Enrichment of anaerobic, sulfide oxidizing denitrifiers from Trunk River sediments in southern Cape Cod, MA.

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Abstract

The present study describes the attempt to isolate organisms that can anaerobically oxidize sulphide and acetate as electron donors with nitrous oxide as the terminal electron acceptor. Sediment and bottom water samples collected from Trunk River and Sippewissett served as the inoculum for the enrichments. The study presents the enrichment procedure, 16S clone library sequences of the cultured *Pseudomonas stutzeri* from Trunk River sediments along with scanning electron microscopy images. Further, growth experiments show that the growth of *P. stutzeri* is dependent on nitrous oxide and to some extend acetate.

Introduction

Denitrification represents the main output of nitrogen from most aquatic environments and thus represents an important part of the nitrogen cycling in these environments (Ward, 1996). The complete reduction of nitrate to dinitrogen is mediated by various organisms reducing the different intermediate forms of nitrogen. In this study, I've chosen to focus on the reduction of nitrous oxide to dinitrogen.

Increased usage of N-fertilizers in agriculture is one of the main reasons for rising nitrous oxide concentrations in the atmosphere. Nitrous oxide is a strong green-house gas and therefor understanding the ecology of the microbes mediating the reduction of N_2O may aid our understanding of how nitrogen fluxes might change in a changing climate perspective (Yoon *et al.,* 2016).

By providing nitrous oxide as the terminal electron acceptor with sulphide and acetate as the electron donors, my hope was to culture organisms that could either use sulphide (I) or acetate (II) or both:

(I) $1HS^- + 4N_2O \rightarrow SO_4^{2-} + 4N_2 + H^+$

(II) $1C_2H_3O_2^- + 4N_2O \rightarrow 2HCO_3^- + 4N_2 + H^+$

Methods

Enrichment

5 samples were collected on August 4th 2016 from the bottom water and the sediment of Trunk River (coordinates) and from the bottom water, sediment and sulfidic sediment of Little Sippewissette. The samples were inoculated in anaerobic media made of a brackish water base with K2PO4 (100mM), trace elements, vitamin solution, sodium sulphide (1mM), thiosulfate (10mM), DCMU, sodium acetate (10mM), BES (10ml/L) and NH_4. Additional bottles with trunk river sediment and bottom water was inoculated without NH_4. 160 ml culture bottles were filled with 34 ml media and the headspace filled with 100% nitrous oxide (N_2O). Approximately 1 ml of water or ~1g of sediment was added to each media bottle.

After 5 days incubation at 30° C, 1 ml was transferred into new media bottles. An un-inoculated bottle containing both media and N_2O in the headspace served as a negative control.

Growth experiments

Growth experiments were initiated to test whether growth was dependent on N_2O (bottles 1 and 2) and sulphide or acetate (bottles 3 and 4). 1 ml of the second passage sample from Trunk river sediment was transferred into 4 different 160 ml culture bottles containing 68 ml media and 92 ml headspace. In bottles 1 and 2 the headspace was filled with argon and 0,6 ml 100% N_2O (1 pressure= atm). After 26 hours the headspace was spiked with 2 ml N_2O and after 31 hours and 66,5 hours with additional 12 ml N_2O. Bottles 3 and 4 had 100% N_2O in the full head-space (1atm) and after 48,5 hour the media was spiked with additional 3mM sodium acetate. The bottles were incubated at 30°C for 72 hours and were sampled 9 times during that period. OD(600nm) was measured on a spectrometer, actetate concentration were measured by high-performance liquid chromatrography (HPLC), ammonium and sulfate concentrations were measured using ion chromatography (IC). Nitrous oxide concentrations were measured using gas chromatography.

Microscopy

Light microscopy images were taken with an Axiocam 503 mono (Zeiss) confocal microscope. 1 ml of the sample was fixed in formaldehyde (4% final concentration) at 4°C over night. 100ul of the fixed samples was mixed with 10 ml molecular grade water and filtered onto a 0.2um membrane filter (MF-Millipore™ Membrane Filters). The filter was dehydrated in a series of ethanol rinses (30%, 50%, 70%, 95%, 98,9% ethanol) and went through critical point-drying before being spotter coated with platinum. Images were done on an environmental scanning electron microscopy (TM3030 Tabletop Microscope, Hitachi).

16S Clone library

6ml of the sample was pelleted and DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega). 16S was amplified using primers 8f and 1391R. PCR cycling conditions were 95 °C 2 min followed by 25 cycles of 95 °C 30 sec, 55 °C 30 sec,72 °C 1.5 min.And final 72 °C 10 min. The amplicons were ligated into the pGEM®-T Easy Vector (Promega) and cloned into E. coli cells. The cells were grown on plates containing ampicillin and X-gal over night and 12 colonier were subsequently picked and sequenced using the sanger method. Sequences were blasted agains the NCBI database.

