Conclusion

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Activated sludge process is the most widely used treatment system where the microbial communities are maintained as planktonic assemblages (flocs). However, the usage of attached-growth or biofilm reactors has increased over suspended growth systems (i.e., activated sludge systems) due to its conferred advantages such as low space requirement, operational flexibility, reduced hydraulic retention time, resilience to changes in the environment, increased biomass, resistance to dehydration, enhanced ability to degrade recalcitrant compounds and lower sludge production. Biofilm reactors run in different modes show mechanical failures, instability, need for frequent backwashing and moreover were not volume-effective. To overcome the operational problems faced by biofilm reactors, MBBR was developed which lacked these drawbacks and additionally possessed the advantages of both fixed film and suspended growth reactors. In MBBR, the biofilm grows on specifically engineered carriers that move freely in the reactor, making its performance highly effective. MBBR inoculated with activated sludge frequently exhibits poor reactor performance due to lack of functional microorganisms, as well as rapid start-up issues. However, the bioaugmentation of biofilm producing denitrifying bacteria in the MBBR has not only the advantages of rapid start-up and stable operation of the MBBR but also increases the functional population in the reactor. Moreover, the bioaugmentation of specific denitrifying bacteria in MBBR is a cost-effective and eco-friendly approach and promising alternative technique for the treatment of high nitrate containing wastewater.

For the development of special microbial seed, enrichment of denitrifying bacteria was carried out in Winogradsky column by using activated sludge as inoculum, since large number of denitrifying bacteria was reported to be present in activated sludge from wastewater treatment plants. Amyloids are extracellular surface proteins produced by bacteria that are important components of bacterial biofilms and are widely present in wastewater sludge. Therefore, it was envisaged that amyloid producing bacteria could be good biofilm forming bacteria. Finally five isolates selected upon screening based of biofilm forming ability and denitrification efficiency because biofilm is the key feature of MBBR and denitrification efficiency is an important process for nitrate removal. Denitrifiers that accumulated nitrite that is more toxic than nitrate leading to

further pollution were eliminated. A consortium was developed with five isolates which showed the highest denitrification efficiency, good biofilm forming ability with no nitrite accumulation. Selected five isolates identified as *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp.V9, *Pseudomonas* sp.V11 and *Thauera* sp.V14, comprised of consortium DC5. Thioflavin T staining giving bright green fluorescent cells under fluorescence microscope further confirmed amyloid production by the five isolates. The studies here lead to development of consortium DC5 with amyloid producing denitrifiers possessing good biofilm forming ability which was tested in further studies.

The literature on reactor studies show that microbial consortia are more effective compared to individual isolates in degradative performance. Stable coexistence within a single microbial consortium is a prerequisite for the construction of a microbial consortium. The growth curves of all the five isolates almost coincided with each other and the coculture assay suggested that the isolates did not harm each other and coexisted in mutual association. These properties of the isolates demonstrated the development of stable consortium. Flask level denitrification studies also concluded high nitrate removal efficiency and lack of nitrite accumulation, which is an important attribute for denitrifying consortium. Denitrifying consortium of selected five isolates was named as consortium DC5. Thus, results of flask level studies along with gas chromatography and kinetics studies demonstrated that all the isolates of consortium DC5 were denitrifiers and showed synergistic effect on each other to enhance nitrate removal efficiency. Nitrogen gas production by all the isolates and consortium DC5 was confirmed by comparing the peak at 1.373 with nitrogen gas as standard demonstrated that all the isolates showed complete denitrification process. Important parameters significantly affecting biofilm formation and denitrification efficiency that included $MgCl_2$, $CaCl_2$, K_2HPO_4, inoculum size and sodium acetate as an exogenous carbon source were optimized to further enhance the performance of the consortium DC5. Acetate was chosen as exogenous carbon source throughout the studies since enrichment studies were carried out with acetate and it is preferred by majority of denitrifiers.

Nitrate removal studies in dMBBR developed with consortium DC5 concluded that optimizing various process parameters such as C/N ratio, HRT, type of carriers, filling

ratio and nitrate loading improved dMBBR performance. Comparative studies of MBBR inoculated with consortium DC5 and reactors run in suspended growth mode or inoculated with activated sludge or a control reactor i.e., without inoculum again established that MBBR bioaugmented with consortium DC5 enhanced nitrate removal efficiency. It can be concluded from the comparative reactor studies that the dMBBR bioaugmented with consortium DC5 produced superior performance. Biotreatment of different industrial effluents with consortium DC5 pointed towards the versatility of the bioaugmented dMBBR with consortium DC5 as it was able to reduce nitrate and COD from various industrial effluents such as dye industry, domestic wastewater and pharma industries. These studies showed that consortium DC5 has potential in nitrate removal from effluents of varied composition.

