Development of a microbial process leading to the conversion of ammonia to molecular nitrogen: Characterization and optimization of the process

Summary And Conclusion Of

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Guided by

Prof. Anjana Desai

Submitted by

Radhika S. Yadav

Department of Microbiology and Biotechnology Centre
Faculty of Science
The Maharaja Sayajirao University of Baroda
Vadodara – 390002, Gujarat, India

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SUMMARY

Chapter 2a

Comparison of polymorphism in ammonia monooxygenase and hydroxylamine oxidoreductase genes for analyzing *Nitrosomonas* spp. diversity

- Amongst the 22 samples, 6 samples DnrA, DnrB, CETP, DN, N and PF showed higher nitrite production.
- Presence of AOB in the 6 enriched samples was confirmed by amplifying *amoA* gene.
- Twelve kinds of isolated red colored colonies were obtained. These contained heterotrophs but only one kind of autotroph per colony.
- *Nitrosomonas* spp. were observed to be present in 11 of the 12 colonies based on 16S rRNA gene cloning and sequencing.
- *amoA* gene fragment digested with *Hhal* and *HaeIII* restriction enzyme showed 4 distinct patterns with resolution similar to 16S rRNA gene fragments from the same AOB.
- *hao* gene fragment when digested with *MspI* restriction enzyme showed only 2 patterns and therefore a more sensitive method was required to get proper resolution with *hao* gene.
- Through SSCP analysis *amoA* and *hao* gene fragments were resolved into six and four groups respectively which were statistically analyzed and showed significant difference with P value < 0.001.
- Overall 53% resemblance was observed between both the genes in fingerprinting the enriched AOB.
- HAO activity staining was used for the first time for differentiating AOB.
- Validation experiments proved that the obtained bands were of hydroxylamine oxidoreductase.
- Zymogram pattern HAO enzyme was analyzed and compared with *amoA* and *hao* gene fragment SSCP and showed 61.5% and 46.1%
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similarly with *amoA* and *hao* gene fragments SSCP respectively in resolving AOB.

- Simpson’s index of diversity was calculated for the three methods and was found to be 0.64, 0.85 and 0.68 for HAO zymogram, *amoA* gene fragment SSCP and *hao* gene fragment SSCP respectively whereas the concerted use of the three methods could differentiate the 13 AOB with Simpson’s index of diversity 0.95.

- The three methods used to differentiate *Nitrosomonas* sp. followed the following order: *amoA* gene fragment SSCP > *hao* gene fragment SSCP > HAO activity staining.

- Better primers are required to be designed for *hao* to be used analogous to *amoA* gene fragment as a molecular marker for identifying AOB.

Chapter 2b

Assessing *hao* as a molecular and phylogenetic marker in comparison with *amoA* and 16S rRNA genes for analyzing autotrophic Ammonia Oxidizing Bacteria

- 16S rRNA, *amoA* and *hao* gene fragments considered in the present study, used to test *hao* as a phylogenetic and functional marker, were such that all three genes were obtained from the same AOB. These were obtained from NCBI as well as those obtained earlier in Chapter 2a.

- Phylogenetic trees constructed using the three genes showed similar tree topologies which were statistically analyzed and paired t-test was applied to the Shannon’s index of diversity. As the values obtained by the t-test were less than the table value and p-value for the two-tailed test were high null-hypothesis that there is no significant difference in the diversity of AOB with respect to the three gene pairs, was considered true.
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- Magnitude of the genetic variation in AOB was studied with respect to the three genes and it was found that rates of transition substitutions were more than transversion in 16S rRNA than amoA and hao genes. Ti/Tv rate ratios and p-distances calculated for the genes indicated lower sequence divergence in 16S rRNA gene than amoA and hao genes.
- Correlation plots based on sequence similarity showed co-evolution of amoA and hao genes with linear regression $r^2$ value 0.9. This was further confirmed by Pearson’s correlation coefficient $r$ 0.949 with p value <0.001 for the two genes.
- Patterns of mutations were also similar for amoA and hao genes dN:dS ratio were found to be 1.92 and 1.56 respectively for amoA and hao genes indicating positive Darwinian selection was going on in both the genes.
- 70.5% mutations observed in the hao gene were nonsynonymous mutations therefore effect of these mutations was checked on the structure of the protein by comparing all the structures with HAO of *Nitrosomonas europaea* pdb ID: 1FGJ
- Their RMSD values were between 0 to 0.04 indicating no significant variation in the structure of the proteins amongst *Nitrosomonas* spp. but distinct differences were observed in the structure of *Nitrosococcus oceanii* HAO.