Results and Discussion

Enrichments

Out of the initial 7 bottles of enrichment cultures only the Trunk River sediment bottle (with NH_4) had noticeable growth. Using light microscopy and SEM the cells observed were highly motile, rod-shaped cell of approx. 2-4um length (figure 1,2,3). Alle cells seemed to be similar in shape and size, suggesting that the selectiveness of the enrichment was strong enough to favour the growth of a single organism.

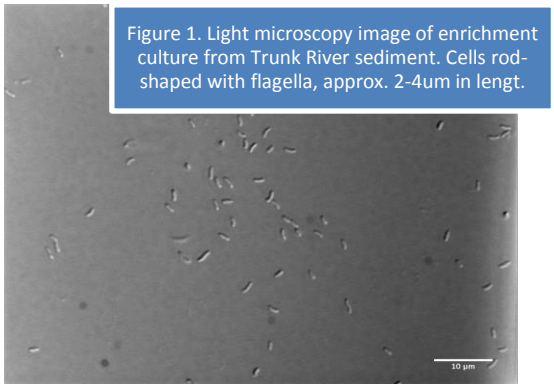

Figure 1. Light microscopy image of enrichment culture from Trunk River sediment. Cells rod-shaped with flagella, approx. 2-4um in lengt.

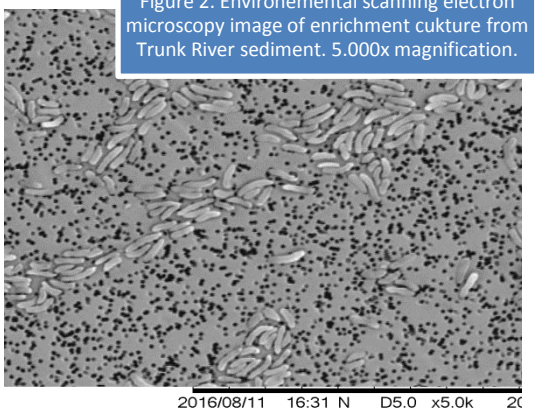

Figure 2. Environemental scanning electron microscopy image of enrichment cukture from Trunk River sediment. 5.000x magnification.

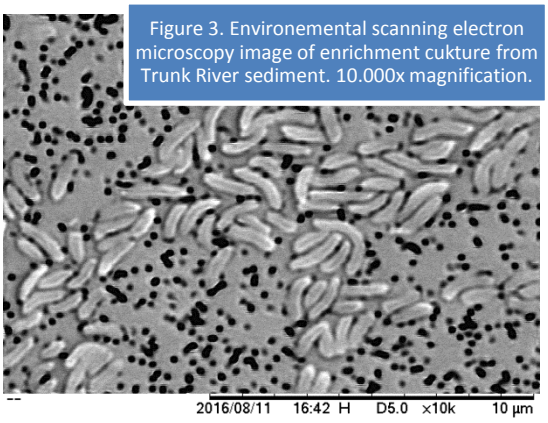

Figure 3. Environemental scanning electron microscopy image of enrichment cukture from Trunk River sediment. 10.000x magnification.

16S sequences of *Pseudomonas stutzeri*

12 clones picked and sequenced from the 16S clone library obtained from the Trunk River sediment sample all had 97-99% identity to *Pseudomonas Stutzeri* when blasted against the NCBI nucleotide database. Pseudomonas is a well-studied denitrifier that also possess the ability to oxidize sulphide (Mahmood *et al.,* 2009). The fact that all 12 sequences turned out to belong to the P. stutzeri further shows that the selectiveness of the media has successfully favoured one organism. I cannot be certain that *P. stutzeri* is the only organism growing in this media, therefor isolation by plating or shake-tubes will be necessary for further isolation of this organism. *P. stutzeri* is a gamma proteobacteria harbouring the clade I *nosZ* (nitrous oxide reductase gene). Studies have shown that organism belonging to clade I are the main groups responsible for controlling N_2O emission from soils thereby determining the N_2O sink of soils (Yoon *et al.,* 2016).

Growth Experiments

Bottles 1 and 2 did not show any growth until spiked with 12 ml N_2O. After the addition of 12 ml N_2O the optical density increased rapidly in both bottles indicating that N_2O was limiting the growth (figure 4).

