Chapter 2

Selection of special biofilm forming denitrifying bacteria from activated sludge

2.1 Introduction

Bioaugmentation of functional microorganisms can increase the efficacy of biological treatment systems by increasing activities of functional microbes (Quan et al., 2005). Use of specific contaminant degrading microorganisms is the most effective strategy for the removal of various toxic pollutants from wastewater treatment (Schauer-Gimenez et al., 2010; Duque et al., 2011; Zhang et al., 2012b). Use of specific microorganisms that carry out the same functions and cooperate in a synergistic way is an important approach for successful bioaugmentation (Herrero and Stuckey, 2015). In the literature, various consortia have been used for the removal different pollutants from wastewater (Moosvi et al., 2006; Sorokin et al., 2007; Okamoto and Eltis, 2007; Vaidya et al., 2018; Kachieng and Momba, 2018). Removal of high nitrate from wastewater is only possible if microorganisms are able to tolerate it and able to remove it from contaminated wastewater (Pinar et al., 1997). Biological nitrogen removal is the method of choice for wastewater denitrification and denitrifying bacteria have been widely used for the treatment of nitrate containing wastewater. *Rhodocyclus*, *Thauera*, *Alcaligenes*, *Achromobacter*, *Azoarcus*, *Aquaspirillum*, *Bacillus*, *Paracoccus, Brachymonas* and *Pseudomonas* were commonly found in denitrifying mixed cultures (Kniemeyer et al., 1999; Sakano et al., 2002; Leta et al., 2004; Yoshie et al., 2004; Rehfuss and Urban, 2005; Thomsen et al., 2007; Wang and Lee, 2007).

Higher removal efficiency could occur when functional bacteria are immobilized in biofilm, which are used for pollution removal. Biofilm is the best way to keep functional bacteria in bound form together in wastewater treatment plants. Pollutant removal by biofilm forming bacteria without any functional attributes is not possible. Moreover, simply mixing of pollutant degrading bacteria with biofilm-forming bacteria without functional attribute may cause slow biofilm development, mutual inhibition of growth and difficult operation of bioreactors (Li et al., 2016). Biofilm-forming bacteria contribute via specific surface molecules that play an important role in the formation of multispecies biofilms in a various environments. One of them is bacterial functional amyloids. In bacteria, amyloids are surface associated proteins widely present in natural biofilms and flocs of activated sludge (Larsen et al., 2007; Larsen et al., 2008). Amyloid proteins are folded as β-sheet rich fibrils, stacked perpendicular to the fibrillar axis

(Chapman et al., 2002). They have been studied well in *Salmonella, Bacillus, Streptomycetes*, *E. coli* species, fungi, etc. (Gebbink et al., 2005; Sarang and Nerurkar, 2020). Since amyloids are the basis of most biofilm structure of bacteria, amyloid producing bacteria should be biofilm formers. Hence amyloid producing bacteria were isolated. Biofilm formation by bacteria can be affected by different environmental factors such as pH, dissolved oxygen, nutrition concentration, temperature, carbon sources, etc. (Renner and Weibel, 2011; Bassin et al., 2012; Ansari et al., 2012; Barwal and Chaudhary, 2014). In this perspective, the study presented in this chapter was focused on the development of a denitrifying bacterial consortium with biofilm forming attribute to be used as special seed in a biofilm reactor, viz., MBBR (Moving Bed Biofilm Reactor). Effect of different factors on denitrification and biofilm formation by the denitrifying consortium was checked to improve the efficiency of the denitrifying consortium.

2.2 Materials and Methods

2.2.1 Enrichment and isolation of denitrifying bacteria

Enrichment of denitrifying bacteria was carried out in Winogradsky columns. Winogradsky columns were set up using the activated sludge samples collected from wastewater plants.One fourth column of 1L measuring cylinder was packed with garden soil mixed with activated sluge.The rest of the cylinder was topped with tap water. The columns were supplemented with sodium acetate/methanol as carbon sources and potassium nitrate as nitrogen source. Aliquots were taken from the columns every 3 days to check the presence of denitrifiers and to enumerate them by Most Probable Number test.

2.2.1.1 Most Probable Number (MPN) of denitrifiers

To estimate the density of denitrifiers in enriched columns 0.1 ml and 1 ml of enriched sample was inoculated in respective 10 ml single strength Peptone nitrate broth (PNB) tubes (0.5 g Peptone, 0.3 g Beef extract, 0.1 g Potassium nitrate in 100 ml distilled water) and 10 ml of enriched sample in 10 ml double strength PNB tubes. After 48 h of incubation, the ability of the consortia to denitrify the nitrate present in the PNB tubes was detected by bubble formation in Durham's tubes (Gamble et al., 1977). The number

of denitrifiers present in the sample was determined by the MPN calculation table (MLG Appendix 2.05).

2.2.1.2 Isolation of amyloid producing bacteria

Isolation of biofilm forming denitrifying bacteria was carried out from enriched Winogradsky columns. Samples were first subjected to serial dilutions using 0.85 % saline. Then 100 µl of each dilution was spread on CPNA (Congo red peptone nitrate agar) plates (0.5 g Peptone, 0.3 g Beef extract, 0.1 g Potassium nitrate, 2 g Agar, 0.005 g Congo red in 100 ml of distilled water (D/W)) and incubated for 48 h at 37 °C. Isolates showing red colored colonies indicative of amyloid production were selected for further studies and sub-culturing was done on fresh CPNA plates until pure colonies were obtained. The obtained pure isolates were preserved at 4-8 °C on the CPNA agar plate after 48 h of incubation at 37 °C. Isolates were maintained through sub-culturing on the same media.

2.2.2 Screening of biofilm forming denitrifying bacteria

As discussed earlier amyloids are the structural components of bacterial biofilm hence it was envisaged that amyloid producing bacteria would be good biofilm formers. Therefore, screening of selected isolates was done based on their amyloid producing ability and denitrification activity.

2.2.2.1 Denitrification test

Qualitative denitrification test was performed by inoculating 100 µl of an isolate culture in 5 ml of PNB with Durham's tube and incubated at 37 °C for 48 h in static condition. The presence of gas in Durham's tube was considered a positive test.

2.2.2.2 Screening on basis of nitrite accumulation

Nitrate is reduced to nitrite by denitrifiers and further to Nitric oxide, Nitrous oxide and N_2. Nitrite accumulation was detected qualitatively by the Nitrate Reduction Test (MacFaddin, 1999). For this test 0.5 ml aliquots were taken from PNB media inoculated with the isolates and 0.1 ml of α- naphthylamine and 0.1 ml of sulphanilic acid were added. Accumulation of nitrite was detected by the formation of red to deep red color depending on the amount of nitrite present.

2.2.2.3 Measurement of denitrification efficiency

The denitrifying bacterial isolates were grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. The cell pellet obtained was washed twice with phosphate buffered saline (PBS) and resuspended in PBS with absorbance set at 0.5 OD_{600nm}.Then 1 % of this was inoculated in 100 ml of flask containing 50 ml MM2 medium prepared from dissolving 0.2 g $MgSO_4.7H_2O$, 0.2 g K_2HPO_4, 0.05 g $FeSO_4.7H_20$, and 0.02 g $CaCl_2.2H_2O$, 0.002 g $MnCl_2.4H_2O$, 0.001 g $NaMoO_4$ $.2H_2O$, 1 g KNO_3, 0.6 % Sodium acetate and 0.5 g Yeast extract in one liter of distilled water and was adjusted to pH 7. Experimental flasks were incubated at 37 °C for 48 h in static conditions; aliquots were drawn at 12 h intervals and assayed for Nitrate, Nitrite and Ammonia (Srinandan et al., 2011). Denitrification efficiency was calculated by the following formula

$$\text{Denitrification Efficiency (\%)} = \frac{[No_3]In\text{-}[No_3]Out}{[No_3]In} \times 100$$

2.2.2.4 Microtiter plate assay for biofilm formation

For microtiter plate assay, the isolates were grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. The cell pellet was washed twice with PBS, resuspended in PBS till the absorbance was adjusted to 0.5 $OD_{600\ nm}$. Then 40 µl of cells were inoculated in a 24 wells microtiter plate with 1960 µl of MM2 medium. After 48 h of incubation to allow biofilm formation, the wells were rinsed five times with 1.5 ml of sterile PBS to remove any adhering planktonic cells. The biofilm formed on the plate was then stained with 1.5 ml of 1 % crystal violet for 45 min, rinsed five times with 1.5 ml of distilled water and destained with 70 % ethanol for 15 min. The absorbance of the decanted solution was measured in Tecan (InfiniteM200pro) microtiter plate reader at 595 nm (Srinandan et al., 2010).

2.2.3 Identification of selected isolates

Genomic DNA was extracted from 16-18 h old culture of finally five selected isolates (grown in PNB) by using the modified CTAB (Cetyl trimethylammonium bromide) method (Andreou, 2013). For identification of the bacterial isolates genomic DNA of the five cultures was isolated. For genomic DNA isolation 2 ml of 16-18 h culture grown in

PNB was centrifuged at 8000 rpm for 5 min. Pellet was resuspended in 330 µl of 25 mM TrisCl (pH 8), 25 mM EDTA (pH8) and 300 mM Sucrose. Then 4 µl of lysozyme was added to the system and incubated at 37 °C for 1 h. To this 10 % SDS was added and the tubes were inverted to mix properly and kept in water bath at 60 °C for 1 h. Tubes were cooled down to room temperature and 100 µl of 5 M NaCl was added in the tubes. Then 50 µl of 10 % CTAB was added in the same tubes and incubated at 65 °C for 10 min. Then equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and the mixture was centrifuged at 10,000 rpm for 10 min. This step was repeated twice. Upper aqueous phase was collected and equal volume of Chloroform: Isoamylalcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. Aqueous phase was extracted and $1/10^{th}$ volume of 3 M chilled sodium acetate was added in the tubes. In the same tubes double volume of absolute alcohol was added and incubated at chilled temperature for 1 to 24 h. After incubation the tubes were centrifuged at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was air dried and resuspended in 25 µl of milliQ water. Then 1 µl of 0.01 % RNase was added to it and incubated at 65 ° C for 10 min and preserved in cold condition. Presence of genomic DNA was detected by agarose gel electrophoresis.

2.2.4 16S rRNA gene amplification and analysis

16S rRNA gene of five selected isolates was amplified from their respective genomic DNA using the Polymerase Chain Reaction (PCR) with bacterial universal primers 8F (AGA GTT TGA TCC TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T). The 16s rRNA gene sequencing was outsourced to AgriGenome Labs (India) and the sequence similarity search was done by using EZBioCloud. For phylogenetic analysis sequences for *Diaphorobacter* sp., *Pannonibacter* sp., *Thauera* sp., *Pseudomonas* sp. and *Thauera* sp., genera were obtained from GenBank and phylogenetic tree was constructed along with the sequences of the isolates by using (Distance matrix) was constructed by using neighbor joining method in MEGA X.

2.2.5 Confirmation of amyloid production by Thioflavin T staining

Thioflavin T stains specifically the amyloid coated on the surface of the bacterial cells, thereby identifying the amyloid producing bacteria. Amyloid production by the bacteria was qualitatively detected by the Thioflavin T staining method. Stock solution was prepared by dissolving 32 mg of Thioflavin T in 1 ml of distilled water, which was stored in the dark at 4 °C. The working solution was freshly prepared by diluting the stock solution 1:2 before immediate use. The smears of the bacterial isolates were treated with Thioflavin T solution for 5 min in the dark, rinsed with distilled water and air dried before observing under an Olympus BX41 fluorescence microscope (Berg et al., 2010).

2.2.6 Co-culture growth inhibition

Co-culture growth inhibition was tested by cross streak assay method with modifications. Single streak (4–6 mm diameter) of one isolate was streaked on the surface of a CPNA plate, and the other four isolates were streaked on the same plate separately at perpendicular direction and incubated at 37 °C temperature for 48 h to observe growth inhibition.

2.2.7 Antibiotic Sensitivity Test

Antibiotic sensitivity of all the isolates was checked using HiMedia octadisc. Antibiotics used for the test were Amoxycillin, Cloxacillin, Erythromycin, Tetracyclin, Ciprofloxacin, Ofloxacin, Penicillin, Co-Trimoxazole, PenicillinV and Cefalexin. 100 µl of culture was added on 0.8 % soft agar. 5 ml of 0.8 % soft agar was overlaid on the plate containing basal Luria Agar (LA). Then the antibiotic disc was kept on the LA, which was incubated at 37 °C for 24 h. Resultant zone of growth inhibition was observed and graded as Sensitive (S) and Resistant (R) based on the analysis reference table given by HiMedia.

2.2.8 Growth profile of selected isolates

All the five selected isolates were grown separately in PNB for 18-24 h and then centrifuged at 8000 rpm for 7 min. The cell pellet was washed twice with PBS and resuspended in PBS to obtain an absorbance of 0.5 $OD_{600\ nm}$. Then 1 ml of the inoculum

was then inoculated in 100 ml flask containing 49 ml Luria Broth (LB) medium and incubated at 37 °C for 48 h at static condition. Aliquots were drawn at every 2 h intervals for 48 h and used for measuring growth in terms of OD_{600nm}.

2.2.9 Flask level denitrification studies

2.2.9.1 Preparation of denitrifying consortium

A consortium, henceforth termed consortium DC5, was prepared by growing all the selected isolates separately in PNB for 24 h. The absorbance of 0.5 OD_{600nm} was set and 400 µl of each isolates were pooled to make 2 ml final volume. The cell pellet obtained after centrifugation at 8000 rpm for 7 min was washed twice with PBS and resuspended in the same volume of PBS. Then 1 ml of suspension of the consortium was then added to a sterile 100 ml of MM2 medium and incubated at 37 °C under static condition for 24 h to be used as inoculum.