To characterize the community structure and functional potential of the carrier associated biofilm, NGS approach was adopted. In whole metagenome sequencing of biofilm after 300 days of continuous operation of dMBBR *Thauera* emerged as the most abundant, most persistent organism. It also showed that *Thauera* spp. and *Pseudomonas* sp. flourished luxuriantly in the reactor after 300 days of operation; notably three out of five consortium DC5 members belonged to these two genera. It also enhanced enrichment of other denitrifying organisms. The functional potential of the biofilm developed in dMBBR demonstrated that genes involved in nitrogen metabolism, especially in the denitrification process were widely present in the developed biofilms emphasizing the dominance of denitrifiers in the reactor. Genes involved in amino acid, fatty acids, carbohydrate metabolism were highly abundant in the developed biofilm as they are involved in generation of electrons for the denitrification process. Abundance of genes involved in other metabolic pathways such as methane metabolism, sulfur metabolism and xenobiotic degradation were widely present in the biofilm. This might be attributed due to the feature of the denitrification process that it is often associated with the decomposition of organic matter under anaerobic conditions and can be used for the treatment of various wastewaters which contain organic pollutants such as nitrate, sulfate and various xenobiotic compounds. Overall metagenomic results revealed that inoculation of special microbial seed of consortium DC5 could maintain the high functionality of microorganisms in the reactor and consequently make the dMBBR

system more efficient for the treatment of nitrate containing wastewater. It is concluded here that a consortium like DC5 was better equipped with degradative abilities to deal with nitrate and other pollutants that may creep in variety of effluents. Broadly, it can be said emphatically that the bioaugmentation of reactors degrading environmental pollutants enhance their performance.

As mentioned above, *Thauera* was the most dominant and persistent organism in continuous dMBBR environment. *Thauera* has been also reportedly most frequently found in denitrifying reactors. Therefore, further investigations were accomplished with one of the consortium member *Thauera* sp. V14. Firstly it was found that *Thauera* sp. V14 showed increased amyloid production and swarming motility at high temperature, which are important parameters for biofilm formation. The Plackett-Burman analysis studies showed ethanol, $CaCl_2$, peptone, DMSO and yeast extract played an important role in biofilm formation and denitrification efficiency of *Thauera* sp.V14. Further, results of Response Surface Methodology (RSM) 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 $CaCl_2$ were found to be most significant components influencing biofilm and denitrification of *Thauera* sp.V14. DMSO and $CaCl_2$ both are known to elicited biofilm formation in many bacteria. *Thauera* sp.V14 alone showed 100 % denitrification efficiency with an initial nitrate concentration of 765 mg L^{-1} at flask level. In continuous MBBR study, it showed 91 % and 90 % of denitrification efficiency with an initial nitrate concentration of 620 and 744 mg L^{-1} respectively within 3 h of HRT. High copy number 2×10^8 copy number/µl of *Thauera* in the MBBR carrier biofilm quantified using real time PCR suggested its prominent presence in the developed biofilm. The robust biofilm forming ability of *Thauera* sp.V14 was due to its 83.8 % auto-aggregation ability and 93 % hydrophobicity. SEM analysis of biofilm revealed bacteria rich biofilm on the dMBBR carrier and the FTIR analysis showed proteins, polysaccharides and humic acids as the major components of the EPS. Thus *Thauera* spp. could be an important component of consortia that may be used in bioaugmentation of denitrifying reactors.

To conclude the entire studies a robust denitrifying bacterial consortium was developed by adopting conventional enrichment approach like Winogradsky column which was

stable in its performance in reactor studies. dMBBR seeded with the consortium DC5 initially performed continuously for 300 days without any further reinoculation and moreover optimization of different relevant parameters enhanced its performance. In the present studies two of the DC5 consortium members were *Thauera* spp. Interestingly the metagenomic studies of the carrier associated biofilm also proved that *Thauera* persisted and dominated in efficient and long term operated acetate-fed MBBR. MBBR studies conducted with *Thauera* sp. V14 also emphasized the role of this genus as a major contributor to denitrification where exogenously added acetate is the carbon source.This asserted the correctness of the approach adopted in the composition of the consortium and operation of the MBBR. The outcome of these studies should give impetus to use of special seed for reactor operation rather than using non specific seed.