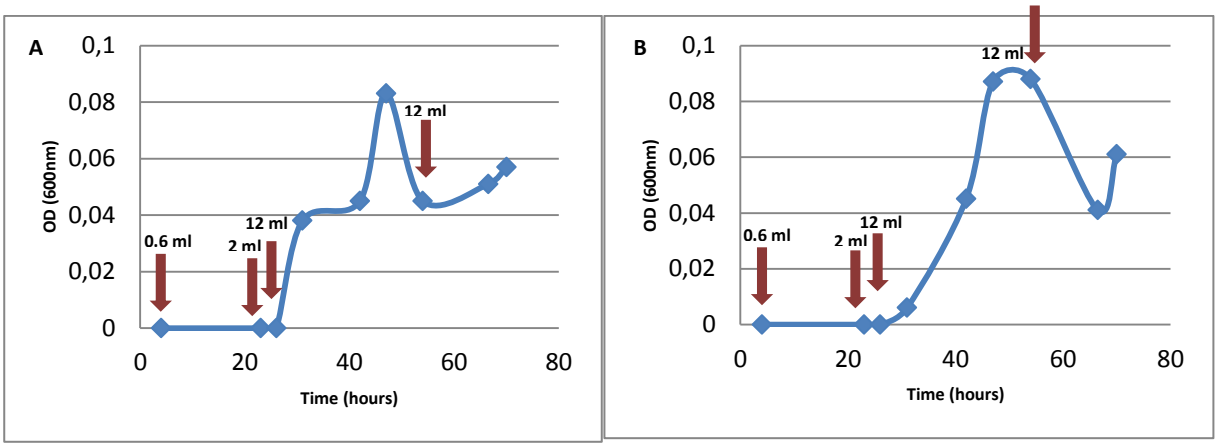

Figure 4. Growth curve of bottle 1(a) and 2(b). Arrows indicating the addition of N_2O to the headspace.

In bottles 1 and 2 the acetate concentration is decreasing slowly during the full 72 hours without being depleted (figure 5 and 6). After the addition of 12ml N_2O after 26 h and 12 ml N_2O after 66,5 hours there is an increase in ammonium concentrations and the appearance and increase of lactate. The reason for these increases are unclear and may be an artefact of measurement techniques.

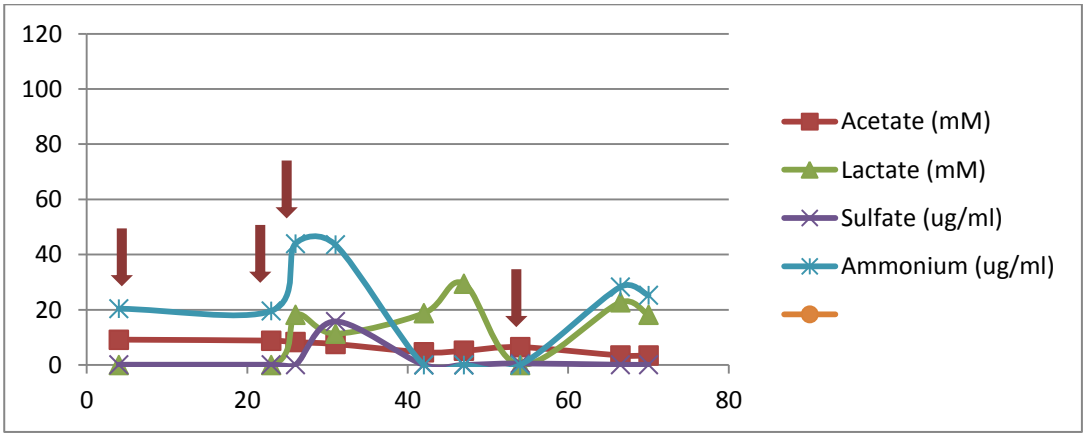

Figure 5. Bottle 1. Concentrations of Acetate (mM), Lactate (mM), sulfate (ug/ml) and ammonium (ug/ml) during the 72 hour growth experiment.