Chapter 3

Identifying physiological significance of heterotrophs co-existing with autotrophic Ammonia Oxidizing Bacteria in an ammonia oxidizing colony

- Heterotrophs were found to be coexisting with autotrophs in isolated colonies.
- These were found to be *Pusillimonas sp.*, *Acidovorax sp.*, *Acromobacter sp.*, *Janibacter sp.*, *Alcaligenes sp.*, *Sphingopyxis granuli*, *Mezorhizoium*
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sp. R2, Thermomonas sp. and Castellaniela defragnans through 16S rRNA gene cloning and sequencing.

➢ DnrA in which such association was observed for more than one year was used to unravel the mechanism of coexistence between the autotrophs and the heterotrophs.

➢ Three kinds of heterotrophs Pusillimonas sp., Acidovorax sp., and Janibacter sp., and an autotroph Nitrosomonas eutropha (designated as Nitrosomonas sp. RA) were found in an isolated red colored colony.

➢ Pure heterotrophs failed to grow alone in the inorganic media which showed their dependence on Nitrosomonas sp. RA in the said media.

➢ Pure heterotrophs could grow and utilize ammonia as nitrogen source in the presence of organic carbon like acetate. Thus, higher ammonia removal was observed in the system.

➢ Serial dilution was carried to separate AOB and heterotrophs. Growth of autotrophs was observed till 10\(^{-9}\) dilution but with the associated heterotrophs.

➢ Heterotrophs were not eliminated even in the presence of copper upto 100 \(\mu\)M concentration.

➢ Growth of autotrophs was observed upto 20 ppm mercury where as growth of heterotrophs was not observed beyond 8 ppm mercury concentration.

➢ Heterotrophs were observed in SEM image of Nitrosomonas sp. RA exposed to 20ppm mercury, suggesting it to be in the dormant state in the presence of mercury. The heterotroph could be resuscitated by giving heat shock and was found to be Pusillimonas sp.

➢ Longer lag phase was observed in the growth of Nitrosomonas sp. RA with reduction in 21.2% in nitrite production.

➢ In the absence of heterotrophs growth of autotrophs did not occur till iron concentration reached 10 \(\mu\)M Fe\(^{2+}\), whereas in the presence of heterotrophs, growth of autotrophs occurred even without providing an external iron source.

➢ Pusillimonas sp. showed highest siderophore production amongst the heterotrophs.
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- The siderophore produced by *Pusillimonas* sp. was found to be hydroxamate type and supplied exogenously at 1 mg/ml concentration to the autotrophs where increase in growth was observed in the presence of the externally supplied siderophores.
- In the absence of heterotrophs, 200 μg/ml siderophore concentration maximally supported the growth of autotrophs.
- Further, the growth and uptake of exogenously supplied siderophores were checked in the presence of heterotrophs. Growth of autotrophs was more in the presence of heterotrophs even in the absence of exogenously supplied siderophores implying sufficient siderophores were been provided by the heterotrophs to support the growth of autotrophs.
- Amplification of TonB dependent siderophore receptor gene showed presence of siderophore uptake system in *Nitrosomonas* sp. RA and increase in the growth of the autotrophs in the presence exogenously supplied siderophores suggested functionality of the siderophore receptors in *Nitrosomonas* sp. RA.
- Increase in SMP levels released by the autotrophs in the presence of mercury suggest dependence of heterotrophs on autotrophs for their organic carbon requirement.
- Nitrite at higher concentration was shown to inhibit the growth of autotrophs.
- Nitrite could be utilized by the heterotrophs in the presence of acetate as carbon source, this would increase the growth of autotrophs as they would be relived from nitrite inhibition.
- Thus, a mutual interdependence amongst the two groups of organisms was established.

