Assessment of Denitrifier Community Composition in Activated Sludge and Studies with *Paracoccus* sp. W1b Biofilm

Summary and Conclusion of the

Thesis Submitted to

The Maharaja Sayajirao University of Baroda

For The Degree of

Doctor of Philosophy
(Microbiology)

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July 2011

Summary

Chapter 2

Composition of denitrifying bacteria in activated sludge and the denitrifying activity of selected isolates

- Four different activated sludge samples were collected to investigate the denitrifying bacterial composition. The sludge samples were designated as DaS (Denitrifying reactor sludge from a fertilizer industry), GS (An aeration tank sludge from a fertilizer factory), NL (An aeration tank sludge from CETP) and WL (An aeration tank sludge from domestic sewage). The proximate composition of the sludge samples was also determined.
- Abundance of culturabledenitrifiers in the four different activated sludge samples tested, were in the range 2.28 X 10^8 to 2.8 X 10^9, as assessed by the MPN assay.
- Abundance of denitrifying functional genes in the sludge samples showed *nir*Sin the range of 10^4-10^5 per ml,*nos*Zwith 10^4-10^6 per ml and 16S rRNA gene in the range 10^9-10^{10} copy number per ml of sludge, as analysed by quantitative real-time PCR.
- The ratio of the *nos*Z and *nir*S genes of 0.5 in the NL sludge sampleindicated that it contains more number of denitrifiers truncated in the *nos*Z gene.
- The abundance of culturabledenitrifiers and the functional genes suggested high number of unculturable denitrifying bacteria to be present in the DaS, denitrifying reactor sludge sample.
- The cultivation of the denitrifying bacteria from the four different activated sludge samples revealed *Pseudomonas* sp. and *Alcaligenes* sp. to be numerically dominant.
- Denitrifying bacterial isolates, possibly truncated in the nitrate reduction step, were also obtained from the activated sludge samples.
- *nos*Z gene library was constructed from the fertilizer factory activated sludge samples (DaS and GS) yielding 114 clones from DaS sludge and 104 clones from the GS sample
- RFLP analysis of the clones with *Alu*I enzyme yielded 10 OTUs in DaS sample and 13 in the GS sample sludge.
- Rarefaction analysis showed that the sludge sample DaS was nearly reaching the asymptote, unlike the GS sample where increasing the clone number would have shown more diversity.

- The Shannon-Weiner and the Simpson's reciprocal diversity were high in GS sludge sample than DaS, refurbishing that the *nosZ* diversity is high in GS sludge sample.
- The translated protein sequences of the *nosZ* gene clones suggested Betaproteobacteria to be numerically dominant in the sludge.
- *Paracoccus*sp., *Comamonas* sp. and *Pseudomonasfluorescens*isolates showed efficient denitrification with negligible amount of nitrite accumulation, while *Diaphorobacter* sp., *Pseudomonas mendocina*, *Pseudomonas stutzeri* and *Brevundimonasdiminuta* accumulated nitrite during denitrification.
- The nitrate reduction rate was 1.5 times more than nitrite reduction in *Diaphorobacter*sp. D1, whereas ratio of the rates of nitrate and nitrite reduction in *Paracoccus* sp. W1b was nearly 1.0, as analysed by the resting-state denitrification kinetics.
- Increasing nitrate concentration upto 10 mMin the medium increased the nitrite accumulation in *Diaphorobacter*sp. D1, but not in *Paracoccus*sp.W1b indicating the presence of a sequential denitrification process in the former and a branched electron transfer during denitrification in the latter.
- *Diaphorobacter*sp. D1 was unable to denitrify at high nitrate concentrations from 1M, but *Paracoccus*sp.W1b could denitrify even upto 2M nitrate.

