A Study of Hyphomicrobium

----Measurement of Denitrification and Examination of Manganese Oxidation

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Abstract

Hyphomicrobium is a denitrifying methylotroph, meaning that they reduce nitrate, and use methane as carbon and energy source. It has been suggested that Hyphomicrobium can oxidize manganese, but whether they can harvest energy from this process is not known. In this study, soil samples are taken from different environments in Woods Hole and enriched for hyphomicrobium. I obtained enrichment cultures containing up to 90% hyphomicrobium and measured the rates of denitrification under different conditions. Manganese concentration had some effect on cell growth and respiration, but the mechanism is inconclusive at this level of resolution.

Introduction

Hyphomicrobium is a dimorphic α-Proteobacteria. They reproduce by budding and form two daughter cells that are morphologically and behaviorally different: one nonmotile prosthecate that has elongated cylindrical appendages, and the other becomes a motile flagellated swarmer cell. This morphological separation results in the spatial separation of the daughter cells to increase the efficiency in nutrient acquisition (Garrity et al., 2006; Moore, 1981). Cell morphology varies with growth conditions. In media with low nutrient concentration, hyphae may elongate up to 300μm. The unique morphological characteristics facilitate the relatively easier recognition and identification of hyphomicrobium from environmental samples.

Hyphomicrobium are ubiquitous in the environment, but their distribution is dependent on nutrient levels (Staley et al., 1980; Staley, 1971; Stanley et al., 1979). In a polluted stream in Michigan, these prosthecate bacteria accounted for 0.62 to 1.1% of the total bacterial population(Staley, 1971). Although hyphomicrobium are normally found in these polluted area, they do not prefer eutrophic habitats. In a population study carried out in lakes of differing trophic states, these prosthecate, budding bacteria accounted for as much as 15% of the total bacterial population in an oligotrophic lake and up to 35% in a mesotrophic lake (Staley et al., 1980). These results suggest that changes in the population size may reflect changes in nutrient levels and that these organisms could be used as indicator of water quality.

Hyphomicrobium are metabolically variable. They are facultative anaerobes, meaning they can switch between oxygen and nitrate as electron acceptors. They can use a variety of carbon compound as both energy and carbon source, including

methylamine, di- and trimethylamine, dichloromethane and methylsulfate (Garrity et al., 2006; McDonald et al., 2001). Methanotrophy is possible with low concentration of oxygen (Amaral & Knowles, 1995). Their capability of coupling methanol oxidation and denitrification is of special interest in waste water treatment, which removes nitrate from eutrophic environments at the expense of methanol(Green et al., 2010; Liessens et al., 1993). Their function in the nitrogen cycle is not limited to denitrification. Genes coding for nitrogen fixation enzymes has been shown by genome sequencing and Southern or dot blot hybridization with gene probes specific for nitrogenase reductase (nifH) (Kloos et al., 1995; Vuilleumier et al., 2011). It has long been suggested that hyphomicrobium are capable of oxidizing manganese since they are always found to be present in manganese deposits (Tyler, 1970). However, whether they are capable of oxidizing manganese or using manganese oxidation for energy source has not been studied.

The goal of this study is to enrich and isolate hyphomicrobium strains from different environments in Woods Hole, and examine its morphology and growth. Another objective is to measure rate of denitrification of hyphomicrobium in culture, assessing it's ecological importance in the nitrogen cycle. The third interest is to test whether some hyphomicrobium stains are capable of manganese oxidation and whether the energy is harvested to promote cell growth.

Method and Materials

Soil samples were collected from four different locations in Woods Hole: near School St. swamp (swamp), near fire hydrant by Stoney Beach (pond), under pine tree by garbage beach (GB), tarmac driveway by fire hydrant (tarmac). A small scoop of soil was inoculated to a Pfenning bottle containing selective medium. The medium contains following components: 171 μM NaCl, 19.7 μM $MgCl_2$, 6.8 μM $CaCl_2$, 67.1 μM KCl, 20 mM pH 7.2 MOPS buffer, 1X HCl-dissolved traced elements, 200 μM Na_2SO_4, 100 μM pH 7.2 K_3PO_4, 5mM NH_4Cl, 50mM KNO_3, 2.5ml methanol, 1X multivitamin solution. Samples were incubated at 30 $^{\circ}$C. Enrichments were achieved through series transfers of 2ml of culture into fresh medium. Enrichment medium was prepared anoxically and all procedures were performed inside anaerobic chamber. Liquid cultures were streaked onto agar plates containing same components as the liquid medium after three transfers.