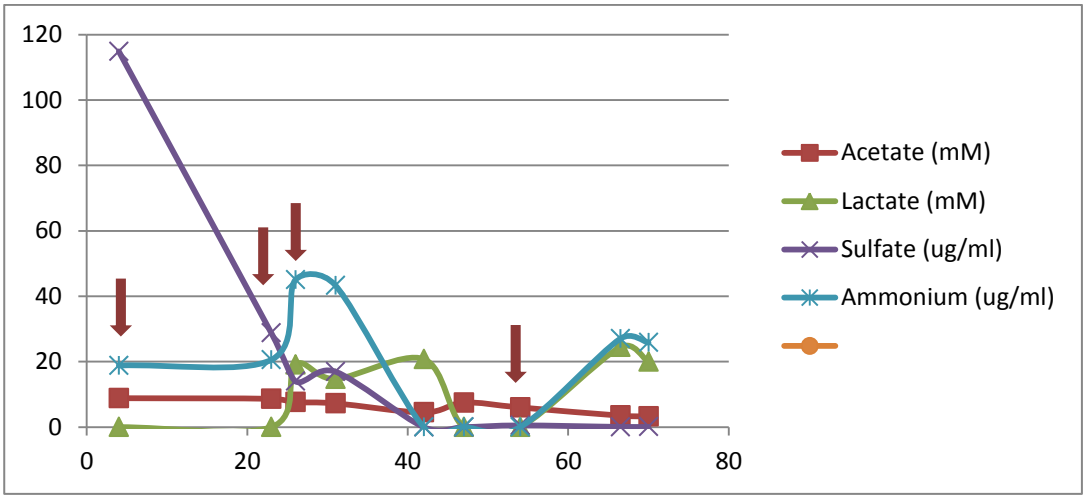

Figure 6. Bottle 2. Concentrations of Acetate (mM), Lactate (mM), sulfate (ug/ml) and ammonium (ug/ml) during the 72 hour growth experiment.

N_2O consumption was measured after 4h, 26h, 31h and 42h. After the addition of 0,6ml (initialy) and 12ml N_2O (after 31h). The N_2O concentration decreased in both cases in both bottles confirming that N_2O is being depleted (figure 7).

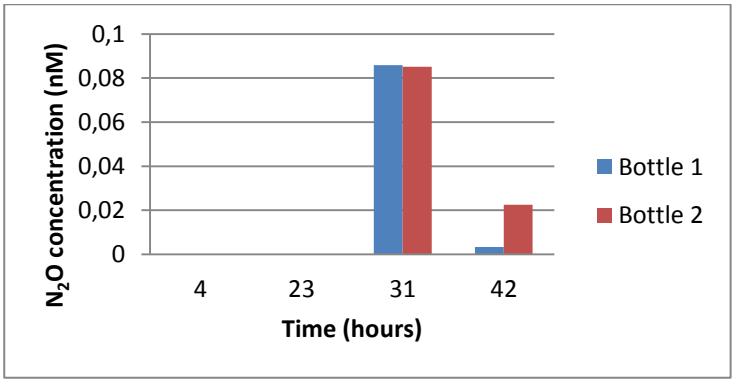

Figure 7. N_2O concentration measured after 4h, 23h, 31h and 42h. From 4h to 23h N2O conc. decreased. From 31h to 42h the conc. decreased in both bottles.

Bottle 4 showed very rapid growth for the first 26 hours. After the addition of 3mM sodium acetate at 48,5 h the OD increased in both bottles 3 and 4 suggesting that the growth at this time was limited by acetate (figure 8). Further growth would have been needed to show dependency of sulphide for growth. A possibility would be to change the carbon source of the media and increase the concentration of sulphide in the media.

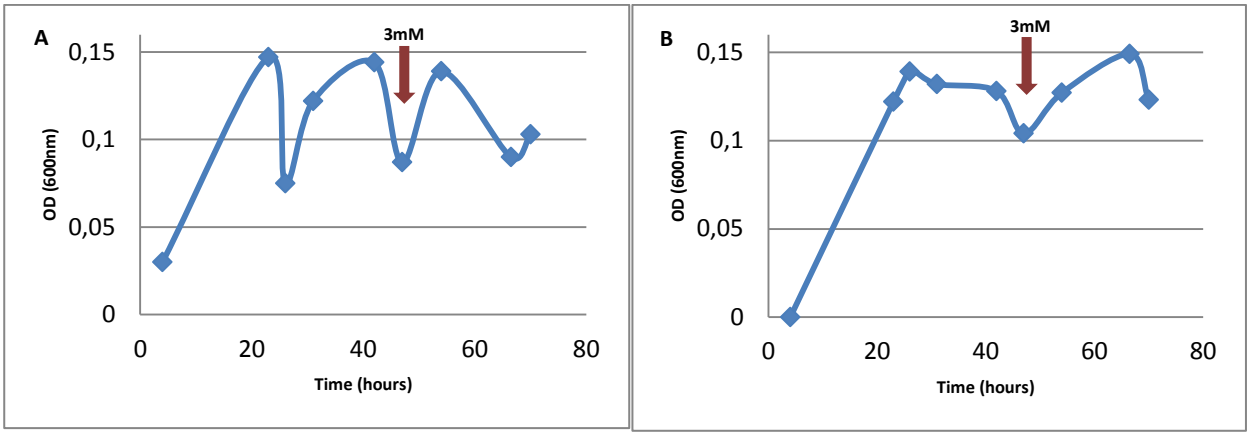

Figure 8. Growth curve of bottle 3(a) and 4(b). Arrows indicating the addition of 3mM sodium acetate.