2.2.10 Gas chromatography

Cells of the selected isolates grown for 24 h in PNB were harvested by centrifugation at 10,000 rpm for 5 min, washed twice with PBS and resuspended in PBS. 500 µl of this was inoculated into PNB and tubes were sealed with suba-seal rubber stoppers and incubated for 48 h. After 48 h, 1 µl (gaseous sample) collected from these tubes sample was injected into the GC. Dinitrogen (N_2) content was determined by gas chromatography (GC) using Sigma Instruments Ltd. Equipped with Flame Ionization Detector (FID). The temperature of the column, the injector port, and the FID were 250 °C, 260 °C and 260 °C, respectively. CR-624 column with a mesh size of 3 µm was used, and nitrogen gas was used as a carrier gas.

2.2.11 Resting cells kinetics

Nitrate and nitrite reduction rates by resting cell suspension of the selected isolates were performed as follows. Cells grown for 24 h in PNB were harvested by centrifugation at 10,000 rpm for 5 min, washed twice with PBS and resuspended in PBS. Sodium acetate and nitrate were added as electron donor and acceptor respectively and the reduction of nitrate and formation of nitrite were estimated for 48 h after every 12 h time intervals.

2.2.12 Effect of various parameters on denitrification and biofilm of consortium DC5 in microtiter plate assay

2.2.12.1 Effect of metal ions

Different concentrations of $MgCl_2$ (1, 2.5, 5, 7.5 and 10 mM), K_2HPO_4 (0.5, 1, 5, 7.5 and 10 mM), $CaCl_2$, (0.5, 1, 5, 7.5 and 10 mM) and $FeCl_3$ (0.125, 0.250, 0.5 and 1mM) were taken.40 µl of consortium DC5 was inoculated in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of consortium DC5 were checked.

2.2.12.2 Effect of inoculum size

Different concentrations of inoculum 1 %, 2 %, 3 %, 4 %, 5 % and 6 % were taken and inoculated in 2 ml of MM2 media in microtiter well. After 48 h of incubation, denitrification efficiency and biofilm forming ability of consortium DC5 were checked.

2.2.12.3 Effect of different carbon sources

0.6 % of different carbon sources like glucose, ethanol, methanol, molasses, sodium acetate, and glycerol with 40 µl of consortium DC5 were added in 2 ml of MM2 media. After 48 h of incubation, denitrification efficiency and biofilm forming ability of consortium DC5 were checked. Denitrification efficiency and biofilm formation of consortium DC5 were performed according to sections 2.2.2.3 and 2.2.2.4. Growth of individual isolates of the consortium was checked by semi quantitative PCR analysis of the consortium gDNA as follows.

2.2.12.4 Semi-quantitative PCR analysis

After 48 h gDNA was extracted from the consortium grown on different sugars individually using the CTAB method according to section 2.2.3. PCR amplification with genus specific 16S rRNA primers was carried out to check presence of isolates. Primers used for the isolates are listed in Table 2.1. After amplification, the products were confirmed on 2 % agarose gels at 75V.

Table 2.1 16S rRNA gene specific primer sequences of individual isolates of consortium DC5 used for PCR analysis

Isolates	Primers
Diaphorobacter sp.	Forward: GGCCGCTCCGTCCGC Reverse:CGCTCGTTGCGGGACTTAACC
Pannonibacter sp.	Forward:AGTCGAACGCATCGCAAGAT Reverse:ATTACCGCGGCTGCTGGCA
Thauera sp.	Forward: TGCATTGCTGCTCCGAAC Reverse;CGCTCGTTGCGGGACTTAACC
Pseudomonas sp.	Forward: CAATCAGTGTCAGTATTAGC Reverse:CGCTCGTTGCGGGACTTAACC

2.2.13 Analytical Methods

Nitrate, Nitrite, Ammonia methods were performed according to the APHA manual, 1998.

2.2.13.1 Standardization of nitrate, nitrite and ammonia estimation methods

KNO_3 for nitrate estimation, $NaNO_2$ for nitrite estimation, and NH_4Cl for ammonia estimation were used as standards. Standard graphs are depicted in Fig.2.1.

2.2.13.1.1 Nitrate estimation by UV-Spectrophotometric method

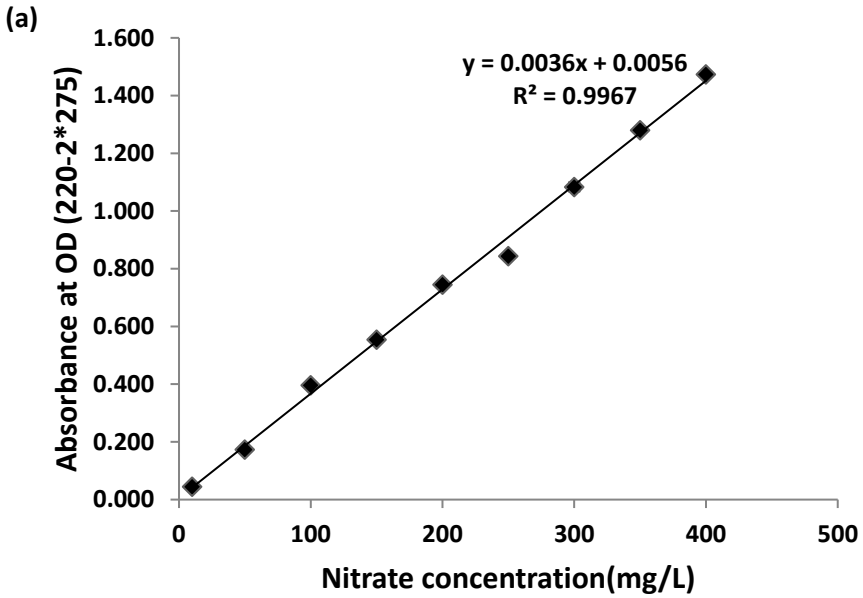
40 µl of samples were added to 1960 µl D/W to make a system of 2 ml. 40 µl of 1N HCl was added to the system and absorbance was measured at $OD_{220\ nm}$ and $OD_{275\ nm}$. UV absorbance at 220 nm determines the concentration of NO_3 but dissolved organic matter is also absorbed at 220 nm and NO_3 does not absorb at 275 nm, second measurement was taken at 275 nm used to correct the NO_3 value. Finally (220-2*275) formula was used to determine the final concentration of nitrate.

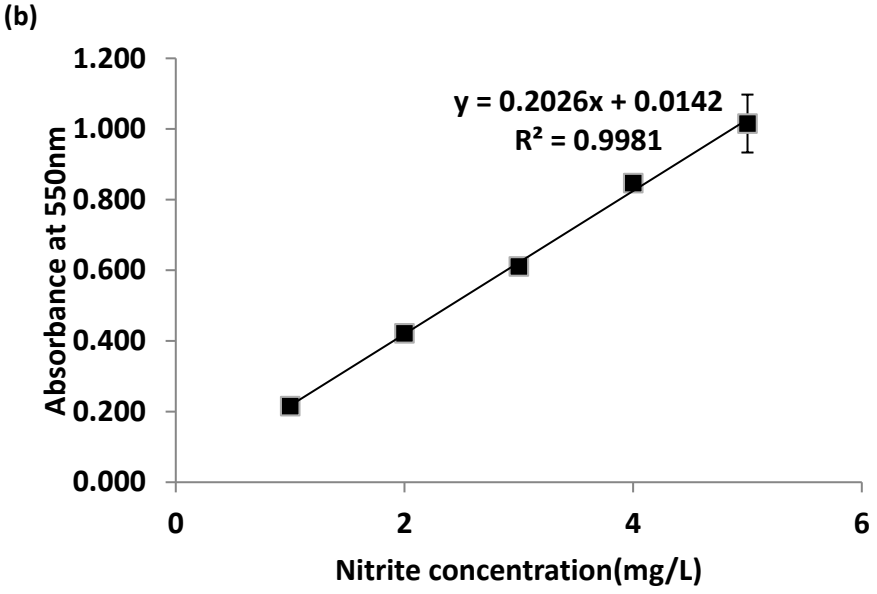
2.2.13.1.2 Nitrite estimation by Diazotization method

40 µl samples were mixed in 1960 µl D/W to make 2 ml system. To this system, 40 µl of Griess Reagent (1 % Sulphanilamide + 2 % Phosphoric acid + 0.1 % Naphthylethtlediaminedihydrochloride) was added. Pink color developed was measured at $OD_{540\ nm}$ after incubation of 50 min.

2.2.13.1.3 Ammonia estimation by Phenate method

2 ml sample (40 µl Sample + 1960 µl D/W) was mixed with 20 µl Phenol + 20 µl Sodium Nitropruside solution + 40 µl oxidizing solution. After incubation for 60 min in the dark at room temperature, blue color developed was measured at $OD_{640\ nm}$.





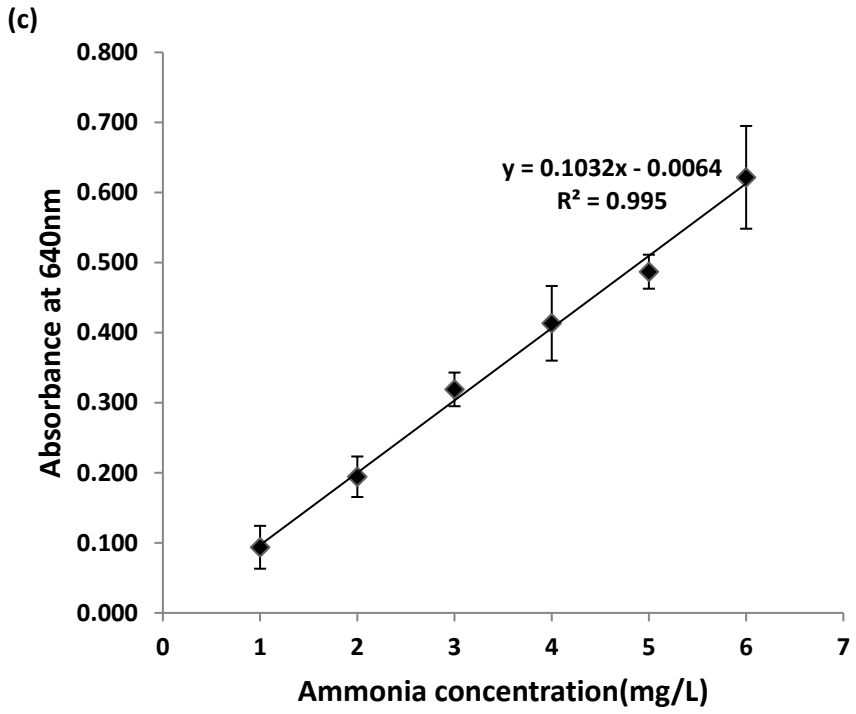


Figure 2.1 Standard graphs for (a) nitrate (b) nitrite and (c) ammonia estimation

2.2.14 Data analysis

All the experiments were performed in triplicate. The statistical significance was determined using one way ANOVA analysis with Tukey's multiple comparison test. Error bars represent standard deviations in GraphPad Prism 6.0 (San Diegao, CA, USA).

2.3 Results and Discussion

Microorganisms from specific environments can better colonize and survive in the same environment than nonnative microorganisms (Bouchez et al., 2000; Fantroussi and Agathos, 2005). Therefore, microorganisms used for bioaugmentation processes are generally isolated from contaminated sites and are native to that site (Gentry et al., 2004). Similarly, the aim of present studies was to isolate special strains of denitrifying biofilm forming denitrifying bacteria for bioaugmentation studies. For this, denitrifying Winogradsky column was developed by inoculating activated sludge so as to enrich denitrifiers. Acetate was added as carbon source since denitrification process requires exogenous carbon source. Denitrifying and amyloid

producing bacteria were isolated from the enriched column.Amyloid is an important component of biofilms therefore amyloid producing ability was also checked so as to select a good biofilm former. Furthermore, the selected isolates were screened for denitrification efficiency and biofilm formation.

2.3.1 Enrichment of denitrifying bacteria from activated sludge

It has been reported that most of the bacterial populations in activated sludge systems are capable of denitrification (Nielsen and Nielsen, 2002). Most efficient denitrifying strains in activated sludge can perform the complete pathway of denitrification (Magnusson et al., 1998). Therefore, looking at the omnipresence of amyloid producing and denitrifying bacteria in activated sludge, sludge samples were collected from different sewage treatment plants. Isolation, screening, identification of the specific bacteria and development of the consortium DC5 composed of biofilm forming denitrifying bacteria is presented in this chapter.

2.3.1.1 Development of denitrifying Winogradsky columns

Winogradsky columns allow enrichment of specific physiological group of bacteria by providing particular source of inoculum, nutrients and incubation conditions. A variety of complex heterogeneous samples can be enriched for a desired group of microorganisms. For the development of denitrifying Winogradsky columns, high concentration of nitrate and preferred carbon source for denitrification i.e. acetate was supplemented to the column medium with the aim of enriching denitrifiers (Van rijn et al., 1996). Activated sludge from different wastewater plants was used as inocula for the columns. Regular monitoring of the appearance, turbidity and bubble formation in Winogradsky columns was carried out. As shown in Fig. 2.2 cloudiness was observed on day 1 due to suspended soil particles which settled down subsequently to give clear water column. Increase in turbidity due to bacterial growth was observed on day 7. There was significant increase in turbidity of water and pigmentation due to presence of nitrate and environmental condition by 18^{th} day. On 21^{st} day pigmentation of water decreased while turbidity increased along with bubble formation on the water-soil interface. This indicated that denitrifying Winogradsky

column was developed since the medium and inoculum selected were specific for enrichment of denitrifiers.