After three transfer, 1 ml of each of the four liquid cultures were transferred into four sets of selective medium containing different Mn^{2+} concentration: trace, 10μM, 50μM and 100μM Mn^{2+}. The liquid cultures were also streaked on selective agar plates with same series of Mn^{2+} concentrations.

Subsamples were taken from each enrichment bottle from the Mn^{2+} matrix everyday after inoculation. Growth conditions were monitored by measuring optical density of the subsamples at 600 nm wavelength. 75 μL of subsamples were mixed with 25 μL ethanol to fix the cell. 1.5 μL of the mixture were added to 5 ml PBS and filtered onto 0.2 μL membrane filter. Filters were stained with DAPI for total cell count. Mixture were also analyzed using phase contrast microscope for relative

abundance of hyphomicrobium in the culture based on morphology. Remaining subsamples were filtered through 0.2 µL syringe filter and stored at -20 °C for nitrate analysis. Nitrate concentrations were measured using NOx box in Wankel lab at WHOI as described (Braman & Hendrix, 1989; Garside, 1982).

Results and Discussions:

Two days after transfer into the Mn^{2+} enrichment matrix, all cultures turned turbid. Bubbles rose in the liquid when loosening the bottle caps (Fig 1). After 72 hours, cell density in all enrichment exceeded 10^8 cells / ml. Relative abundance of hyphomicrobium were counted based on morphology using a light microscope (Fig. 2). The flagellate cells accounted for between 64.5% and 89.6% of the total

Fig. 1 Nitrogen gas forming upon loosening the cap

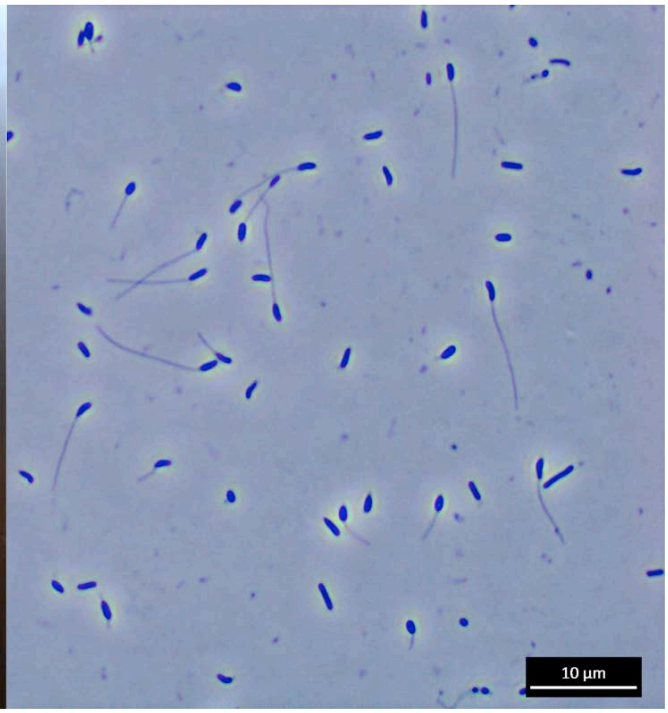

Fig. 2 Wet mount of liquid culture used for examining relative abundance of hyphomicrobium

population (Fig. 3). Enrichment from soil near garbage beach contained fastest growing cells and highest relative abundance of hyphomicrobium. Cell density of enrichment tarmac and pond were lower, but relative abundances of hyphomicrobium were comparable to the other two sets of enrichments. After 72 hours of incubation, nitrate in the enrichments were mostly depleted, thus not able to support continuous growth of hyphomicrobium. The plateaus in growth curves were probably due to lack of nitrate instead of stationary growth of the culture. In future enrichment, this problem could be avoided by increasing starting nitrate concentration, or spiking nitrate into medium during the incubation. Nitrates consumption occurred at a much higher rate than normally measured from the field. Considering the ubiquitous distribution of hyphomicrobium and high abundance in the environment, they can be important players in the biogeochemical cycling of nitrogen.

