Summary

Chapter 2: Selection of special biofilm forming denitrifying bacteria from activated sludge

- Isolation of denitrifying bacteria was carried out from enriched denitrifying Winogradsky columns. Denitrifying Winogradsky columns were developed using potassium nitrate and sodium acetate as nutrient sources and activated sludge from domestic sewage as inoculum source.
- Total 33 morphologically different isolates were obtained, which were further screened based on their nitrate reduction ability, denitrification efficiency, amyloid production, biofilm forming ability and nitrite accumulation.
- Out of 33 isolates, 24 isolates showed nitrate reduction ability. Out of 24 isolates, 10 isolates showed the highest denitrification efficiency, i.e., above 80 % and good biofilm forming ability. 12 isolates that showed nitrite accumulation during denitrification studies were discarded.
- Finally, five isolates were selected for further studies based on 1) denitrification efficiency 2) amyloid and biofilm formation and 3) lack of nitrite accumulation.
- Production of surface amyloid protein by the selected five isolates was confirmed by Thioflavin T staining.
- BLAST analysis of 16S rRNA gene sequencing of five selected isolates R4, V5, V9, V11 and V14 revealed 99 %, 99 %, 100 %, 100 % and 99 % sequence similarity with *Diaphorobacter* sp., *Pannonibacter* sp., *Thauera* sp., *Pseudomonas* sp. and *Thauera* sp., respectively.
- Results of the growth curve and co-culture growth inhibition assay suggested that the selected five isolates did not show any mutually harmful effect on each other and can be part of a consortium, which was termed consortium DC5 (5 membered consortium from denitrifying column).
- Flask level denitrification studies with consortium DC5 showed 100 % nitrate reduction with initial nitrate concentration of 200 mg L^{-1} in 10 h without accumulation of nitrite.

- Nitrogen gas production by all the isolates was confirmed by comparing the peak at 1.373 with standard nitrogen gas, which suggested that all the selected isolates were denitrifiers.
- Nitrate removal kinetic constants showed that compared to individual isolates, consortium DC5 showed the highest nitrate removal efficiency compared to individual isolates.
- Various parameters optimized by OFAT approach for biofilm formation and denitrification efficiency of consortium DC5 were as follows:

Parameters	Amount
Magnesium Chloride	5 mM
Calcium Chloride	5 mM
Potassium hydrogen phosphate	0.5 mM
Inoculum size	1 %
Carbon source	Sodium acetate (0.6 %)

Chapter 3: Performance of bench scale Moving Bed Biofilm Reactor (MBBR) with the consortium of the selected biofilm forming denitrifying bacteria and its evaluation.

The OFAT dMBBR studies results were as follows:

- C/N ratio of 0.3 showed 100 % of nitrate (620 mg L^{-1}) and COD below permissible range of 250 mg L^{-1}.
- HRT of 3 h showed 100 % nitrate removal efficiency and COD below permissible range.
- 20 % filling ratio of Pall ring carriers showed the highest nitrate removal efficiency, COD reduction below permissible range and the biofilm developed on carriers contained highest EPS components compared to Fluidized media and Kaldness media.
- Consortium DC5 was able to reduce nitrate up to 2400 mg L^{-1} with the optimized parameters summarized below.

- **Optimized parameters for dMBBR were as follows:**

C/N ratio	0.3
HRT	3
Carriers	Pall ring
Filling ratio of carrier	20 %

- The kinetic constants of nitrate removal KB (saturation value constant) and Umax (maximum nitrate removal rate constant) were 17.10 mg L^{-1}.day and 20.54 mg L^{-1}.day, respectively.
- Results of comparative studies carried out with different modes were as follows:

Reactor mode	Nitrate removal (%)	COD
dMBBR with consortium DC5	100 % at 620 mg L^{-1}	Below permissible range (i.e. 250 mg L^{-1})
Suspended growth reactor inoculated with consortium DC5	60 % at 620 mg L^{-1}	Below permissible range (i.e. 250 mg L^{-1})
MBBR developed with activated sludge	56 % at 620 mg L^{-1}	Below permissible range (i.e. 250 mg L^{-1})
Control MBBR(without any inoculum)	38 % at 620 mg L^{-1}	Below permissible range (i.e. 250 mg L^{-1})

- Results of treatment of different industrial effluents with consortium DC5 in dMBBR were as follows:

Effluents	Nitrate removal (%)	COD removal (%)
Dye industry	75 %	60 %
Pharma industry 1	85 %	60 %
Pharma industry 2	100 %	60 %
Pharma industry 3	76 %	69 %
Domestic wastewater spiked with nitrate (200 mg L^{-1})	80 %	80 %

Chapter 4: Characterization of the biofilm produced by the selected bacterial isolates.

Metagenomic analysis studies of biofilm developed in continuous dMBBR for 300 days with acetate as the carbon source revealed the following.