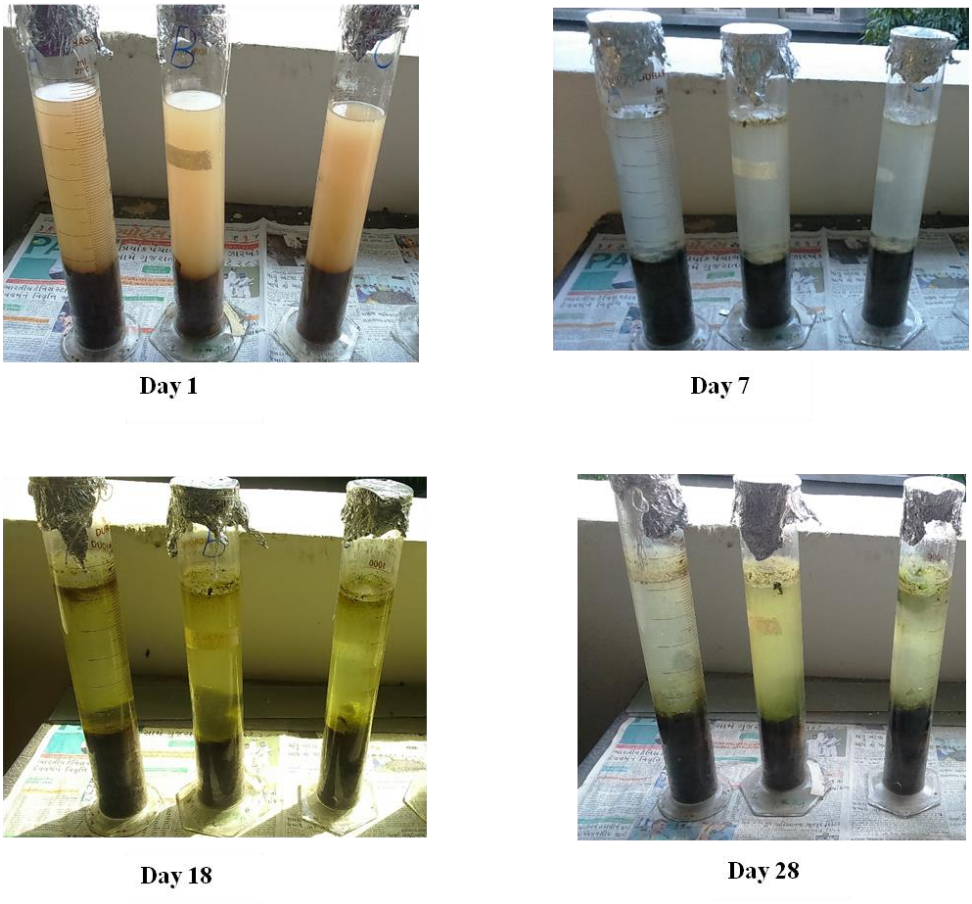


Figure 2.2 Changes observed in appearance of Winogradsky columns spiked with nitrate and activated sludge from three different sources

In order to follow the enrichment of denitrifying bacteria in the Winogradsky columns, MPN technique was used. For MPN, aliquots from the column were taken from the interface of the soil and media (anaerobic) region every 3rd day. MPN index was obtained from the combination of positive tubes of PNB with bubble formation. Table 2.2 shows that as the number of days increased, the number of denitrifiers increased. In case of columns A and C number of denitrifiers increased on day 10 and in column B on day 6. On day 21, 1100 MPN index i.e. maximum was achieved in all the three columns which suggested that there are large numbers of denitrifiers

enriched inside the Winogradsky columns from three different activated sludge samples tested.

Table 2.2 Enumeration of denitrifying bacteria in Winogradsky column (MPN index)

Days	Column A	Column B	Column C
4	11	36	7.2
6	7.4	1100	16
10	240	27	1100
14	3.6	27	28
18	1100	160	38
21	1100	1100	1100

2.3.1.2 Isolation of amyloid producing bacteria from denitrifying Winogradsky columns

Isolation of biofilm forming denitrifying bacteria was carried out from the developed denitrifying Winogradsky columns. Amyloids are extracellular surface proteins produced by bacteria that are important components of bacterial biofilms and are widely present in wastewater sludge (Larsen et al., 2007; Larsen et al., 2008). Therefore, it was envisaged that amyloid producing bacteria could be good biofilm forming bacteria. Isolation of amyloid producing bacteria from the denitrifying Winogradsky column yielded 33 morphologically different isolates on CPNA plates. CPNA was used to detect the presence of amyloid producing bacteria. Amyloids take up Congo red and its incorporation in CPNA helped to distinguish amyloid producing bacteria from non amyloid producing ones. Congo red in CPNA specifically binds to the β-sheets of the amyloid proteins imparting red color to the colonies of amyloid producers. Chapman et al., (2002); Gebbink et al., (2005); Larsen et al., (2007) have also used Congo red dye to identify amyloid producing bacteria based on their colony color. Out of 33 total 19 isolates (R2, V3, R4, R19, V5, R6, R7, V8, V9, V11, V14,V15, V16, B17, B20, V18, R22, R23, R24) showed red colonies on CPNA. 3 isolates (V1, R10 and V12) showed yellowish colonies and R6, B20, B13 isolates showed orange colonies on CPNA plates.

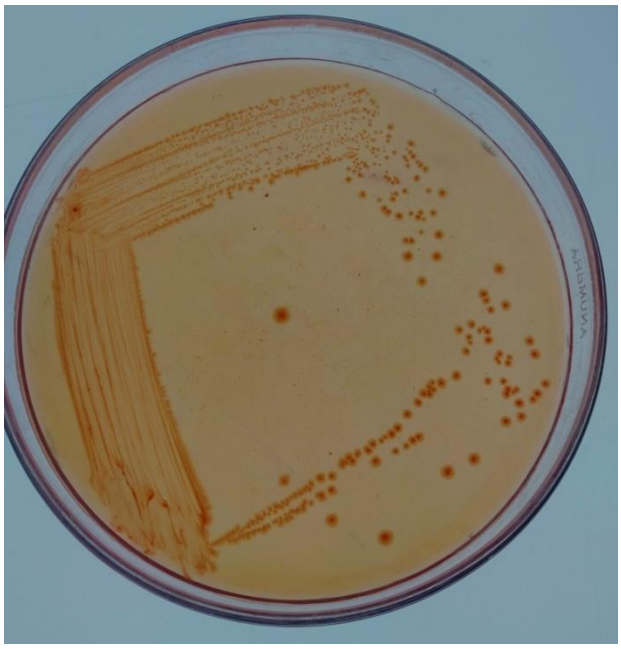


Figure 2.3 Amyloid producing bacteria showing red colonies on CPNA plates

2.3.2 Screening of amyloid producing isolates for denitrification ability

2.3.2.1 Screening based on nitrate reduction biochemical test

The same 33 isolates from Winogradsky column were further tested for qualitative biochemical test for denitrification activity. Ability to produce gas in PNB medium by bacteria is mostly due to the ability of denitrification. The gas accumulated in Durham's can be nitrous oxide, nitric oxide or nitrogen, with nitrogen gas being the major gas produced (Gamble et al., 1977). Out of 33 isolates, 24 isolates showed gas production in PNB. Therefore, it was concluded that the 24 isolates were potential denitrifiers (Fig.2.4).

2.3.2.2 Screening of the isolates based on their denitrification efficiency and biofilm forming ability

Next step in screening included quantitative assays for biofilm forming ability and denitrification efficiency in order to select efficient bacteria for biofilm based wastewater treatment system studies. Therefore, identifying such bacteria and their practical application studies were carried out. Denitrification efficiency and biofilm forming ability of all the selected 24 amyloid producing isolates were checked.

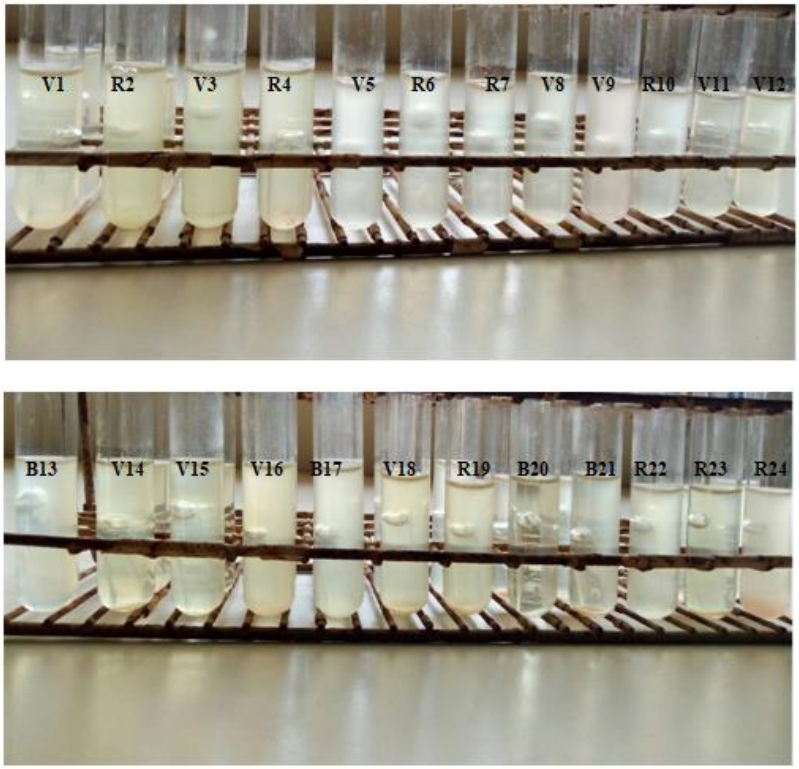


Figure 2.4 Gas production in PNB by the amyloid producing isolates

Table 2.3 Morphological and microscopic characteristics of the amyloid producing denitrifying isolates from Winogradsky columns

Serial No.	Isolates	Gram nature	Size (mm)	Shape	Margin	Elevation	Consistency	Opacity
1	V1	+ve	1	circular	entire	convex	moist	opaque
2	R2	-ve	3	irregular	undulate	convex	moist	opaque
3	V3	-ve	3	irregular	undulate	convex	moist	opaque
4	R4	-ve	2	circular	entire	convex	moist	opaque
5	V5	-ve	3	circular	entire	convex	moist	opaque
6	R6	+ve	10	irregular	undulate	convex	moist	opaque
7	R7	+ve	2	irregular	entire	convex	dry	opaque
8	V8	-ve	4	circular	entire	convex	moist	opaque
9	V9	-ve	2	circular	entire	convex	moist	opaque
10	R10	-ve	3	circular	entire	convex	moist	opaque
11	V11	-ve	4	irregular	spiny	flat	moist	opaque
12	V12	-ve	3	circular	entire	convex	moist	opaque
13	B13	-ve	3	circular	entire	convex	moist	opaque
14	V14	-ve	2	circular	entire	convex	moist	opaque

15	V15	-ve	2	circular	entire	convex	moist	opaque
16	V16	-ve	10	irregular	undulate	convex	moist	opaque
17	B17	-ve	2	circular	undulate	convex	moist	opaque
18	V18	-ve	2	irregular	entire	convex	moist	opaque
19	R19	-ve	1	circular	entire	convex	moist	opaque
20	B20	+ve	10	circular	undulate	convex	dry	opaque
21	B21	-ve	6	fusi form	undulate	convex	moist	opaque
22	R22	-ve	6	circular	undulate	convex	moist	opaque
23	R23	-ve	10	irregular	undulate	flat	moist	opaque
24	R24	-ve	10	Irregular	undulate	flat	moist	opaque

Out of 24 isolates, V3, R4, V5, R6, R7, V9, V11, V14, B17 and V18 showed highest denitrification efficiency, i.e. above 80 % (Fig. 2.5a). Isolates V1, R4, V5, V8, R10, V11, B13, V14, B17 and R23 showed good biofilm forming ability (Fig. 2.5b).

Nitrate (NO_3^-) when reduced to nitrite (NO_2^-) gets accumulated and is even more toxic than nitrate. Nitrite is also harmful to humans to a certain level and has been reported to be a carcinogen, teratogenic and mutagenic especially the N-nitroso compounds (Yılmaz et al., 2006). Therefore, nitrite accumulation by denitrifiers in wastewater tanks is considered to be an unfavorable trait. In this biochemical test if nitrate is reduced to nitrite by the isolates due to the nitrate reductase enzyme that they possess. After addition of sulphanilic acid nitrite reacts to produce diazotized sulphanilic acid complex (nitrite-sulphanilic acid) which reacts with α-naphthylamine to form a red-colored compound (prontosil, an azo dye). Formation of red colored precipitate in the tube is considered a positive result for formation of nitrite. Out of 24 isolates 12 isolates showed nitrite formation or even accumulation after 72 h in biochemical test. Nitrite accumulation and it was found with isolate V1, R2,V3, R6, R10, V12, B13,V15, V16, B17, R19, B21, R22, R23, R24 (Fig.2.6). Hence these isolates were discarded from further studies.

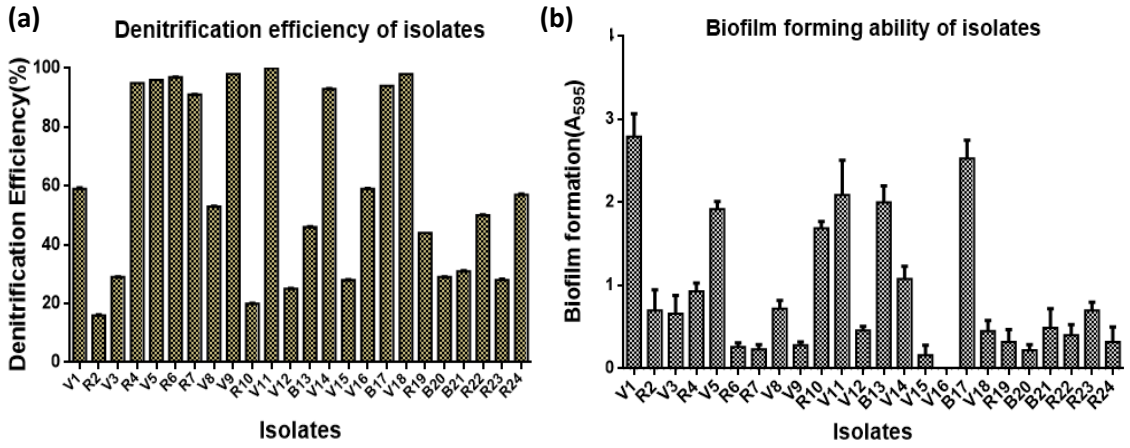


Figure 2.5 (a) Denitrification efficiency and (b) Biofilm forming ability of selected 24 amyloid producing denitrifying bacterial isolates

Nitrite accumulation test negative Nitrite accumulation test positive

Figure 2.6 Nitrite accumulation test of amyloid producing denitrifying isolates

2.3.3 Development of denitrifying consortium

Five isolates were selected for further studies based on 1) Denitrification efficiency 2) Amyloid and biofilm formation and 3) Lack of nitrite accumulation

2.3.3.1 Identification of the selected 5 biofilm forming denitrifying bacterial isolates

Identification of the selected isolates R4, V5, V9, V11 and V14 in two separate analysis showed 99 %, 99 %, 100 %, 100 % and 99 % sequence similarity with *Diaphorobacter* sp., *Pannonibacter* sp., *Thauera* sp., *Pseudomonas* sp., and *Thauera* sp., respectively and its sequences were deposited in the GenBank database with the accession number MN880203, MN880206, MN880207, MN880204 and, MN880205, respectively. All the selected isolates namely, *Diaphorobacter* sp. (Chakravarthy et al., 2011), *Pseudomonas* sp. (Srinandan et al., 2011), *Thauera* sp. (Lu et al., 2019), *Pannonibacter* sp. (Bai et al., 2019) are known denitrifiers.