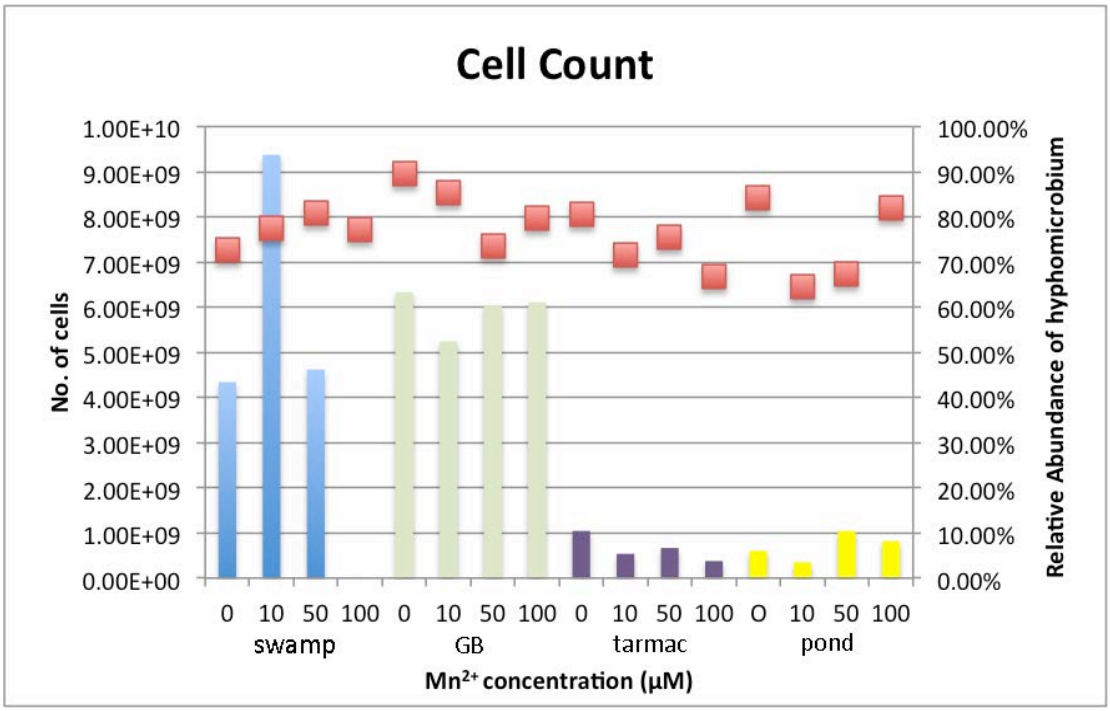

Fig.3 Total DAPI cell counts and relative hyphomicrobium abundance

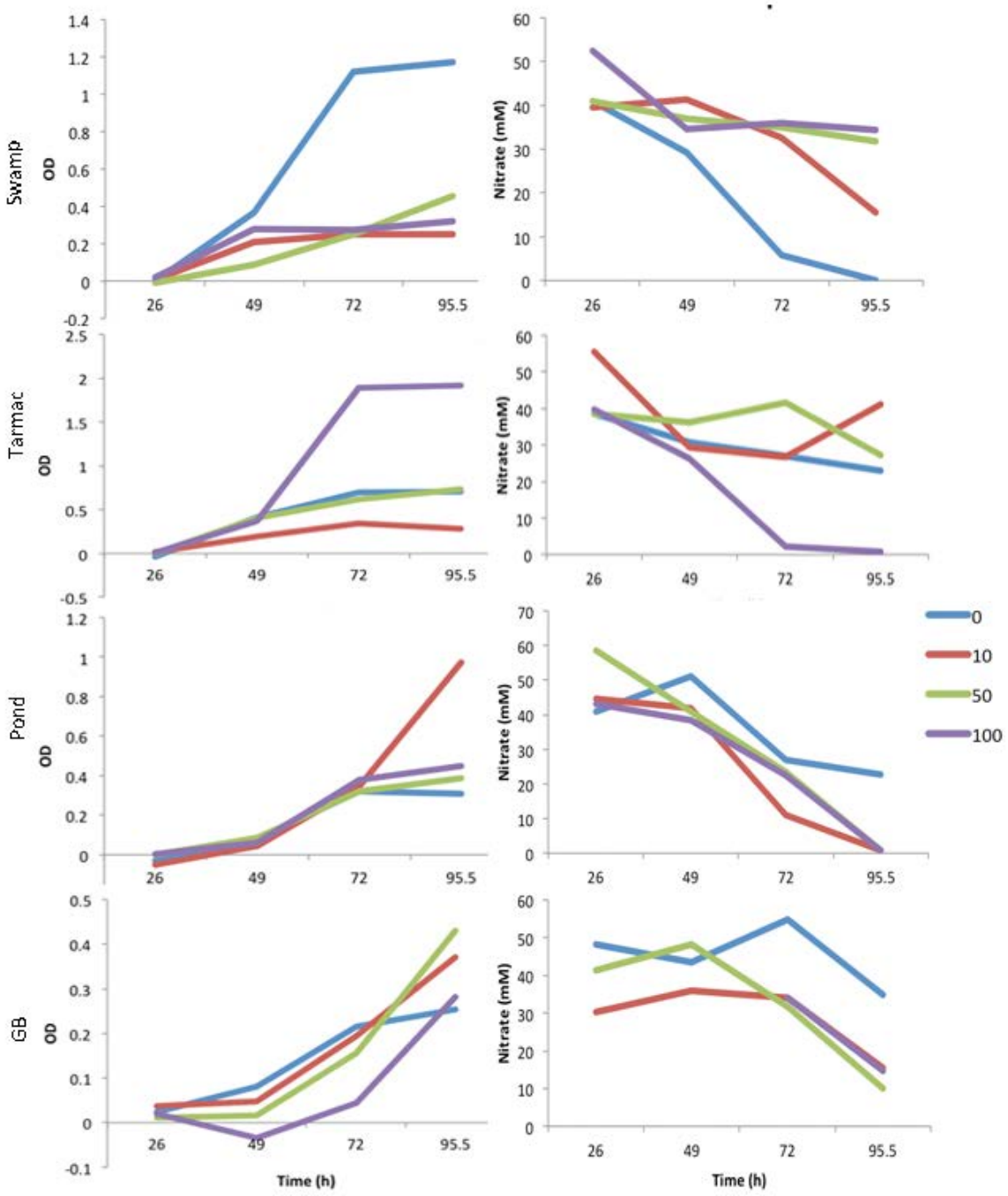

Fig.4 Enrichment growth curve (left) and Nitrate concentrations (right) over time for four different enricnments series with varying manganese concentrations

In swamp enrichments, medium without addition of Mn^{2+} supported highest growth of cells and depletion of nitrate (Fig. 3). Addition of Mn^{2+} in the medium seemed to inhibit the cell growth and denitrification. In contrast, in GB enrichments, 100 μM Mn^{2+} promoted cell growth. Nitrate was depleted fastest with 100 μM Mn^{2+} medium across all experiments. For pond and tarmac enrichments, addition of Mn^{2+} into the medium catalyzed cell growth and denitrification to some extent, but the differences weren't as apparent.

Manganese oxides weren't visible in liquid enrichments and plates. However, Mn^{2+} was very low in concentration, and the total amount manganese in each enrichment bottle was very low for detection of the manganese oxides (Francis et al., 2001; Mandernack et al., 1995). Assays for measuring Mn^{2+} consumption or MnO_2 formation are needed to confirm the occurrence of Mn^{2+} oxidation (Francis et al., 2001; Krumbein & Altmann, 1973).