Chapter 3

Characterization of *Paracoccus* sp. W1b biofilm

- Brightfield and scanning electron microscopy confirmed biofilm formation by *Paracoccus* sp. W1b on polystyrene slides.
- The Plackett-Burman design was shown to be useful for detecting the influence of nutrients on biofilm formation, and the nutrients were also shown to affect the architecture of biofilm.
- In the Plackett-Burman experiment, higher concentrations of succinate, Mg^{++}, Ca^{++} and Mn^{++} enhanced biofilm formation, whereas higher concentration of iron decreased biofilm formation of *Paracoccus* sp. W1b.
- Confocal image quantification of the biofilm formed by *Paracoccus* sp. W1b at high succinate concentrations tested, showed more roughness with high surface to biovolume ratio. The data also suggested a possible production of increased EPS with high succinate concentration.

- Higher Mg^{++} or Ca^{++}concentrations of 10 mM in the medium, induced cohesion of biofilm cells, but contrasting biofilm architectures were detected. Biofilm with subpopulations of pillar-like protruding cells were distributed on a mosaic form of monolayer cells in medium with 10mM magnesium, while10mM calcium induced a dense confluent biofilm
- Denitrification activity was 5.9 and 6.3 folds increased respectively in the magnesium and calcium induced biofilm of *Paracoccus* sp. W1b.
- Chelatortreatment of various biofilm ages indicated that divalent cations are important in the initial stages of biofilm formation of *Paracoccus* sp. W1b.
- EDTA treatment of the magnesium-induced biofilm of *Paracoccus* sp. W1b indicated the presence of subpopulations which was confirmed by the FAME analysis, where the composition of the cellular fatty acids were different in the pillar-like cells from that of the mosaic monolayer.
- The nitrogenous oxides, nitrate, nitrite and nitric oxide at various concentrations did not affect the *Paracoccus* sp. W1b biofilm significantly.

Chapter 4

Influence of carbon sources on the biofilm community grown in a 1L laboratory-scale bioreactor in denitrifying conditions

- Acetate-fed biofilm community showed the highest denitrifying activity with an emergent biofilm structure showing a high thickness and diffusion distance.
- Glucose-fed biofilm community accumulated 213% more ammonium than the influent including accumulation of nitrite was observed, although 99% nitrate was reduced.
- Methanol-fed biofilm accumulated high nitrite during nitrate removal and formed a confluent biofilm without characteristic voids.
- Ethanol-fed biofilm showed relatively higher ratio of denitrifiers and a biofilm of lower thickness and diffusion distance was formed.
- DGGE analysis showed *Pseudomonas* sp. to dominate the acetate and ethanol-fed biofilm, while *Enterobacter* sp. and *Methylobacillus* sp., dominated glucose and methanol biofilms respectively.
- FISH analysis revealed *Pseudomonas* sp. to dominate the biofilm community, possibly due to the colonization of the substratum surface at the early stage of the biofilm development.

- Increasing nitrate concentrations in the influent of the reactor increased the abundanceof *Paracoccus* sp. relative to *Pseudomonas* sp. However, *Pseudomonas* sp. was founf to dominate the substratum surface.

Conclusion

Biological denitrification is used widely to remove high nitrate concentrations from the wastewaters. Higher nitrate concentrations are produced by some fertilizer, chemical and explosive industries. When these nitrates are untreated and released into the environment, it causes eutrophication of lakes, or the nitrate percolates into the drinking water. Consumption of high level of nitrates causes various diseases including methemoglobinamia in the infants. The nitrates are removed by different processes like the activated sludge process (suspended growth process) or the biofilm-based reactors (attached growth process). However, there is very little understanding of the microbiology in these processes although the degradation job is done by these organisms, and more importance is generally given to the engineering design of the reactor. Knowing the denitrifying community composition of these nitrate removal processes will reinforce a different perspective of the reactor design to increase its efficiency. A top-down to bottom-up approach was used in this study, where the denitrifying bacterial community in different activated sludge were assessed and single population biofilm as well as community biofilm structure of denitrifying reactors were analysed with an attempt to correlate it with activity.

The denitrifying bacterial composition in the four different activated sludge samples were studied by both culture-dependent and independent approaches. *Pseudomonas* sp. and *Alcaligenes* sp. are found to be numerically dominant by the culturable methods, whereas the culture independent techniques targeting the *nos*Z gene shows betaproteobacteria to dominate the sludge environment. Denitrification was observed to vary among the cultures isolated from sludge, with some accumulating nitrite while reducing nitrate, and the others not accumulating. Difference in denitrification pattern among the cultivated bacteria, and presence of truncated denitrifierswith high numbers in the sludge as observed in this study could possibly be one of the reasons for inefficient nitrate removal and accumulation of intermediates during denitrification in wastewater treatment processes.

