removal.

growth, a 2-liter volume of a single-cell “*Candidatus Kuenenia*” culture at an optical density at 600 nm (OD_{600}) of 1.0 was harvested from an active continuous reactor. Biomass was concentrated to 500 ml and manipulated in a manner identical to that described for the batch tests. The preservation method with the best results (Fig. 1) was chosen: freezing at -80°C in mineral medium with 5% DMSO (vol/vol), 1% trehalose (wt/vol), and 0.3% TSB (wt/vol) as the CPA. After 23 weeks, the biomass was resuscitated as described above and used to seed a 2-liter continuous reactor with an internal membrane unit. The reactor was operated at 33°C and pH 7.3 as previously described (8). After inoculation, the culture started to grow immediately as determined by optical density measurements at 600 nm (Fig. 2). Within 8 days, it was possible to increase both ammonium and nitrite concentrations in the influent medium from 3 mM (13 mg of N load/liter/day) to 36 mM (159 mg of N load/liter/day), while all available nitrite was consumed. In our experience, this is almost identical to the time necessary for starting up a new reactor from active cells, suggesting that most of the cells were viable after preservation. The nitrite concentration in the effluent was below the detection limit ($10\ \mu\text{M}$) throughout the operation of the reactor; the nitrite-and-ammonium conversion ratio increased from ~ 1 (day 3) to 1.22 (day 41), similar to the previously reported anammox stoichiometry (23). This indicated that other nitrite-reducing processes (i.e., denitrification) did not contribute significantly to substrate conversion. A doubling time of 12 days was calculated, assuming that the cells grew exponentially from the startup of the reactor, between days 6 and 16 ($R^2 = 0.98$). Moreover, growth of “*Candidatus Kuenenia stuttgartiensis*” was still stable, with a constant washout of cells (starting on day 29) at a flow rate of 120 ml/day. This growth phase (after day 29) could be described with a solids retention time (SRT) of 18.3 days, corresponding to a doubling time of 12.7 days. In this phase, the reactor was stable for longer than 3 doubling times (data after day 60 not shown). These combined results showed that the continuous culture of preserved anammox cells had no apparent loss of activity and that their activity could immediately be coupled to growth after resuscitation.

Currently, only one successful stable preservation protocol is described for the (aggregated) anammox culture method using lyophilization (17), resulting in $\sim 11\%$ activity recovery after preservation. Unfortunately, lyophilization is a very complex procedure where seemingly trivial changes in a protocol can render the process unsuccessful (16), making it not easily applicable. Here we demonstrate for the first time that a simple and rapid cryopreservation procedure is suitable for stable and long-term storage of both aggregated and single-cell anammox cultures and results in both activity and growth recovery. Our choices for cryoprotective agents appear to be crucial for this success, as activity recovery after previous cryopreservation of aggregated anammox cultures at -20°C or -60°C with glycerol and/or skim milk (17, 26) failed. We therefore propose freezing at -80°C using 5% DMSO (vol/vol), 1% trehalose (wt/vol), and 0.3% TSB (wt/vol) as the CPA as the primary cryopreservation condition. However, preservation success is always strain dependent, and preliminary tests should be performed to confirm applicability on other cultures.

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