Based on this experiment, the catalytic effect of Mn^{2+} oxidation on hyphomicrobium growth was inconclusive. The different responses to addition of Mn^{2+} in different enrichments could be attributed to strain-specific metabolisms. Furthermore, the Mn^{2+} concentration in the medium was orders of magnitudes lower than methanol, the competing electron donor. The energy yield of Mn^{2+} oxidation is lower than methanol oxidation (Fig.4). When methanol is replete in the medium, it would be thermodynamically less favorable for cells to oxidize Mn^{2+}. However, harvesting energy from Mn^{2+} oxidation would spare part of the methanol

from energy source for carbon assimilation and promote cell growth. Due to low concentration of Mn^{2+} in the medium compared to methanol availability, the effect of Mn^{2+} oxidation would be overshadowed by methanol oxidation. In addition, a better method for measuring cell yield is needed. Hyphomicrobium tends to grow attached to surface, thus taking subsamples from the liquid medium couldn't account for all the growth inside the bottle.

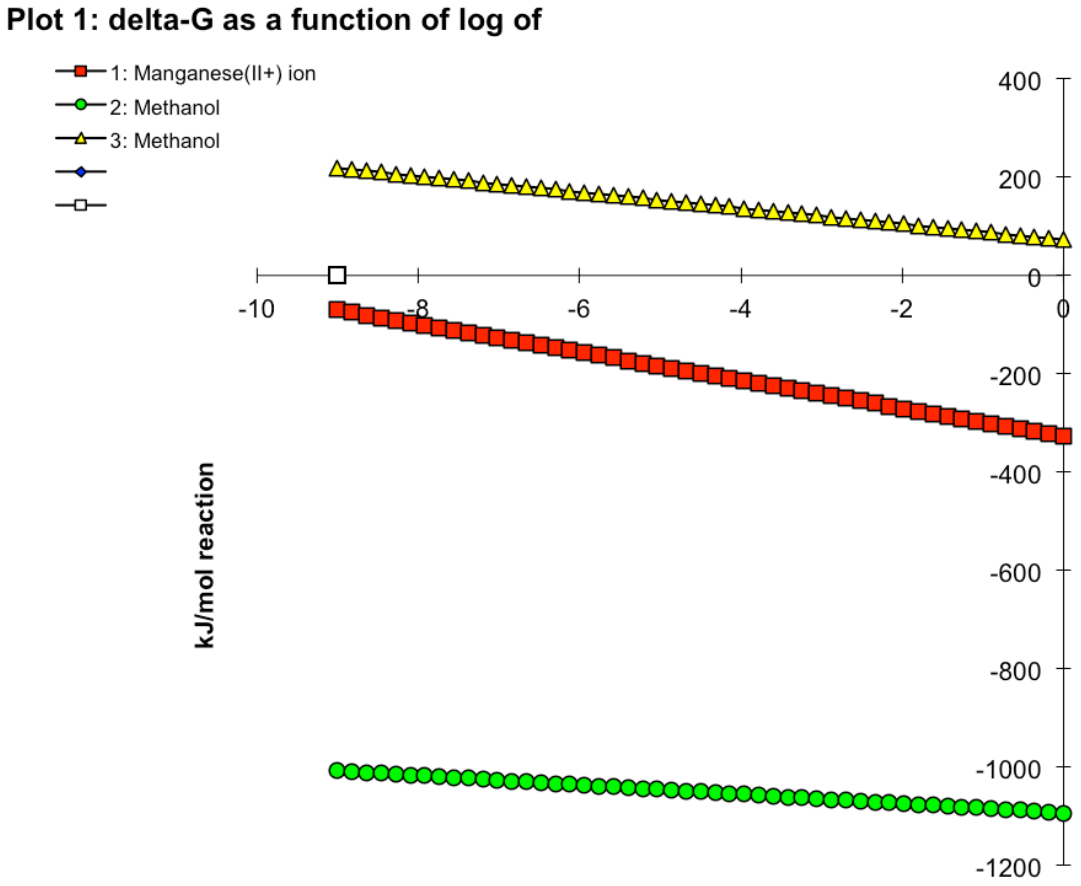

Fig. 4 Thermodyn calculation of energy yield/requirement for methanol oxidation (green), manganese oxidation (red) and methanol assimilation (yellow).