Phylogenetic analysis of the selected denitrifying and other related sequences from public dataset was performed using 16S rRNA gene sequences. The results obtained indicated that *Diaphorobacter* sp. R4 was phylogenetically most closely related to *Diaphorobacter* sp. MK027365.1, *Pannonibacter* sp. V5 to *Pannonibacter* sp. KT380507.1, *Pseudomonas* sp.V11 to *Pseudomonas* sp. MH773384.1, *Thauera* sp.V9 and *Thauera* sp.V14 to *Thauera* sp. EU841543.1 and *Thauera* sp. MK954173.1 (Fig. 2.7).

Diaphorobacter sp. has been isolated from wastewater (Zhong et al., 2020). It has nitrate and nitrite reductases (Chakravarthy et al., 2011) and it has been also identified as the dominant denitrifying bacterial population in different solid phase denitrification reactors (Chu and Wang, 2013; Shen et al., 2013). It was characterized for denitrification using PHBV as carbon source where it showed high nitrate removal efficiency (Qiu et al., 2015; Zhang et al., 2017a).

Pannonibacter phragmitetus has been shown to survive in extreme environments such as hot springs (Bandyopadhyay et al., 2013). It has bioremediation potentials that includes reduction of chromium and detoxification of polycyclic aromatic compounds (Xu et al., 2011; Shi et al., 2012; Wang et al., 2013; Wang et al ., 2016).

Pseudomonas sp. is commonly present in activated sludge processes and wastewaters. It has been shown to reduce nitrate and nitrite producing inert nitrogen gas as the end product (Srinandan et al., 2011). It also carries out important metabolic activities such as degradation of xenobiotic compounds and metal cycling in addition to removal of nitrate (Lalucat et al., 2006).

Thauera spp. has been reported to be most commonly found organism in wastewater treatment plants, polluted freshwater, wet soil and water contaminated with aromatic or aliphatic organic compounds (Heider and Fuchs, 2015). Most of species from this genus are known as nitrate reducing bacteria and denitrifiers (Albertsen et al., 2011; Zhao et al., 2013b).

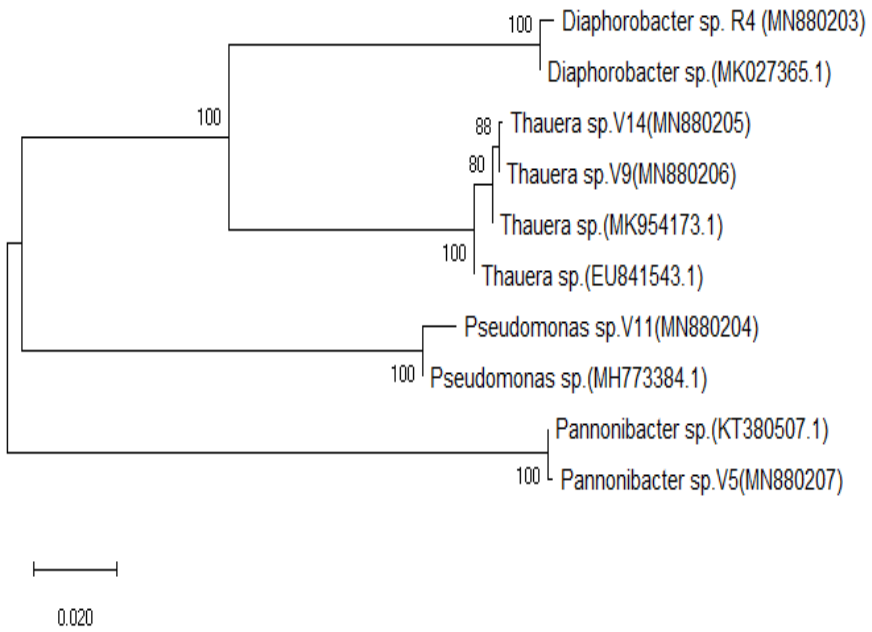


Figure 2.7 Neighbor joining phylogenetic tree depicting phylogenetic relationship of the selected five isolates on the basis of 16S rDNA sequences

2.3.3.2 Confirmation of amyloid producing ability of the selected 5 biofilm forming denitrifying bacterial isolates by Thioflavin T staining

Confirmation of amyloid production by the 5 isolates was carried out using Thioflavin T staining. It is the most widely used "gold standard" for selectively staining and identifying amyloid produced by the bacteria. The amyloid proteins are produced by the bacteria extracellularly. They coat the cell surface and take up Thioflavin T stain and display green fluorescence all over the bacterial cell. All the five isolates were stained fluorescent green by Thioflavin T (ThT) thereby confirming production of extracellular surface amyloid protein. Fig. 2.8 shows microscopic images of Thioflavin T stained isolates. The fluorescence of ThT is considerably enhanced upon interaction with amyloid fibrils. Upon binding to amyloid fibrils, ThT displays a dramatic shift of its excitation maximum (from 385 to 450 nm) and emission maximum (from 445 to 482 nm) indicating that fluorescence is due to the dye bound to amyloid fibrils. ThT staining confirmed that for all the five isolates produced surface amyloid protein.

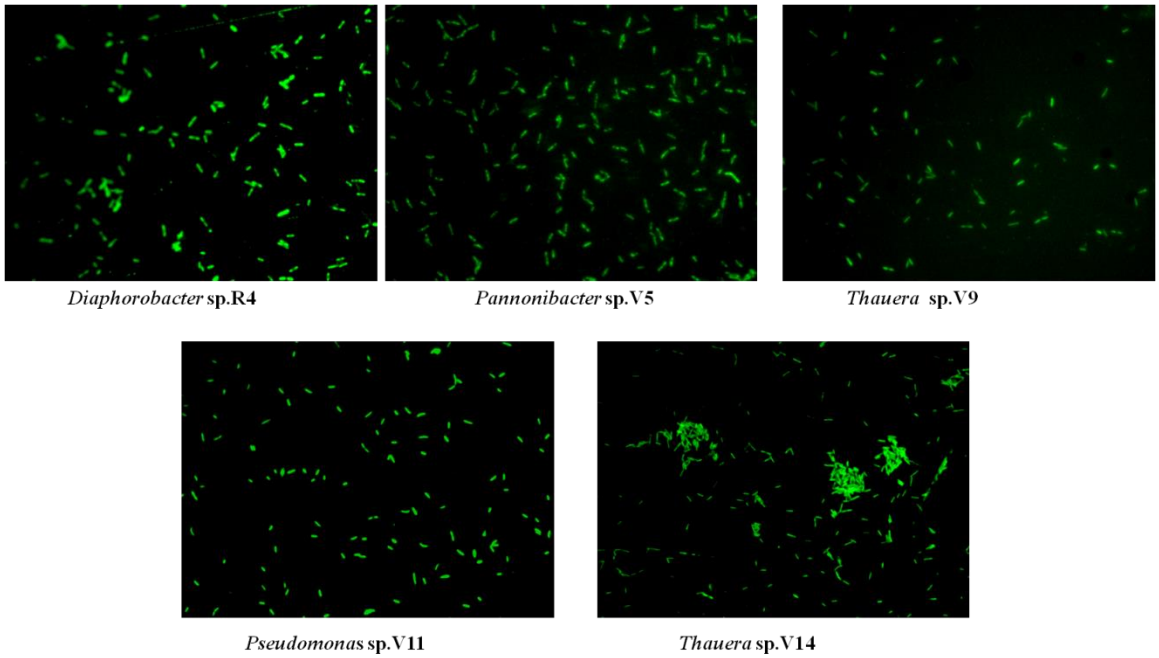


Figure 2.8 Surface amyloid staining with Thioflavin T of the individual isolates of consortium DC5 viz. isolates R4, V5, V9, V11 and V14 (1000 X magnification)

2.3.3.3 Growth curve and co-culture growth inhibition of the 5 selected biofilm forming, denitrifying isolates

In view of the development of denitrifying consortium, growth profile of all the selected isolates was checked. As shown in Fig. 2.9 growth curves of all the 5 isolates almost coincided with each other, which is an important attribute the individual isolates should have to coexist in a consortium. Further, inhibitory capability of all the isolates on each other was checked by cross-streaking assay. Streak assay showed that none of the isolates showed growth inhibition of other isolates when growing on CPNA agar plates (Fig. 2.10). Results of these assay suggested that the isolates did not show any mutually harmful effect on each other and can be part of a consortium which was termed as consortium DC5 (5 membered consortium from denitrifying column).

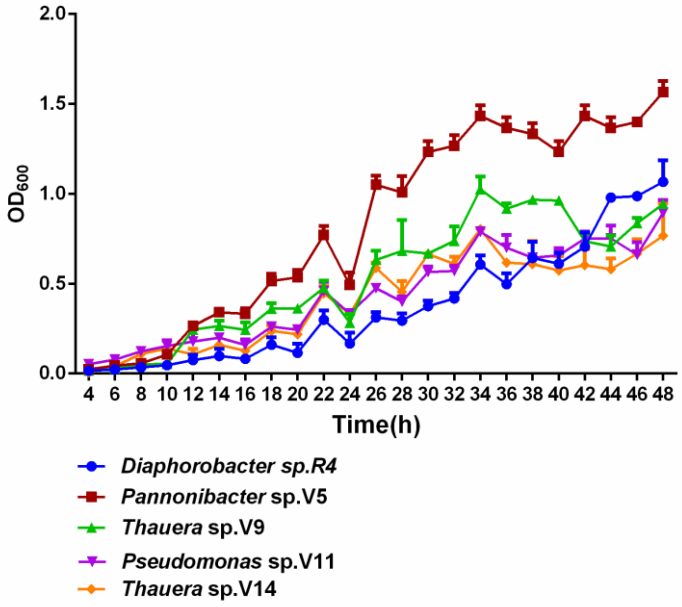


Figure 2.9 Growth profiles of the selected five isolates of consortium DC5.
Error bars represent standard deviation from the mean, (n = 3)

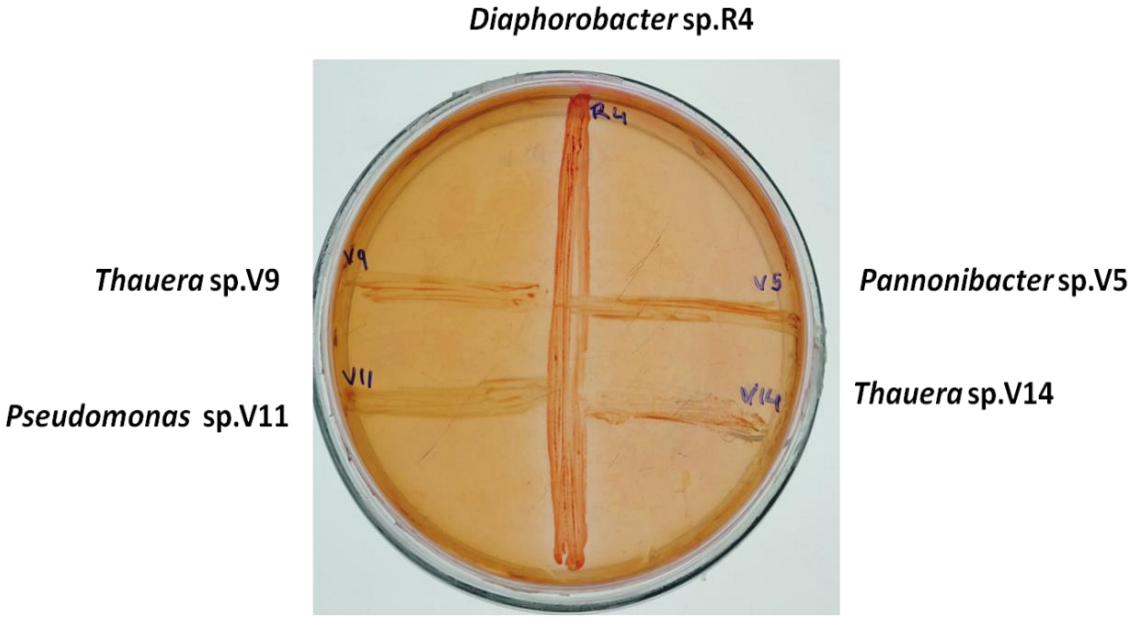


Figure 2.10 The selected five denitrifying isolates showing lack of inhibition by each other in co-culture growth inhibition assay

2.3.3.4 Antibiotic sensitivity test of the 5 selected isolates of consortium DC5

Effect of different antibiotics was checked on the selected 5 biofilm forming, denitrifying isolates using antibiotic sensitivity test. It showed that *Diaphorobacter* sp.R4 was sensitive to Erythromycin, Tetracyclin, Co-Trimoxazole and Cefalexin, *Pannonibacter* sp.V5 to Erythromycin, Tetracycline, *Thauera* sp.V9 to Erythromycin, Tetracyclin, Co-Trimoxazole, *Pseudomonas* sp.V11 to Erythromycin, Tetracyclin, Penicillin, Penicillin V, Co-Trimoxazole, and *Thauera* sp.V14 was sensitive to all the antibiotics (Table 2.4). The antibiotic sensitivity showed the strain differences between the *Thauera* spp.