Chapter 4

Development of a simultaneous partial nitrification, anaerobic ammonia oxidation and denitrification (SNAD) bench scale process for removal of ammonia from effluent of a fertilizer industry
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- PF-anammox obtained from the paddy-field soil sample and N4-anammox obtained from municipal wastewater treatment plants showed highest anammox activity with ammonia and nitrite removal in the ratio similar to the reported stoichiometry for anammox bacteria (1:1.33). Gas formed by the system was also identified to be nitrogen through gas chromatography.
- Addition of hydrazine in the system led to increase in anammox activity of both N4 and PF-anammox.
- Amplification of Planctomycetes and anammox specific amplification confirmed presence of anammox bacteria in both the system.
- These were maintained in small rubber tubes.
- Ammonia removing ability was checked under anoxic conditions by mixing enriched AOB and anammox biomass in 1:5 ratio using synthetic effluent. Gas formed in the system was confirmed to be nitrogen through GC. Further, seed culture was developed by mixing PF and N4 AOB-anammox bacterial biomass in 1:1 ratio.
- An upflow SNAD type bioreactor was run continuously for 125 days. 98.9% removal of ammonia from effluent of a fertilizer company was achieved using the developed system.
- Molecular analysis of the biomass carrying out the anammox activity showed presence of nitrifiers, denitrifiers in the upper part of the reactor where presence of anammox bacteria and denitrifiers was also observed in the lower part of the reactor.
- AOB were dominant in the upper part of the reactor whereas denitrifiers and anammox bacteria were majorly found in the lower anoxic region of the reactor.
- Presence of budding coccoidal shaped cells also suggested presence of anammox bacteria through SEM.
- Twenty eight distinct sequences suggesting 28 different kinds of bacteria mainly nitrifiers, denitrifiers and Planctomycetes were found to be coexisting in the reactor.
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➢ Presence of flagellated cells observed through SEM would help in forming microbial aggregates and increase settling ability of the sludge in the reactor and thus prevent the entangled cells from getting washed off from the reactor.
➢ Food to microbe ratio calculated was found to be 0.62 day\(^{-1}\) which was indicative of endogenous growth of microorganisms and have better settling ability.
➢ A microbial system was thus developed converting ammonia to molecular nitrogen from effluent of a fertilizer company.

Chapter 5

Kinetics of ammonia removal in a 5.3 L open reactor: An aerobic solution to high strength ammonia containing wastewater of a fertilizer industry