Despite the fact that hyphomicrobium were the dominant species in the liquid culture, different attempts of isolation failed. Hyphomicrobium were outgrown by the minor groups present in the liquid culture on selective plates under both aerobic and anaerobic conditions.

Some background species from tarmac enrichment were able to produce a diffusive pigment that turned agar plates green. This organism grew well on selective methanol plates and 5YE rich medium plates (Fig. 5). The green pigment was stable and apparent on both types of plates, but didn't show up in liquid medium. This pigment is fluorescent under UV light. The maximum absorbance is at around 420 nm (Fig. 6). The absorbance spectrum is similar to that of pyoverdine, a fluorescent siderophore produced by *Pseudomonas sp.* (Parker et al. 2004; Xiao & Kisaalita, 1995). Some strains of *Pseudomonas* have been reported to be methylotrophs (Pacheco et al., 2003). Cell morphology examined through wet mound was also compatible with that of *Pseudomonas* (Fig. 7). More detailed studies are needed to confirm the identity of the pigment and the organism.

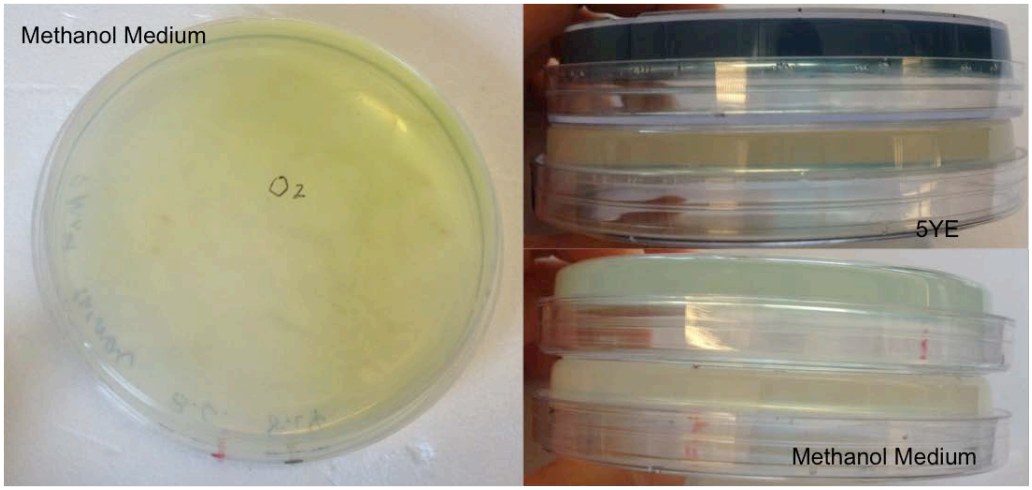

Fig. 5 Original inoculation of liquid medium onto selective agar plate for hyphomicrobium (left), restreaking on 5YE plate (top right), restreaking on methanol plate (bottom right)