Table 2.4 Results of antibiotic sensitivity test for consortium DC5 isolates

Antibiotic name	*Diaphorobacter* sp.R4	*Pannonibacter* sp.V5	*Thauera* sp.V9	*Pseudomonas* sp.V11	*Thauera* sp.V14
Amoxycillin	R	R	R	R	S
Cloxacillin	R	R	R	R	S
Erythromycin	S	S	S	S	S
Tetracyclin	S	S	S	S	S
Penicillin	R	R	R	S	S
Co-Trimoxazole	S	R	S	S	S
PenicillinV	R	R	R	S	S
Cefalexin	S	R	R	R	S

R= Resistant, S=Sensitive

2.3.3.5 Flask level denitrification studies with individual isolates and consortium DC5

Flask level studies depicted in Fig. 2.11 showed that *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp.V9, *Pseudomonas* sp.V11 and *Thauera* sp.V14 gave 78 %, 80 % , 64 %, 100 % and 95.5 % nitrate removal respectively. As indicated in section 2.2.9.1 mixture of all the selected isolates was carried out to construct consortium DC5. Consortium DC5 showed 100 % nitrate reduction with initial nitrate concentration of 200 mg L^{-1} in 10 h. Individually isolates *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5 and *Pseudomonas* sp.V11 showed nitrite accumulation between 12 and 48 h, which decreased after 48 h while the *Thauera* sp.V9, *Thauera* sp.V14 and consortium DC5, showed no nitrite accumulation from the beginning itself. Nitrite accumulation is

undesirable trait in wastewater treatment systems as it is an environmental pollutant. No ammonia was detected in the flask. Results of this experiment suggested that all the isolates showed synergistic effect and cooperated to give enhanced nitrate removal efficiency and no nitrite accumulation, when they were tested as consortium DC5 as compared to individual performance. This clearly showed that there is a distinct advantage in using consortium. As reported in the literature too, stable co-existence of different species within a single microbial consortium is a prerequisite for the construction of a microbial consortium (Qian et al., 2020). Consortium of *Pseudomonas* sp. KW1 and *Bacillus* sp. YW4 (Rajakumar et al., 2008), *Pseudomonas stutzeri* and *Pseudomonas putida* (Cyplik et al., 2013), *Nitrosomonas europaea* and *Paracoccus denitrificans* (Uemoto and Saiki, 2000), pure cultures of *Pseudogulbenkiania* 2002 and *Thiobacillus denitrificans* (Kiskira et al., 2017) have been reported for nitrate and nitrogen removal from wastewater, suggesting that mixed cultures or consortia are highly effective in efficient removal of nitrate from wastewater.

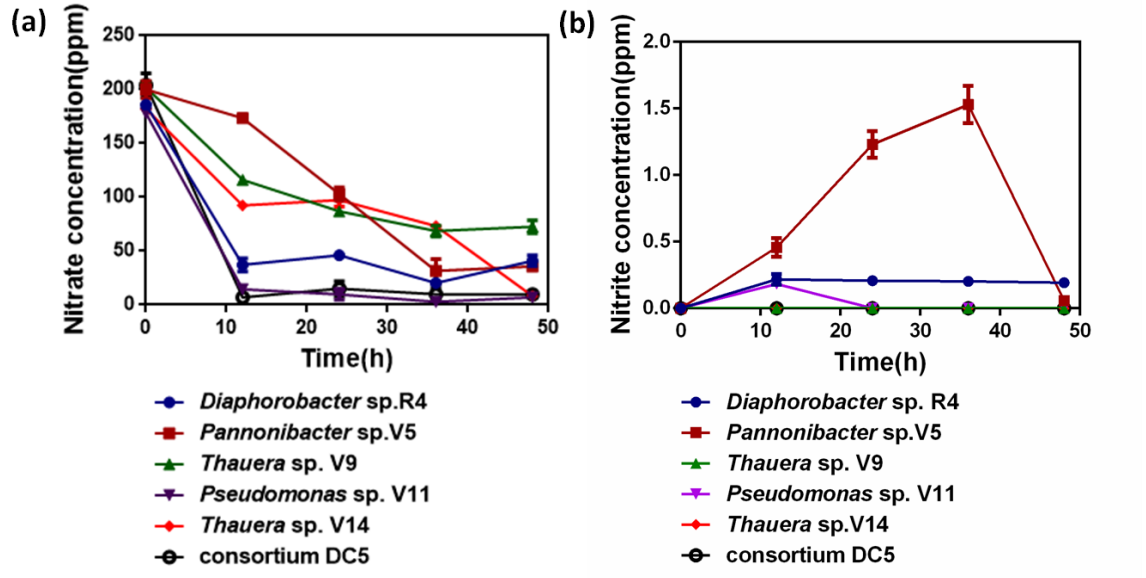
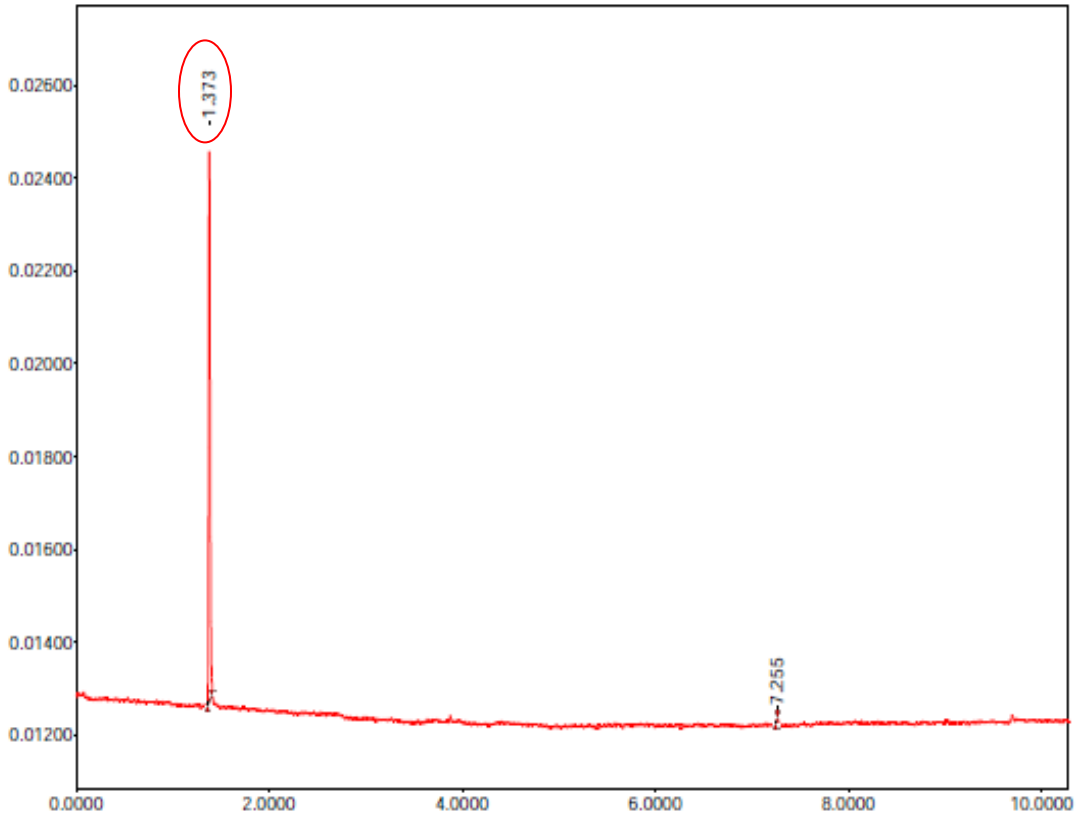


Figure 2.11 Flask level denitrification studies using selected isolates of consortium DC5. (a) Nitrate removal (n = 3), (b) Nitrite accumulation. Error bars represent standard deviations, n = 3.

2.3.3.6 Confirmation of nitrogen gas production by gas chromatography

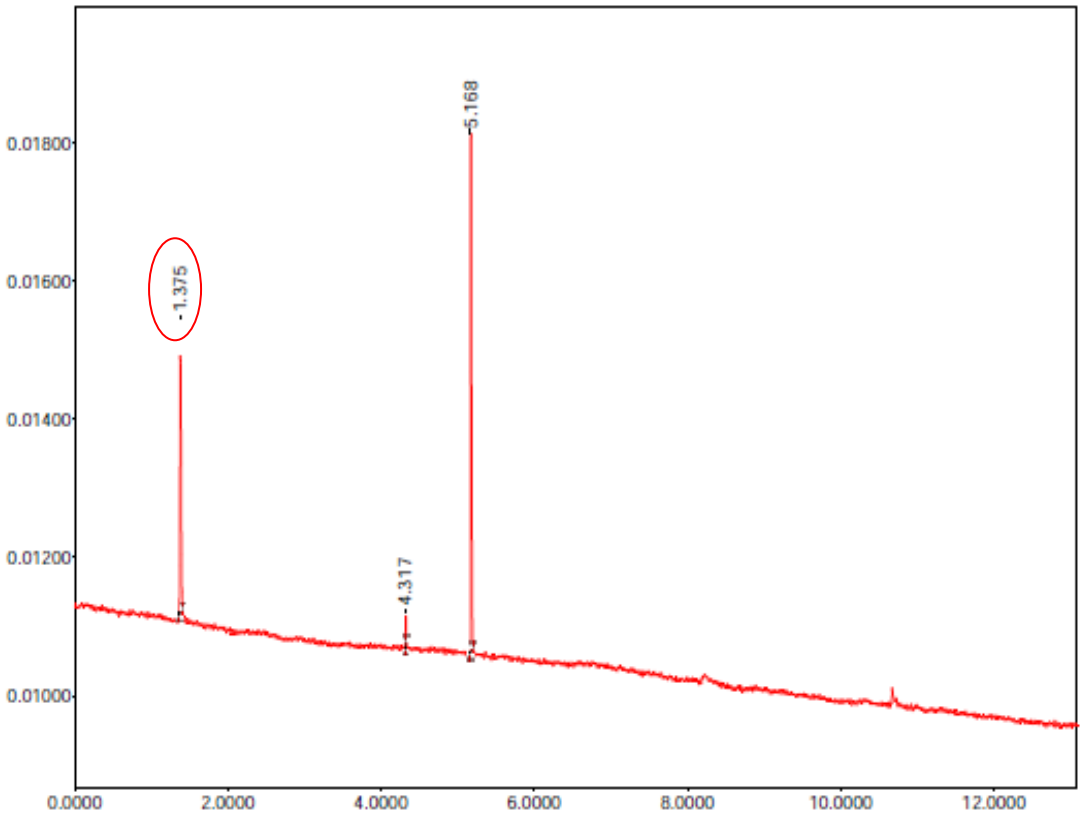
Nitrogen gas production by the 5 isolates was confirmed by gas chromatography. Production of nitrogen gas was confirmed by comparing peak at 1.373 with standard of

nitrogen gas. All the isolates were inoculated in PNB media and after 48 h gas production in media was checked. Isolates *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp. V9, *Pseudomonas* sp. V11 and *Thauera* sp.V14 showed 44.24 %, 50.97 %, 13.01 %, 18.38 % and 64.68 % nitrogen gas production. Compared to individual isolates consortium DC5 showed 81.76 % of nitrogen gas production, which was higher compared to individual isolates (Fig. 2.12).



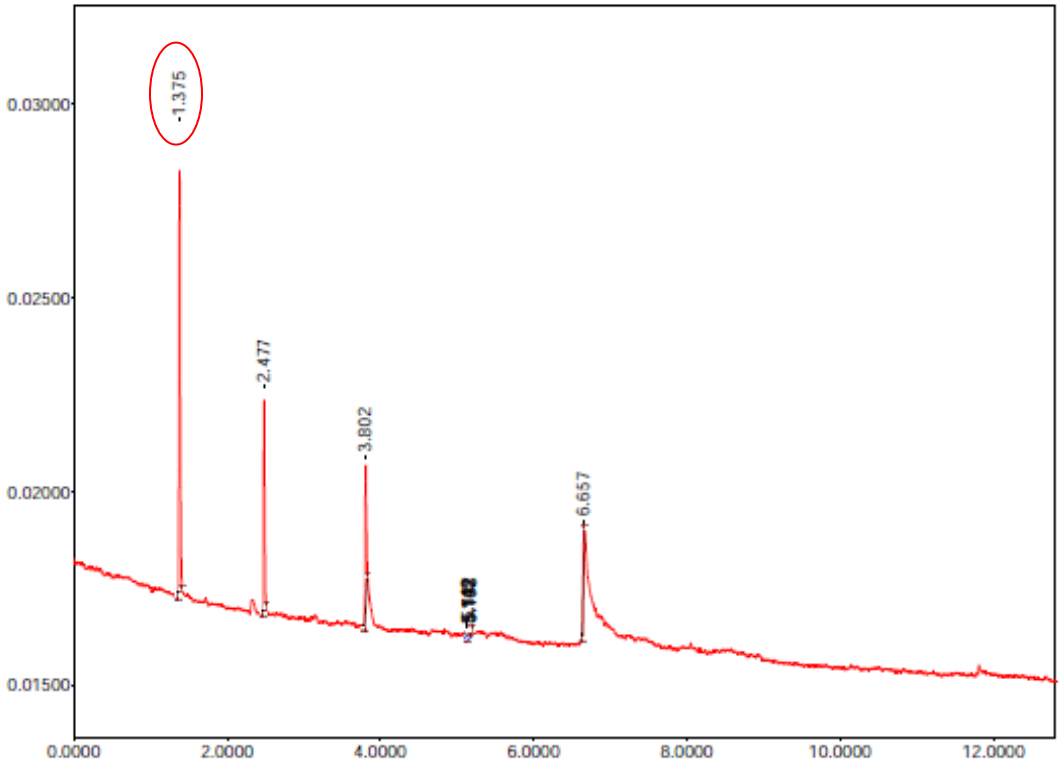
#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	1.373	12.747	BB	3.100	99.068
2	7.255	0.120	BB	0.800	0.932