Denitrification studies between two isolates *Diaphorobacter* sp. D1 and *Paracoccus* sp. W1b, indicated two different kinds of electron transfer to occur in the bacteria. One being a sequential like the *Diaphorobacter* sp. D1, which accumulates nitrite at higher nitrate concentrations, and a branched electron transfer like the *Paracoccus* sp. W1b, which denitrifies efficiently without accumulation of the intermediates. The branched electron transfer strategy also helps the organism to tolerate higher nitrate concentrations, as in the *Paracoccus* sp. W1b.

Biofilm studies on *Paracoccus* sp. W1b showed that the nutrients influence the biofilm formation, as well as its architecture significantly. Plackett-Burman statistical design was used to screen the effect of various nutrients on the biofilm formation, which showed high concentrations of succinate, Mg^{++}, Ca^{++} and $Mn^{++}$to enhance biofilm formation, whereas higher concentration of iron decreased biofilm formation. The divalent cations and succinate were also observed to modulate the biofilm architecture. Moreover, the Mg^{++} and Ca^{++}-induced biofilms, though increased biofilm formation by the cohesion of cells, formed contrasting architectures with subpopulations of pillar-like protruding cells distributed on a mosaic form of monolayer cells in the former andthe latter induced a dense confluent biofilm. However, the subpopulations also differed in the fatty acid composition showing diversification in the *Paracoccus* sp. biofilm. Chelatortreatment of various biofilm ages indicated that divalent cations are important in the initial stages of biofilm formation and the results obtained for *Paracoccus* biofilm also seems apparent for the ecological adaptation model proposed by Klausen et al (2006).Increased biomass and thickness of the biofilm, as induced by magnesium and calcium, increased the denitrification activity, and this could possibly be exploited in nitrate removal processes to provide anoxic conditions for increasing the denitrifying efficiency.

A laboratory-scale biofilm reactor was operated with different carbon sources in denitrifying conditions because exogenous carbon sources, which act as electron donors are provided for denitrification to occur in nitrate removal processes. This study suggests that acetate as a sole carbon source is efficient in nitrate removal by denitrification. Glucose as a carbon source supports the growth of nitrate ammonifying bacteria, which compete with the denitrifiers in anoxic zones leading to the accumulation of nitrite and ammonium. Though methanol is used widely for nitrate removal purposes, the methanol-fed biofilm accumulated high nitrite. Ethanol supports the growth of denitrifying bacteria in comparison to the nitrate ammonifiers and shows higher specific denitrification activity, however, the process of nitrate removal is

relatively slow. *Pseudomonas* sp. dominated the biofilm community in presence of the different carbon sources used, possibly because of its strategy of colonizing the substratum surface from the initial period of biofilm development. Increasing the nitrate concentration increased the population of *Paracoccus* sp., however the *Pseudomonas* sp. were found to be adhered to the substratum refurbishing that they colonize the substratum efficiently.

Denitrification studies with pure cultures have been generally carried out in *Paracoccusdenitrificans, Pseudomonas stutzeri*and*Alcaligenesfaecalis*. Nevertheless, this study found betaproteobacteria to dominate the sludge samples and especially the Comamonadaceae family. It is envisaged that further studies with more members from this group would provide better insight in the process design. Also, an extensive screening for enumeration of truncated denitrifiers in the sludge environments would help to model out their significance in the nitrate removal processes and the impact they have on the nitrogen cycle. This study also showed that the nutrients play an important role in determining the structure of biofilms, which importantly influence the activity. Thus, it would be interesting to develop statistical designs like Plackett-Burman, for high thoroughput screening of environmental impacts on biofilm structure to activity for various applications, including monitoring of substrates qualitatively and quantitatively to improve reactor efficiency in wastewater treatment processes.