Sulfate concentrations in bottles 3 and 4 was initially very high and decreased over time suggesting a switch between oxidizing sulphide to oxidizing acetate, as sulphide is depleted (figure 9 and 10). Acetate concentrations fluctuated over time in both bottles.

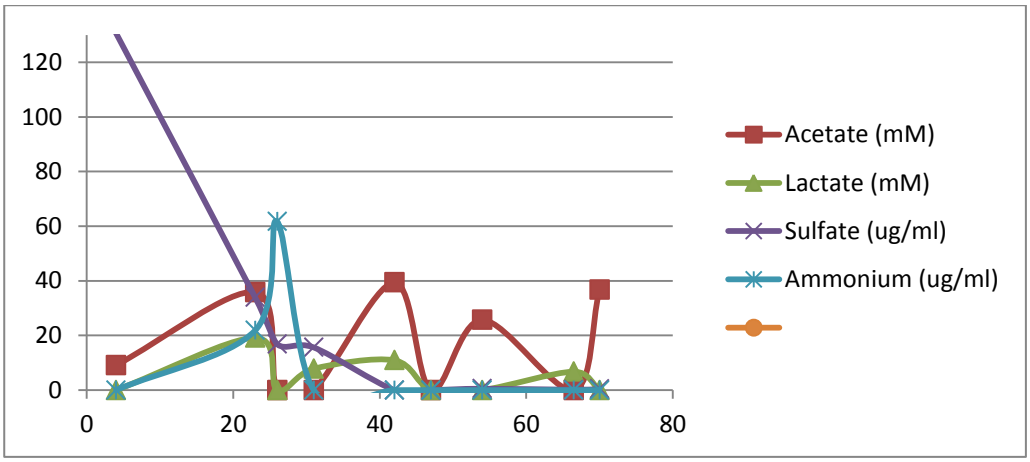

Figure 9. Bottle 3. Concentrations of Acetate (mM), Lactate (mM), sulfate (ug/ml) and ammonium (ug/ml) during the 72 hour growth experiment.

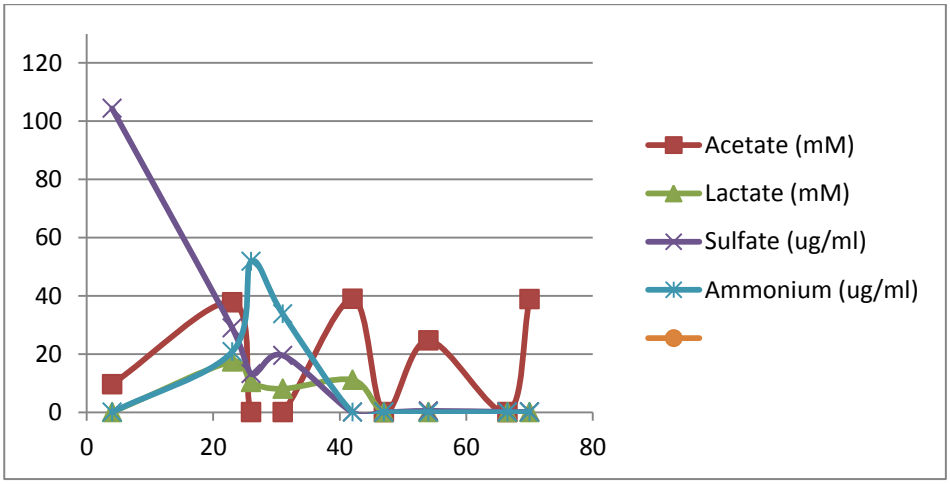

Figure 10. Bottle 4. Concentrations of Acetate (mM), Lactate (mM), sulfate (ug/ml) and ammonium (ug/ml) during the 72 hour growth experiment.

Conclusion

In my attempt to enrich for sulphide oxidizing denitrifiers I managed to promote the growth of, what seems to be a monoculture of *Pseudomonas stutzeri*. The results of the growth experiments showed that the growth of *P. stutzeri* was dependent on N_2O and Acetate. The high levels of sulfate in the initial time-point points to the ability of *P. stutzeri* to also oxidize sulphide into sulfate.

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References

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