(a) Gas chromatograph of standard nitrogen gas



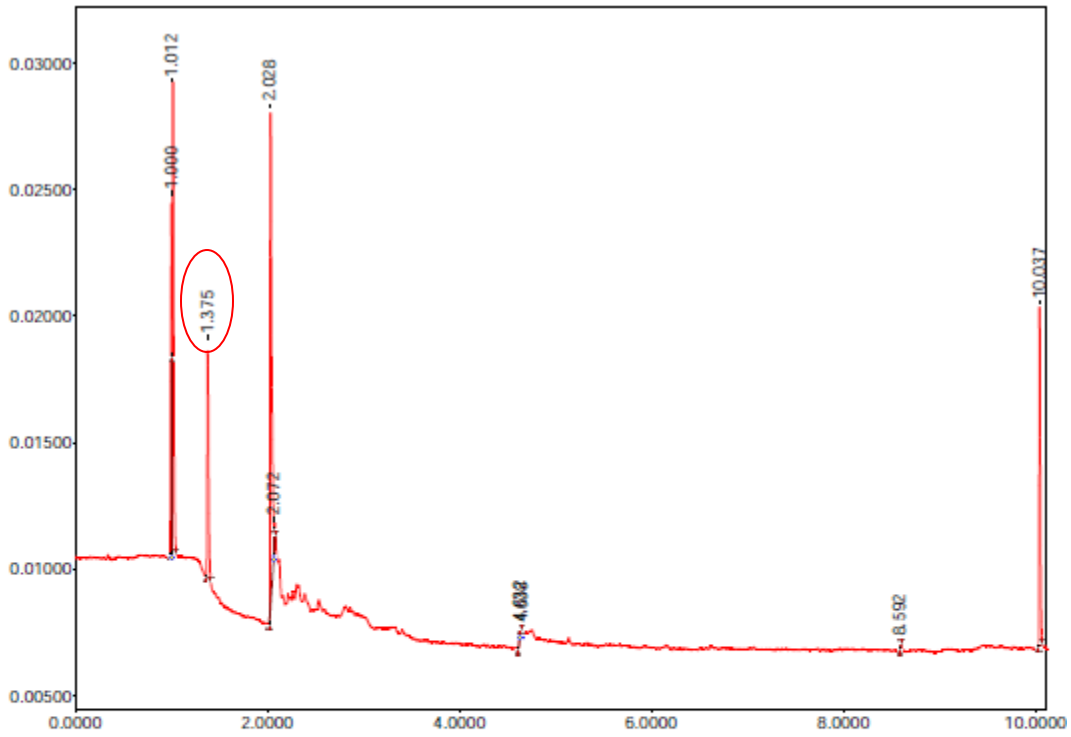
#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	1.375	4.254	BB	2.600	44.424
2	4.317	0.260	BB	1.100	2.714
3	5.168	5.062	BB	2.300	52.862

(b) Gas chromatograph of *Diaphorobacter* sp. R4



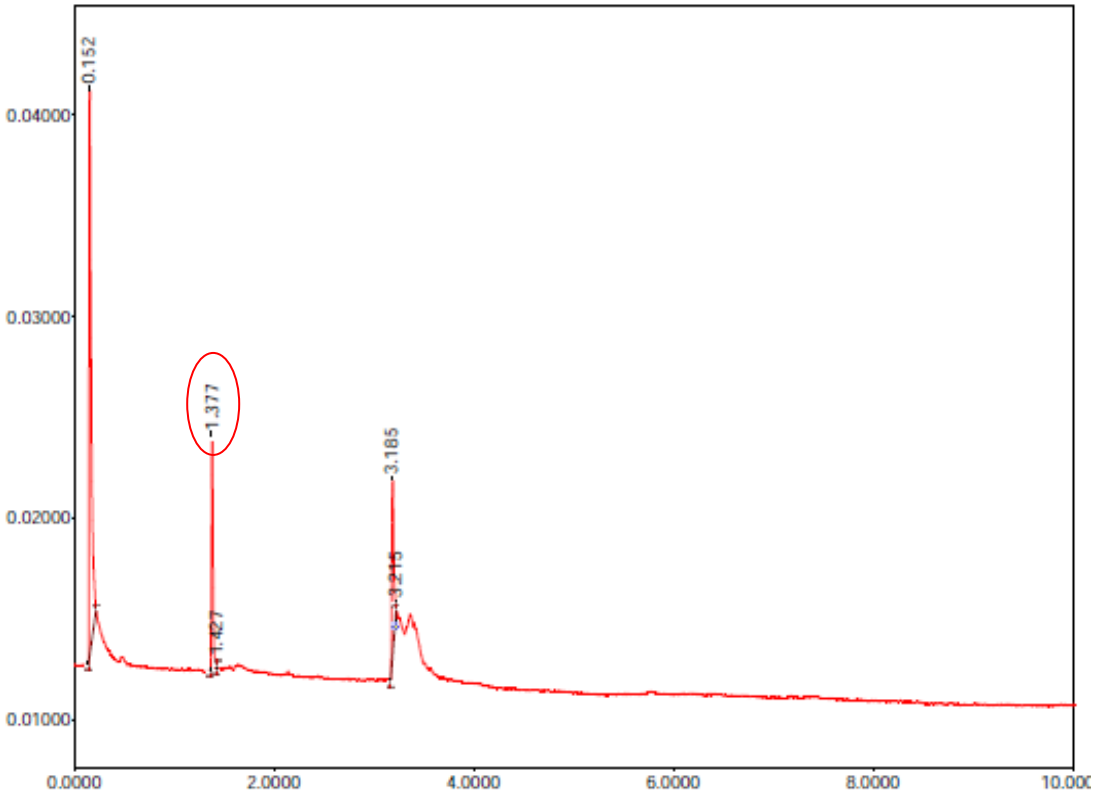
#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	1.375	12.477	BB	3.200	50.977
2	2.477	5.522	BB	2.400	22.561
3	3.802	4.856	BB	2.500	19.838
4	5.132	0.124	BV	0.700	0.506
5	5.142	0.061	VV	0.400	0.249
6	5.145	0.052	VV	0.500	0.211
7	5.172	0.087	VV	1.400	0.357
8	5.183	0.029	VB	0.800	0.120
9	6.657	1.268	BB	1.700	5.180

(c) Gas chromatograph of *Pannonibacter* sp. V5



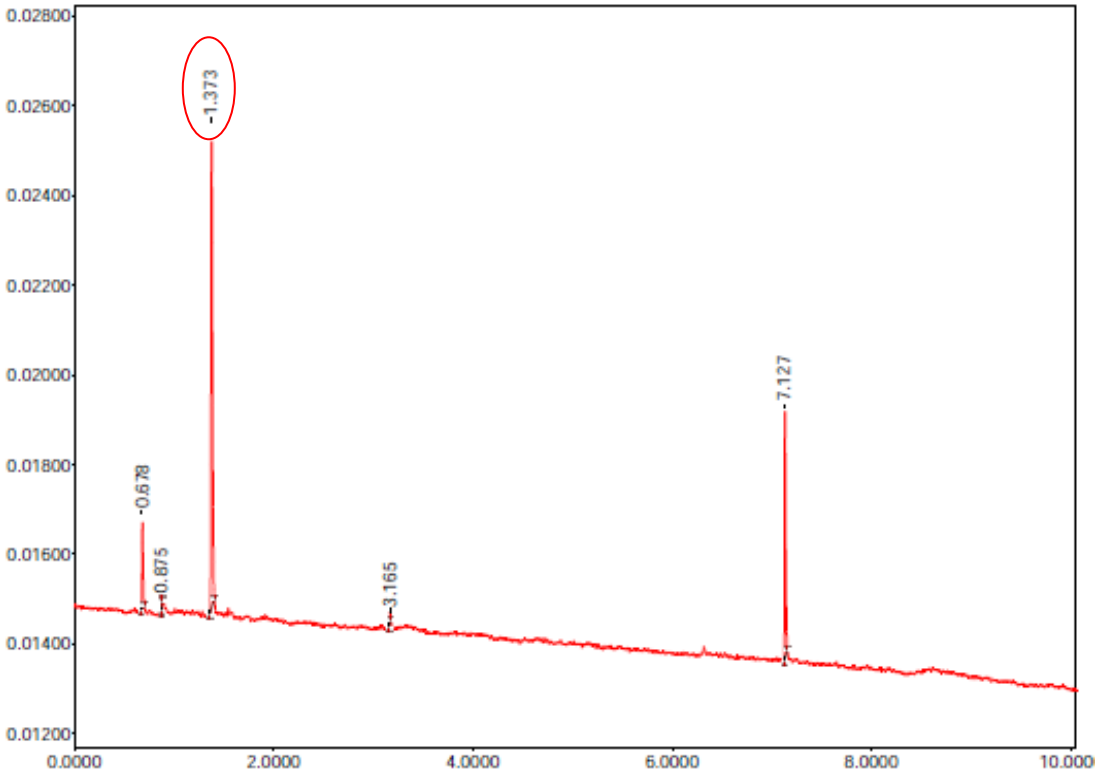
#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	1.000	12.795	BV	1.600	17.634
2	1.012	15.825	VB	2.600	21.809
3	1.375	9.441	BB	2.800	13.011
4	2.028	22.897	BV	2.900	31.556
5	2.072	0.706	VB	0.900	0.974
6	4.632	0.468	BV	1.400	0.644
7	4.638	0.106	VB	0.600	0.146
8	8.592	0.083	BB	0.600	0.115
9	10.037	10.240	BB	2.300	14.112

(d) Gas chromatograph of *Thauera* sp .V9



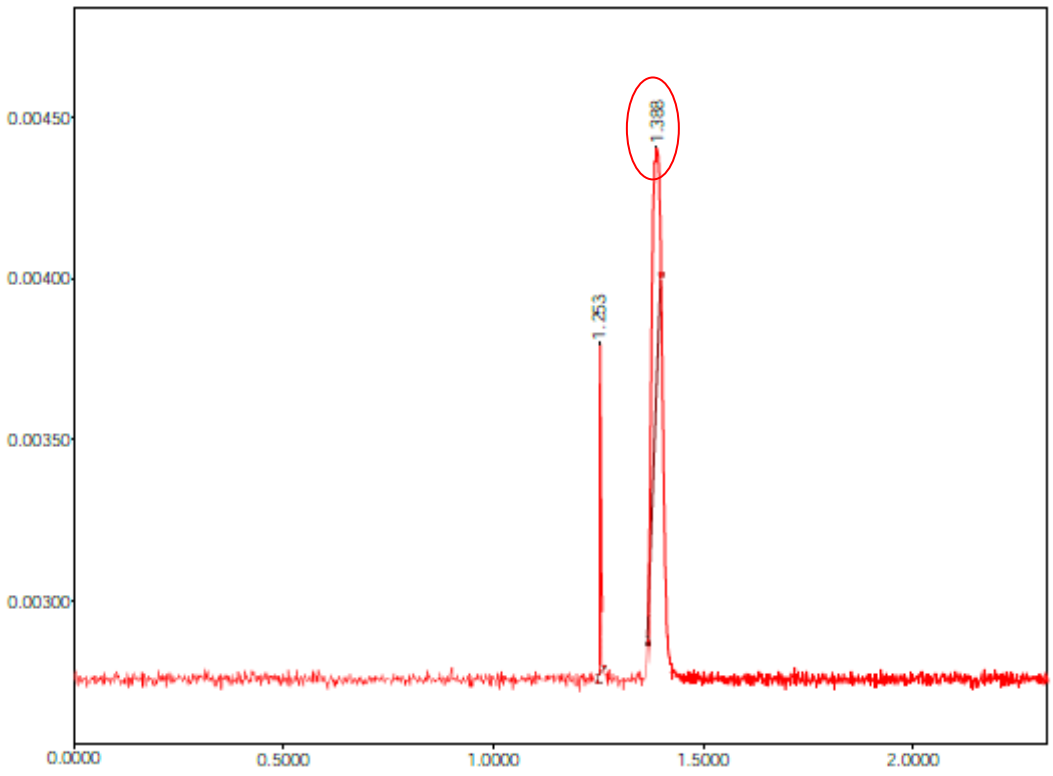
#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	0.152	41.221	BB	4.700	62.364
2	1.377	12.151	BP	3.600	18.384
3	1.427	0.097	PB	0.800	0.147
4	3.185	12.243	BV	2.700	18.522
5	3.215	0.385	VB	0.600	0.583

(e) Gas chromatograph of *Pseudomonas* sp.V11



#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	0.678	1.421	BB	1.700	8.474
2	0.875	0.073	BB	0.700	0.435
3	1.373	10.846	BB	2.800	64.686
4	3.165	0.142	BB	0.900	0.849
5	7.127	4.285	BB	2.100	25.556

(f) Gas chromatograph of *Thauera* sp.V14



#	RT(min)	Area(mV*sec)	Type	Width(sec)	Area%
1	1.253	0.231	BB	0.800	18.232
2	1.388	1.034	BB	1.900	81.768

(g) Gas chromatograph of consortium DC5

Figure 2.12 Gas chromatographs of N2 gas produced by the 5 isolates and consortium DC5 (encircled peak corresponds to N2 gas)

2.3.3.7 Kinetic analysis of nitrate removal by isolates

Nitrate removal rate by all the selected isolates and consortium of DC5 was investigated by using the zero-order kinetic model (Dhamole et al., 2008). The model can be described as follows:

$$C_t = C_i - K_0 t \quad \textbf{1}$$

Where Ci represents the initial NO_3 concentration (mg L^{-1}), Ct (mg L^{-1}) is the remaining NO_3 concentration at time (t), t is the time (h) and K_0 is the rate constant for zero order kinetic.K_0 constant was obtained from the slopes of the plots of Ct versus t for Eq. (1), and the regression coefficients (R^2). R^2 value for *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp. V9, *Pseudomonas* sp. V11, *Thauera* sp.V14 and consortium DC5 were 0.91, 0.90, 0.88, 0.95, 0.92 and 0.95 respectively suggested better fit of zero-order kinetic model (Fig. 2.13).