➢ PF-NOB showed highest nitrite oxidizing activity amongst the enriched NOB.
➢ Microorganisms identified in the NOB enrichment culture were *Nitrobacter winogradsky* Nb-255 (95% identity), Uncultured *Sphingomonas* sp. clone Plot18-2H12 (96%), Uncultured *Acidobacteria* bacterium clone 34 (95%).
➢ Specific growth rate constants for the PF-NOB and PF-AOB enriched biomass were 0.384 and 1.24 with doubling time of 1.8 and 0.56 days respectively.
➢ PF-AOB and PF-NOB were mixed in 1:4 proportion to make the seed culture for the reactor.
➢ The reactor was run in the batch mode for the first 30 days with increasing effluent concentration.
➢ Biomass in the reactor got acclimatized to high ammonia concentration, with reduction of around 90% ammonia and 82.68 mg/L biomass getting accumulated by the end of the run in the batch mode.
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- Beyond 56th day steady state was achieved and 99% reduction in ammonia was achieved by the end of the run.
- Nitrite levels reached permissible limits (0.7 ppm) with no detectable nitrate.
- Settler efficiently retained the microorganisms in the reactor.
- A linear correlation existed between the ammonia added and removed from the reactor with high ammonia conversion efficiency.
- Heterotrophic aerobic denitrifiers were co-enriched with AOB and NOB hence the seed consortium mainly consisted of AOB, NOB and denitrifiers. These would play a major role in the removal of ammonia from the industrial effluent aerobically.
- COD present in the reactor would support the growth of denitrifiers in the reactor.
- Aeration and agitation were kept in the on and off mode for 24 h alternately. Thus, high dissolved oxygen in the on mode would favor nitrification whereas the off mode would favor aerobic denitrification.
- High HRT in the system provided sufficient time for both nitrification and aerobic denitrification to occur simultaneously.
- Higher sludge retention time resulted in larger floc size and density which is suitable for simultaneous nitrification and denitrification to proceed steadily.
CONCLUSION

Ammonia is released in high concentrations in effluent discharged from agriculture based industries and food processing industries. Ammonia at higher concentrations causes eutrophication and oxygen depletion in the receiving water bodies affecting entire aquatic life and causing numerous health hazards. Several reactors have been developed for the treatment of ammonia from industrial effluent. Design of reactors has been given great importance since a long time in the treatment of ammonia, but performance of the reactor greatly depends on the microbial community carrying out the reactions. Hence, knowledge regarding the microbial community composition would help in improving the stability and performance of the reactor. The study aimed, in developing a microbial process for the treatment of ammonia containing effluent of a fertilizer industry, identifying types of microorganisms involved and their diversity and also understanding the ecophysiological significance of the specific groups of organisms found to be coexisting in the process.

Most of the enriched autotrophic Ammonia Oxidizing Bacteria (AOB) were found to have identity with *Nitrosomonas* spp. Diversity studies of these isolated AOB was carried out in two parts a) sequence independent study b) sequencing based study. Hydroxylamine oxidoreductase was explored as a molecular and functional marker in comparison with *amo* gene in differentiating the obtained AOB through SSCP analysis for the first time. The novel use hydroxylamine oxidoreductase (HAO) zymogram showed variation in the studied AOB. These three methods had differentiated *Nitrosomonas* spp. with resolution in the following order; *amo* gene > *hao* gene > HAO enzyme zymogram. Hence, amongst the three novel techniques used in the present study to differentiate AOB belonging to a single genus, *amo* gene fragment SSCP exhibited highest potential and for *hao* gene to be used at par with *amo* gene SSCP in resolving AOB, better primers are required to be designed such that *hao* can be amplified from all AOB. HAO enzyme zymogram technique being simple to perform can be used as a preliminary method to study diversity. The concerted use of these polyphasic
 approaches provided a better understanding of their pivotal role in metabolic and functional diversity of the organisms involved in the process.

Sequence based analysis of *hao* gene as a molecular and functional marker was carried out in comparison with *amoA* gene (a reported functional marker) and 16S rRNA gene (a conventional phylogenetic marker). Phylogenetic trees that were constructed using the three *amoA*, *hao* and 16S rRNA gene sequences were analyzed statistically and were found to have significantly similar topologies. Analysis of AOB genes carried out for the first time showed bias towards transitions over transversions. Higher sequence divergence in functional genes like *amoA* and *hao* compared to 16S rRNA gene was indicative of higher evolutionary rate of the genes compared to 16S rRNA. *amoA* and *hao* gene fragments showed similarity in synonymous and nonsynonymous substitutions pattern. HAO structural analysis carried out revealed that variation in amino acid sequence caused by the nonsynonymous substitutions did not cause major variation in its structure. Co-evolution of *amoA* and *hao* genes involved in the oxidation of ammonia to nitrite and their correlation with 16S rRNA gene were examined for the first time in the present study. Co-evolution of *amoA* and *hao* genes supported that *hao* can also be used at par with *amoA* as an alternative phylogenetic marker in studying diversity and evolution of AOB.