- Proteobacteria was the most abundant phylum, followed by Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Cyanobacteria, Chloroflexi, Euryarchaeota, Deinococcus-Thermus and Verrucomicrobia.
- At both the genus and species level *Thauera* and *Thauera humireducens* and *Thauera* sp. MZ1T were the most abundant in all the biofilm samples.
- Principle component analysis (PCA) of data revealed that at phylum level Cynobacteria, Chloroflexi, Bacteroidetes, Verrucomicrobia, Firmicutes were positively correlated with each other.
- The exogenous carbon source preferred by the denitrifiers *Pseudomonas*, *Thauera* and *Azoarcus* was acetate, they were positively correlated with each other and negatively with *Paracoccus* which prefers methanol as the carbon source
- At the species level, *Thauera* sp. MZ1T, *Thauera humireducens, Thauera aromatic* and *Thauera* sp. K11, were positively correlated with each other.
- dMBBR biofilm was found to possess a high abundance of metabolic functions (57 %) followed by genetic information processing (16 %), environmental information processing (20 %) and cellular processes (7 %).
- Presence of genes involved in oxidative stress, osmotic stress, heat shock, detoxification stress, cold shock and acid stress suggested that biofilm developed with consortium DC5 was resistant to various stress responses.
- Various genes related to antibiotic resistance such as multidrug resistance efflux, Fluoroquinolones, Fosfomycin, Vancomycin, Strptothricin, Erythromycin, Methicillin resistance as in staphylococci and multidrug resistance clusters were annotated in the developed biofilm of dMBBR further reflecting on its sturdiness.
- Genes related to metabolism of toxic compounds such as copper homeostasis, mercury resistance, arsenic resistance, cobalt-zinc-cadmium resistance,

chromium, etc. were also annotated in denitrifying biofilm, which indicates its resilence.

- Various genes involved in nitrogen metabolism majorly involved in the denitrification process such as nap, nir, noz were abundantly present in the biofilm developed inside dMBBR, which was as is expected in high performing reactor.
- Genes involved in the Assimilatory Sulfate Reduction (ASR) pathway were abundantly present such as, Sat, CysND, CysC, CycH and CysJI, etc.
- Genes involved in hydrogenotrophic methanogenesis such as methanohydrogen dehydrogenase, fwdA, fmdA, metF, fae, frmB, ESD and fghA were abundantly present in denitrifying biofilms. Presence of these genes might be due to the anaerobic methane-driven denitrification process that directly utilizes methane as electron donor, which is abundant in biogas from anaerobic digestion.
- Genes involved in degradation of various xenobiotics were also abundantly present in denitrifying biofilm such as 1,1,1-Trichloro-2,2-bis (4-chlorophenyl)ethane (DDT) degradation, Benzoate degradation, Bisphenol degradation, Fluorobenzoate degradation, Dioxin degradation, Xylene degradation, Toluene degradation, Polycyclic aromatic hydrocarbon degradation, Chloroalkane and chloroalkene degradation, Aminobenzoate degradation, Nitrotoluene degradation, Styrene degradation, Atrazine degradation, Caprolactam degradation, Drug metabolism, Steroid degradation suggesting that biofilm developed in dMBBR has potential to degrade wide range of xenobiotic compounds present in wastewaters.

Chapter 5: Studies on most persistent and dominant denitrifying bacterium in continuously operated MBBR

As *Thauera* was found to be most persistent and dominant genus in metabolic studies. Results of studies with one of the members of consortium DC5 namely *Thauera* sp. V14 was as follows.

- *Thauera* sp.V14 showed 93 % auto-aggregation ability and 83.8 % hydrophobicity, which is important for the formation of biofilm in dMBBR.

- Higher incubation temperature 40°C increased amyloid production and swarming motility of *Thauera* sp.V14.
- *Thauera* sp.V14 showed 100 % denitrification efficiency within 72 h with an initial nitrate concentration of 765.89 mg L^{-1} without accumulation of nitrite and ammonia.
- In OFAT studies
    - Inoculum size above 7 % increased denitrification efficiency. Inoculum size 9 % showed highest biofilm forming ability of *Thauera* sp.V14.
    - 10 mM Calcium and 9 mM magnesium ions showed the highest biofilm formation whereas there was no significant effect on denitrification efficiency of *Thauera* sp.V14.
    - Stress agent 0.5 % NaCl showed highest denitrification efficiency and biofilm forming ability.
    - DMSO showed the highest denitrification efficiency at 0.05 % and no significant effect was observed on biofilm formation.
    - Ethanol showed the highest denitrification efficiency at 0.05 % whereas biofilm forming ability was highest at 0.5 % which was further decreased at higher concentration.
- Results of Plackett-Burman analysis revealed that ethanol and $CaCl_2$ showed significant effect on biofilm formation whereas peptone, DMSO and yeast extract showed significant effect on the denitrification efficiency of *Thauera* sp.V14.
- Purified amyloid, NaCl and $MgCl_2$ showed no significant effect on biofilm and denitrification of *Thauera* sp.V14.
- Results of Response Surface Methodology studies suggested that the response yielded a linear model as there was no interaction seen among the components for biofilm formation and denitrification efficiency of *Thauera* sp.V14. DMSO and $CaCl_2$ for biofilm and $CaCl_2$ for denitrification were found to be most significant components influencing the respective abilities of *Thauera* sp.V14 with p-values of < 0.05.
- 3 % DMSO and 0.5 % $CaCl_2$ were found to be most significant parameters affecting the biofilm formation and denitrification in RSM studies.

- Continuous MBBR studies with *Thauera* sp.V14 showed nitrate removal of 91 %, 90 %, 76 %, 66 % and 60 % amounting to 620, 744, 930, 1500 and 2400 mg L^{-1} of nitrate concentration and every time COD reduction was below stipulated permissible range i.e., 250 mg L^{-1}.
- FTIR analysis showed the presence of carbohydrates, proteins and lipid components in the carrier-associated biofilm developed in the dMBBR bioaugmented with *Thauera* sp.V14.
- SEM images of the carriers showed high bacterial density and biofilm was dominated by rod shaped bacteria on the carriers of dMBBR bioaugmented with *Thauera* sp.V14.