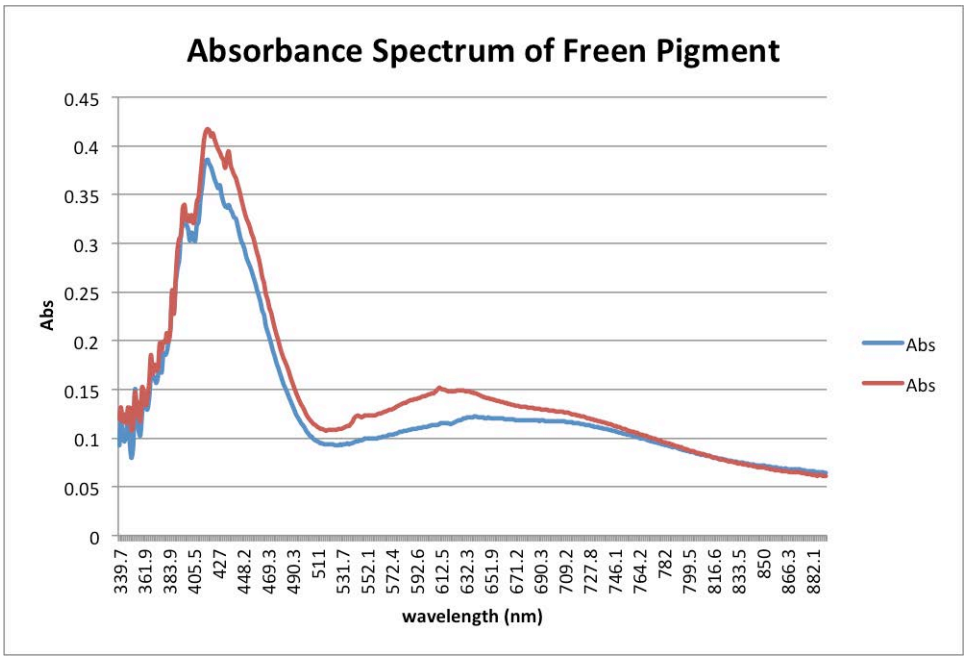

Fig. 6 Absorbance spectra of the green pigment

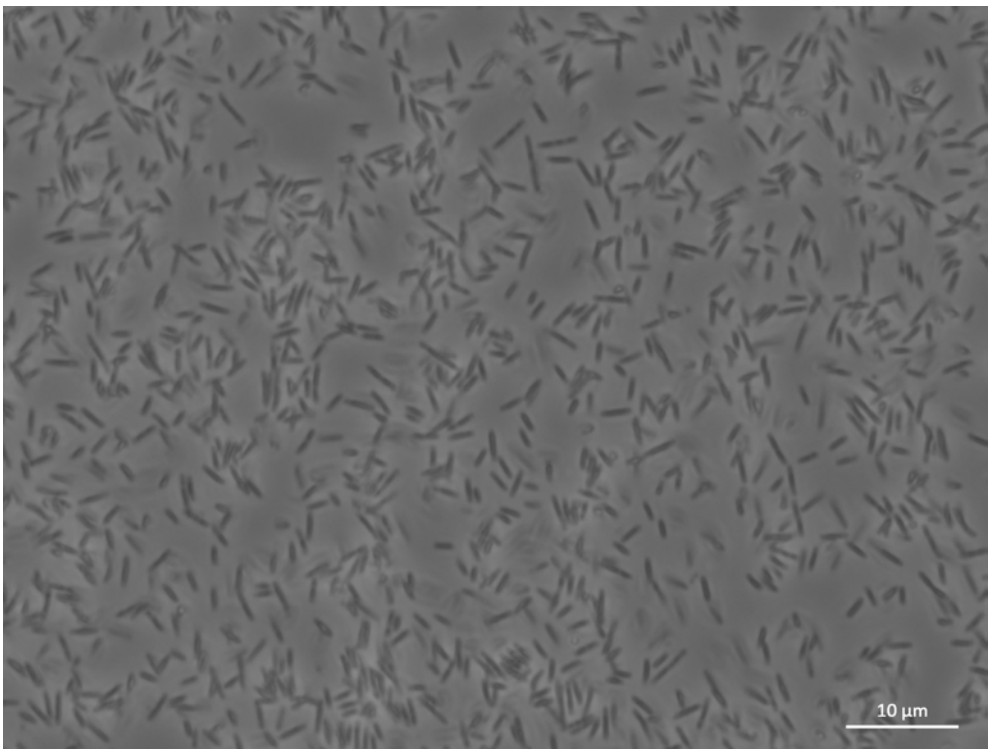

Fig.7 Wet mount made from green pigment producing colony

Conclusion

I was able to enrich for hyphomicrobium from various environmental samples, but attempts to isolate these organisms were not successful. Nitrate consumption were active in all enrichments. Rough estimates of denitrification rate were much higher the denitrification rate measured in similar environment (White & Reddy, 2003), suggesting great potential of these organism in removing nitrate from the environment. Differences in manganese concentrations did affect cell growth and respiration, but data obtained from this study were rather limited, hence not sufficient for clear conclusion. Caveats mentioned previously need to be addressed and improved to better understand these processes.

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