AOB are extremely slow growing with very low growth yield which make their isolation not just difficult but also time consuming. Contaminating heterotrophs tend to build up rapidly in association with AOB, even without an external supply of organic carbon. Intriguingly, it has been reported that aerobic ammonia oxidation proceeds more rapidly along with the contaminating heterotrophs. Knowledge regarding the functional significance of such association is far from complete. Present study reports for the first time a systematic analysis of the interaction between AOB and heterotrophs found closely associated in an ammonia oxidizing colony. *Nitrosomonas* sp. RA and 3 heterotrophs present in a single colony were dependent mutually on each other for growth. A system was developed where in growth of heterotrophs was inhibited by mercury without significantly affecting that of
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*Nitrosomonas* sp. RA thereby a system containing *Nitrosomonas* sp. RA without physiologically active heterotrophs was obtained. This is the first report where *Nitrosomonas* sp. RA has been demonstrated, through bioassay, to utilize siderophores produced by associated heterotrophs towards meeting its iron requirement. Presence of siderophore receptor gene in *Nitrosomonas* sp. RA was shown by the amplification of TonB-dependent siderophore receptor gene fragment and growth of AOB in the presence of exogenously supplied siderophore confirmed it to be functional in the AOB. SMP produced by *Nitrosomonas* sp. RA supported growth of heterotrophs in the inorganic media. Organic carbon sequestration by heterotrophs would consequently facilitate the growth of *Nitrosomonas* sp. RA as organic carbon is reported to inhibit growth of autotrophs. Nitrite produced by the AOB could be utilized by the heterotrophs which in turn would increase the growth of AOB by removing nitrite inhibition. A mutual interdependence amongst the two groups of organisms for growth thus could be established. Mutual interactions and interdependence between different groups of microorganisms as analyzed in the present study are often observed in natural environment and are extremely important for the proper stabilization and functioning of the microbial community.

With the rising demand in environment protection various new technologies have been designed for the treatment of high ammonia containing effluent from different industries. Some of these high ammonia containing effluent also contain low COD like the one in the present study where Simultaneous Nitrification, Anammox and Denitrification (SNAD), which has not been explored in the treatment of effluent from fertilizer industry so far, was applied. The SNAD type bioreactor developed could efficiently remove ammonia from effluent with C:N ratio - 0.066 (much less than that used in the SNAD processes reported till date) and was run continuously for 125 days. 98.9% ammonia removal from the effluent was achieved. Coexisting nitrosifiers, anammox bacteria and denitrifiers were confirmed to be the major microorganisms that were responsible to carry out the reaction in the reactor without supplementation of external organic carbon and without
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accumulation of nitrite or nitrate. Qualitative and quantitative analysis of the biomass generated revealed dominance of AOB in the upper oxic zone of the reactor while anammox followed by denitrifiers dominated in the lower anoxic zone of the reactor. Physiological and molecular studies strongly indicated presence of anammox bacteria in the anoxic zone of the SNAD reactor.

Full scale reactors do not entirely provide uniform environments in the reactor. There are pockets in the reactor that are anoxic in nature and some others that are completely aerobic. It is therefore important to study the mechanism of ammonia removal by the microorganisms and reactions going on in the reactor with varying oxygen conditions. A bench scale reactor with intermittent aeration and agitation was developed and was run for 75 day continuously. Present study demonstrated significance of nitrification and aerobic denitrification occurring simultaneously in a single reactor without addition of organic carbon. The system developed could run with similar nitrification and denitrification rate such that nitrite and nitrate did not accumulate in the system and 99% reduction in ammonia levels was achieved. This system has the benefit of occurring in a single reactor without addition of organic carbon but the only limiting factor for its application in full scale reactor system would be the aeration costs.