Nitrate removal rate constant for *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp. V9, *Pseudomonas* sp.V11, *Thauera* sp.V14 and consortium DC5 were 4.7, 4.6, 4.2, 5.9, 4.6, and 6.2 mg L^{-1} h^{-1} respectively. Among all the isolates *Pseudomonas* sp. V11 and consortium DC5 showed highest nitrate removal rate constant than other isolates. Results of a kinetics study also showed that there is a distinct advantage in using a consortium, where all the isolates showed a synergistic effect on each other, which ultimately increased nitrate removal efficiency.

Further, relative rates (RR) of nitrate reduction was calculated by using following formula

$$\mathrm{RR} = \frac{K_{NO3}}{K_{NO3} - K_{NO2}}$$

In the case of no nitrite build up RR would be equal to 1, and $KNO_2 = 0$. Whereas RR≥1 signifies build up of nitrite (Dhamole et al., 2008). Relative rates of all the selected isolates and consortium of DC5 was 1 suggesting no nitrite build up by the isolate when in consortium which is an important attribute for nitrate reduction by denitrifiers (Table 2.5).

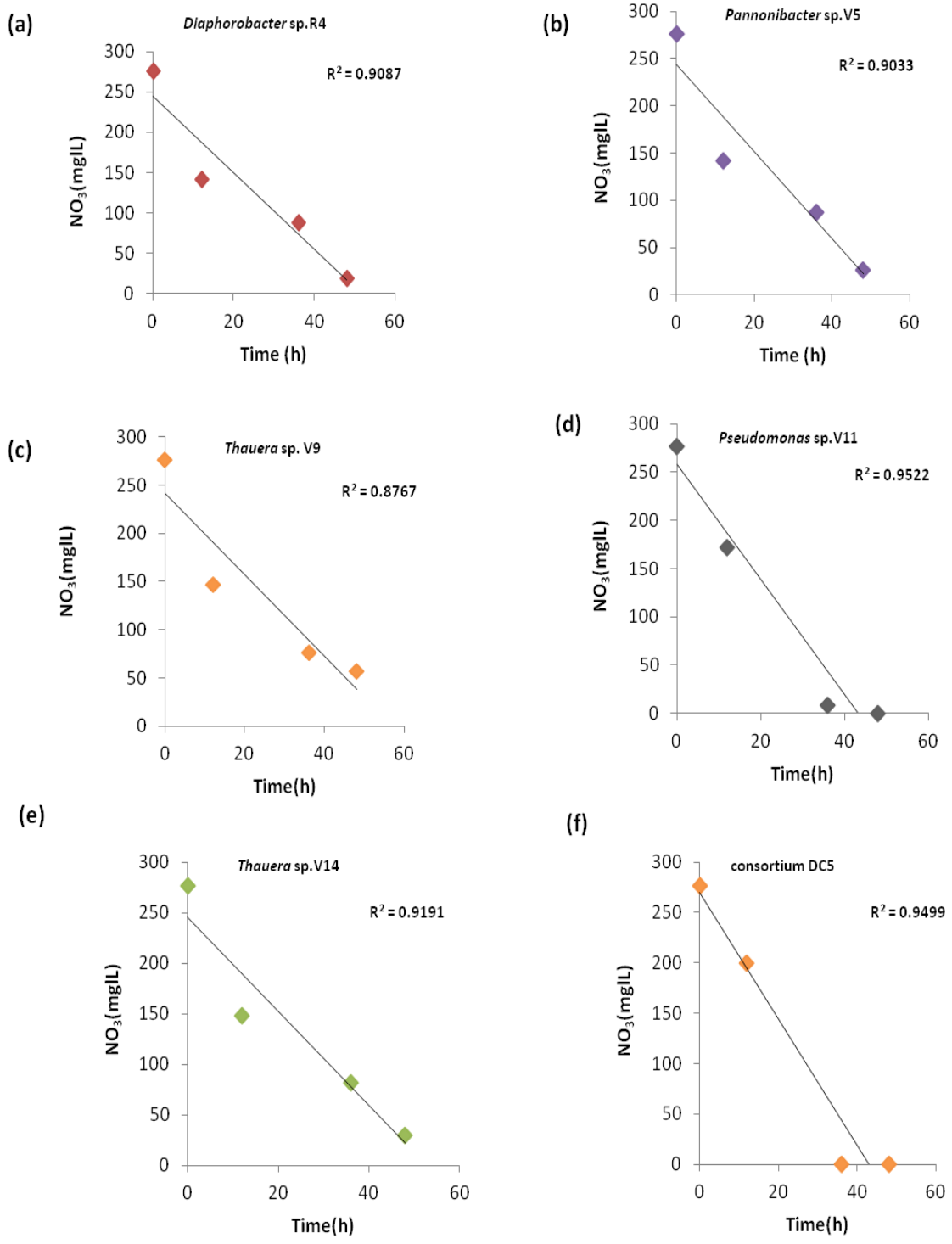


Figure 2.13 Kinetic model plots for the removal of nitrate by denitrifying isolates

Table 2.5 Relative rates of selected five isolates of consortium DC5 for nitrate removal

Isolates	Nitrate reduction rate mg L^{-1} h^{-1}	Nitrite formation rate mg L^{-1} h^{-1}	RR
Diaphorobacter sp.R4	4.75	0.017	1
Pannonibacter sp.V5	4.63	0	1
Thauera sp.V9	4.2	0	1
Pseudomonas sp.V11	5.9	0	1
Thauera sp.V14	4.6	0	1
Consortium DC5	6.2	0	1

2.3.4 Effect of different factors on biofilm formation and denitrification efficiency of consortium DC5 in microtiter plate assay

The consortium DC5 would be further used for bioaugmentation of denitrifying MBBR henceforth termed as dMBBR. Before initiating the reactor studies various parameters influencing the biofilm formation and denitrification ability of the consortium DC5 were studied.

2.3.4.1 Effect of different metal ions

Certain nutritional factors are known to influence biofilm formation. Mainly cations have a role in biofilm development through physio-chemical interactions, gene regulation, signal transmission and protein component function (Wang et al., 2019b). This plays positive role in biofilm structural stability because of the interaction between divalent cations and negatively charged functional groups of EPS (Higgins and Novak, 1997; Mangwani et al., 2014). Based on these studies effect of different metal ions was checked. 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.5 mM K_2HPO_4 increased biofilm forming ability of consortium DC5 whereas $FeCl_3$ did not show any effect on biofilm (Fig. 2.14). No significant effect was observed on the denitrification efficiency of consortium DC5 (Fig. 2.15). Calcium and magnesium were reported to affect the initial attachment via assisting in film conditioning, bridging between molecules, changing cell surface adhesins, and lowering the effect of surface charge and surface potential. They can

influence biofilm formation both directly and indirectly by acting as key cellular cations and enzyme cofactors and by influencing electrostatic interactions (Fletcher, 1988). These cations have an impact on the mechanical properties of biofilms by acting as cross linkers (Koestgens et al., 2001). Results of this study were similar to the results of Srinandan et al., (2010); Somerton et al., (2015) and Haque et al., (2017) who also reported that Mg^{2+} and Ca^{2+} enhanced the biofilm formation. He et al., (2016) reported that cells of *Bacillus* sp. aggregate more rapidly in the presence of 10 mM Ca^{2+} possibly due to the intermolecular interaction between Ca^{2+} and EPS via ionic contact.

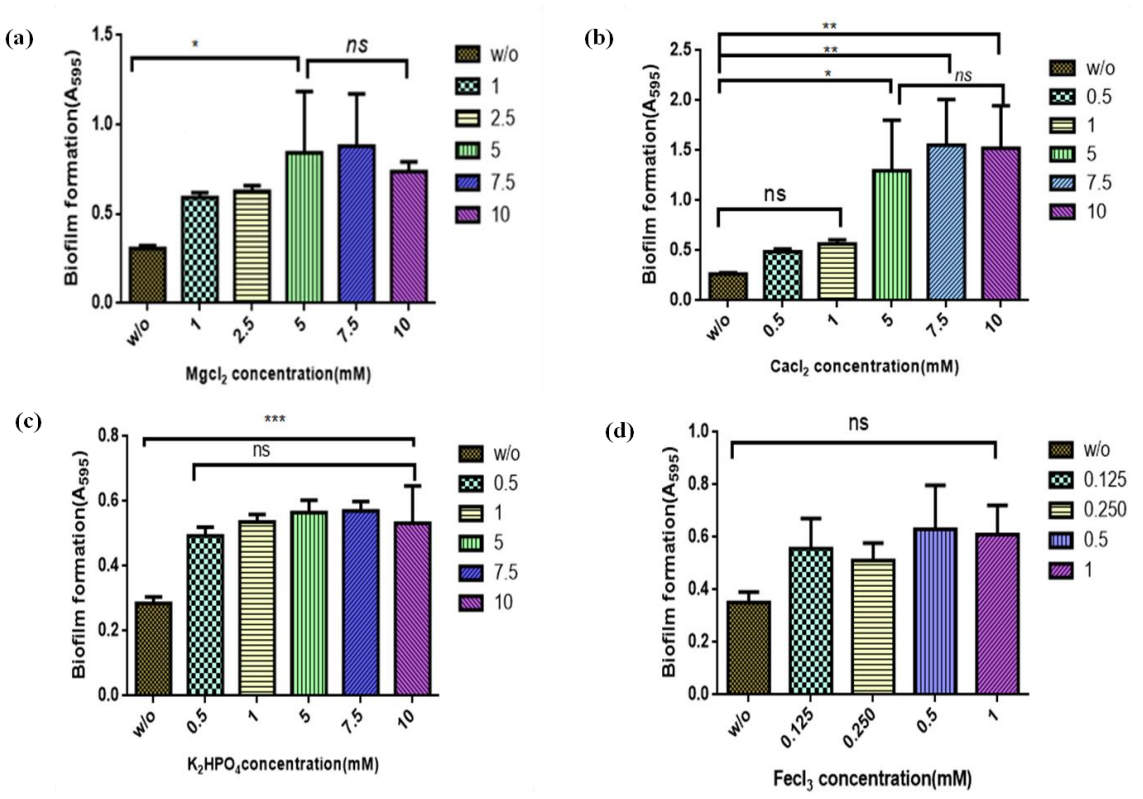


Figure 2.14 Effect of metal ions biofilm formation of consortium DC5. (a) Mg^{2+}, (b) Ca^{2+}, (c) K^{+} , (d) Fe^{3+}.
Error bars represent standard deviations, n=3. * $p<0.05$, ** $p<0.01$, ***<0.001

2.3.4.2 Effect of pH

pH has a significant impact on nitrate removal , bacterial growth , metabolism and is one of the most important factor influencing bacterial denitrification ability (Zhang et al., 2012b). It affects cell-to-cell immobilization and hence, biofilm formation by altering the hydrophobic properties (surface charge) of microorganisms. The optimal pH for

denitrification was 7-9, as observe in case of most environmental denitrifying bacteria (Glass and Silverstein, 1998; Tang et al., 2011; Cai et al., 2015).

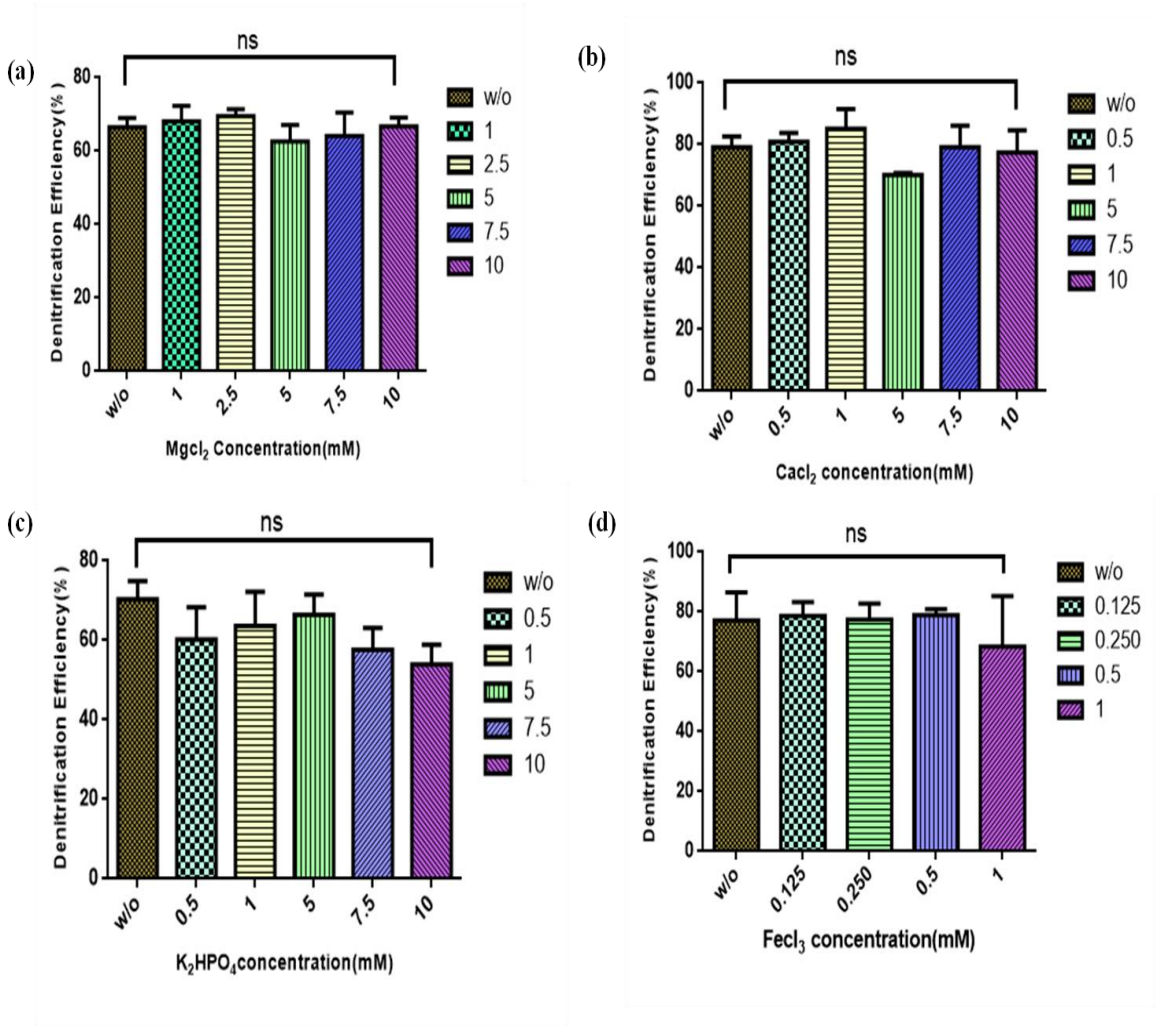


Figure 2.15 Effect of metal ions on denitrification efficiency of consortium DC5.(a) Mg^{2+} (b) Ca^{2+} (c) K^{+} (d) Fe^{3+}.
Error bars represent standard deviations, n=3.

In this study, acidic pH like pH 3 and pH 5 showed around 70 % less denitrification efficiency than alkaline pH and lower biofilm forming ability of the consortium DC5. At higher pH (pH7, pH9, pH12) relatively higher denitrification (80 %) as well as biofilm formation was observed (Fig. 2.16 a, b). Results of these studies were also similar to the pure culture of *Pseudomonas* species, where it was shown that the optimum pH for denitrification is in the range of 7.0 to 7.5 (Knowles, 1982). In an SBR denitrification system, it was reported that the highest denitrification

was achieved at pH values of 7.5-9.0 (Glass and Silverstein, 1998).

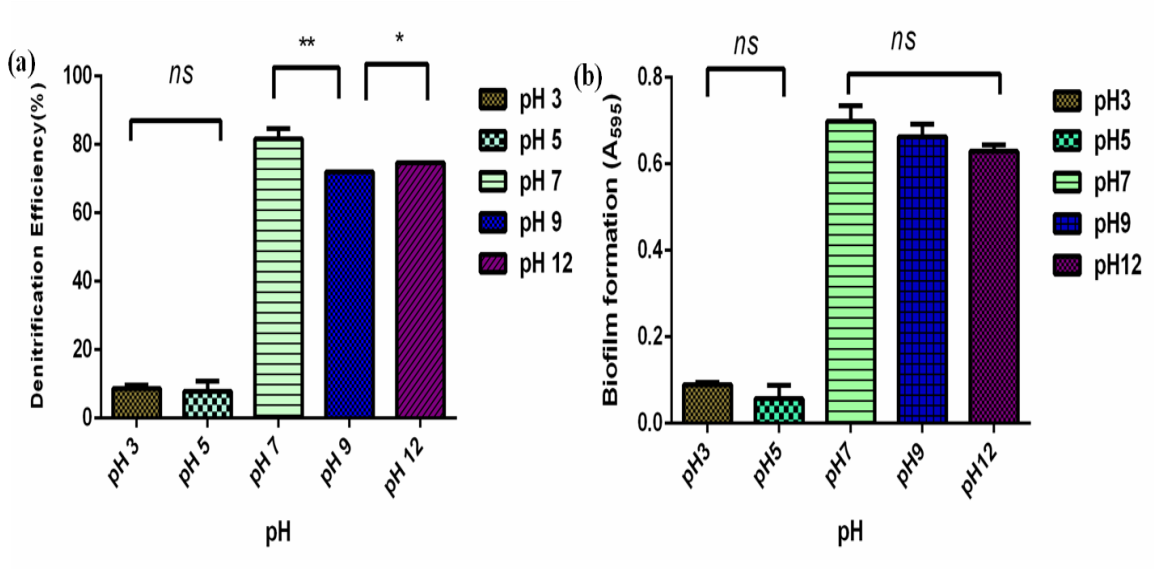


Figure 2.16 Effect of pH on (a) Denitrification efficiency and (b) Biofilm formation of consortium DC5.
Error bars represent standard deviations, n=3. * $p<0.05$, ** $p<0.01$.

2.3.4.3 Effect of different carbon sources

Organic carbon is often limiting substrate for biological denitrification in many wastewater treatment plants. To avoid incomplete denitrification and nitrite accumulation, organic carbon is provided externally as an electron donor. Extra carbon sources are added to balance the denitrification process. The common organic carbon sources (e.g. methanol, acetate and ethanol) and alternative carbon sources (e.g. hydrolysis products of primary sludge and solid waste, glycerin-based byproduct of biofuel production) have been used for denitrification (Peng et al., 2007). These carbon sources influence nitrate removal activity, community structure and biofilm architecture (Srinandan et al., 2012; Li et al., 2016). In the heterotrophic denitrification process, acetate, methanol, glucose and ethanol have been more effective carbon sources. These are simple carbon sources with high nitrate reduction efficiency because they are easy to utilize and provide an adequate amount of electrons for denitrification (Mohan et al., 2016).

As shown in Fig. 2.17a except glucose, all the carbon sources showed similar denitrification efficiency above i.e. 90 %. All the carbon sources produced biofilm equivalent to 1 OD absorbance while ethanol showed biofilm formation less than 1 OD absorbance. In presence of glucose only 6 % denitrification efficiency was observed and in presence ethanol 38 ± 3 % less biofilm was formed compared to other carbon sources by consortium DC5 (Fig. 2.17a,b). Growth of individual members of consortium DC5 in MM2 medium supplemented with different carbon sources was checked by semi-quantitative PCR (Fig. 2.18). All the isolates of the consortium demonstrated growth when glucose, sodium acetate and ethanol were used as carbon sources. Glycerol and methanol selected only *Diaphorobacte*r sp. R4 and eliminated other genera of the consortium while molasses allowed the growth of all other genera and eliminated *Diaphorobacter* sp. R4 (Table 2.6). Sodium acetate as carbon source showed the highest denitrification efficiency, biofilm formation and showed the growth together by all the isolates, therefore it was selected for further studies. In literature, sodium acetate was considered as better carbon source among others (Onnis-hayden and Gu, 2008). Sodium acetate is also known as an easily degradable carbon compound for many bacteria which enhanced nitrogen removal in wastewater by increasing the denitrification rate of organisms (Isaacs et al., 1994; Isaacs and Henze, 1995; Hallin et al., 1996)

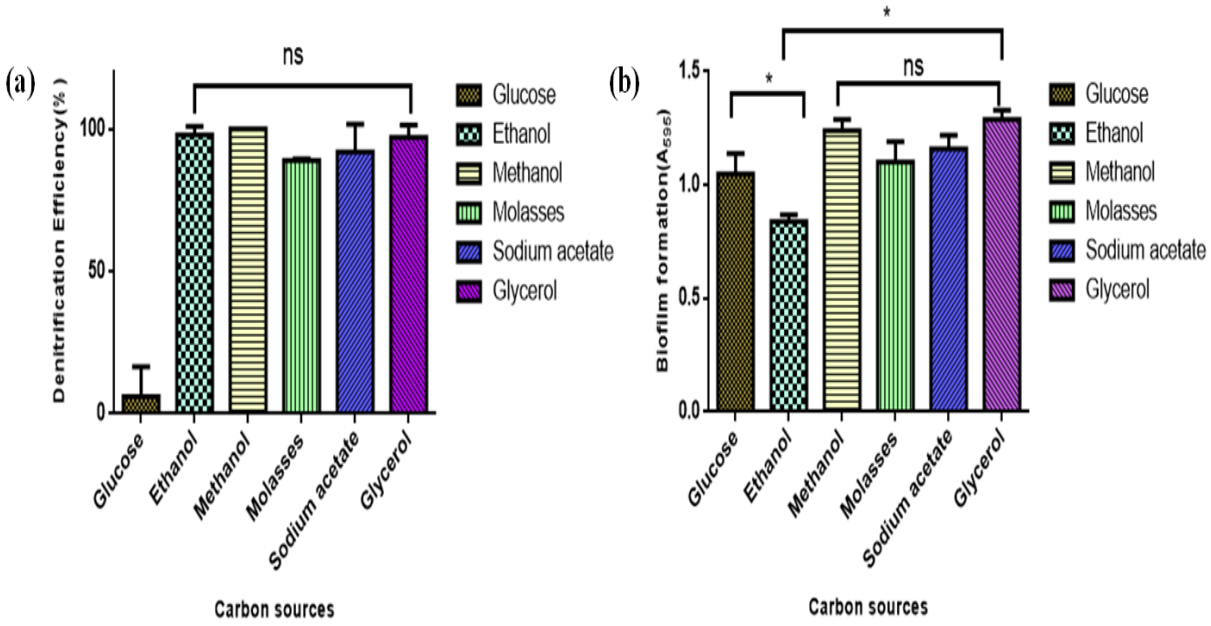
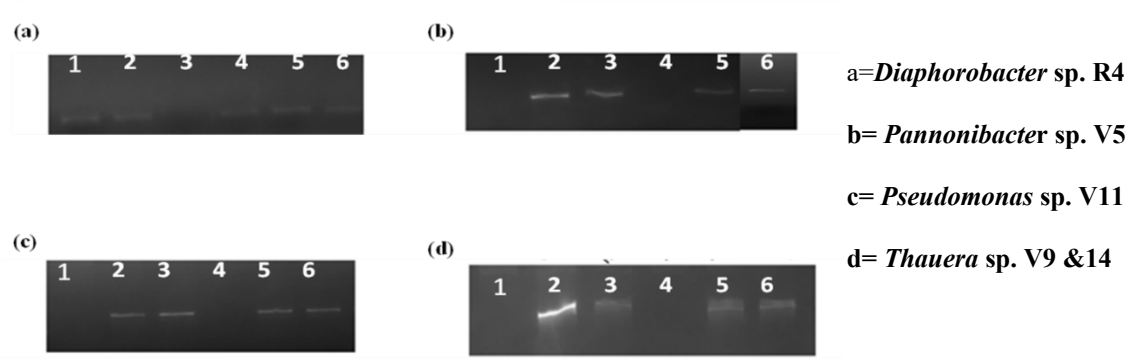


Figure 2.17 Effect of different carbon sources on (a) Denitrification efficiency (b) Biofilm formation of consortium DC5.
Error bars represent standard deviations, n=3 * p< 0.05.



(1. Glycerol, 2.Glucose, 3.Molasses, 4. Methanol, 5. Ethanol, 6.Sodium acetate)

Figure 2.18 Semi-quantitative PCR analysis showing amplicons of individual isolates of consortium DC5 when grown together in different carbon sources

Table 2.6 Growth of individual members of consortium DC5 in MM2 medium supplemented with different carbon sources in terms 16S rRNA gene amplification by semi-quantitative PCR analysis

Carbon sources	*Diaphorobacter* sp. R4	*Pannonibacter* sp. V5	*Thauera* sp. V9 &14	*Pseudomonas* sp. V11
Glycerol	+	-	-	-
Glucose	+	+	+	+
Molasses	-	+	+	+
Methanol	+	-	-	-
Ethanol	+	+	+	+
Sodium acetate	+	+	+	+

(+ presence of organism; - absence of organism)

2.3.4.4 Effect of inoculum concentration

The initial inoculum size has a dramatic effect on the quantity of biofilm formed. According to Su et al., (2017b) when the inoculum size was low (5 %), the concentration of bacteria was not enough in number to remove nitrate. Similarly, when the inoculum size was high (15 %), too many bacteria competed against each other, which affected the removal of nitrate negatively. Different percentage of inoculum was inoculated in the microtiter plate, however, no significant difference was observed in denitrification efficiency and biofilm forming ability of consortium DC5 (Fig.2.19 a, b).

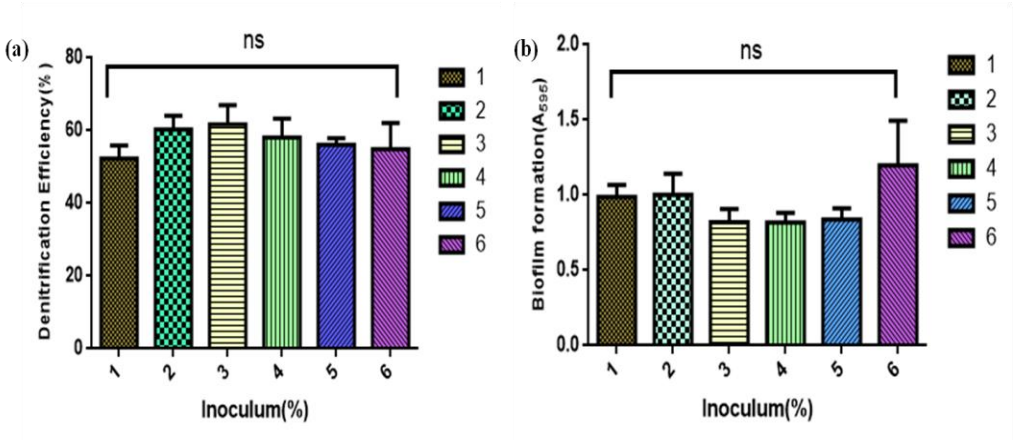


Figure 2.19 Effect of different inoculum (%) on (a) Denitrification efficiency (b) Biofilm formation of consortium DC5.
Error bars represent standard deviations, n=3.

Table 2.7 Optimized parameters affecting biofilm forming ability and denitrification efficiency of Consortium DC5

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 %)

To summarize, the studies here include use of conventional enrichment approach to isolate biofilm forming denitrifying bacteria to develop a consortium (DC5) as a special seed for the dMBBR studies undertaken in subsequent chapter. The parameters optimized for biofilm formation and denitrification efficiency of consortium DC5 listed in Table 2.7 were taken into account in the dMBBR studies.