Chapter 4

Characterization of the biofilm produced by the selected bacterial isolates

4.1 Introduction

Microbiota monitoring is important for the efficient performance of biological treatment systems. Recent developments in Molecular Biology have made new molecular tools available, such as next generation sequencing (NGS) to characterize the total microbial diversity in wastewater treatment processes (Shomar et al., 2020). Use of NGS technologies such as whole metagenomic sequencing has made it possible to get a large number of sequences, which is usually enough to indicate the complexity of a microbial community in a given sample. It also provides a great opportunity and new insights to reveal the composition of microbial communities, the diversity of functional genes and enzymes responsible for the treatment of various wastewaters. Most studies have been focused on the bacterial community structure and only a few have studied the effect of bioaugmentation on prokaryotic microbial community structure (Sul et al., 2016). Santhanarajan et al., (2021) suggested that augmentation aided indigenous microbial community successions and allowed successful bioaugmentation. It also promotes particular microbial communities, suggesting that these metabolic pathways efficiently functioning in positive correlations with each other. It is also used to monitor the diversity of microbial communities involved in nitrogen fixation processes such as, nitrification, ammonia oxidation and denitrification in the nitrogen removal processes (Yu and Zhang, 2012; Shchegolkova et al., 2016; Sul et al., 2016; Fang et al., 2018). From the above perspective, this chapter characterizes the carrier associated biofilm wherein the results of the effect of bioaugmentation of consortium DC5 on community structure and functional potential of biofilm developed in dMBBR are discussed. Here, the diversity of bacteria in biofilm grown on carrier media in dMBBR after 300 days of continuously working was analyzed using metagenomics approach. The dMBBR was showing efficient performance even after 300 days and the inoculum was added only once at the starting of the dMBBR. Therefore, it was also of interest to study the carrier biofilm community structure in dMBBR that was seeded with inoculum of specially selected bacterial consortium DC5.

4.2 Materials and Methods

4.2.1 Microbial community analysis of biofilm developed in optimized denitrifying MBBR

4.2.1.1 gDNA extraction from denitrifying biofilm

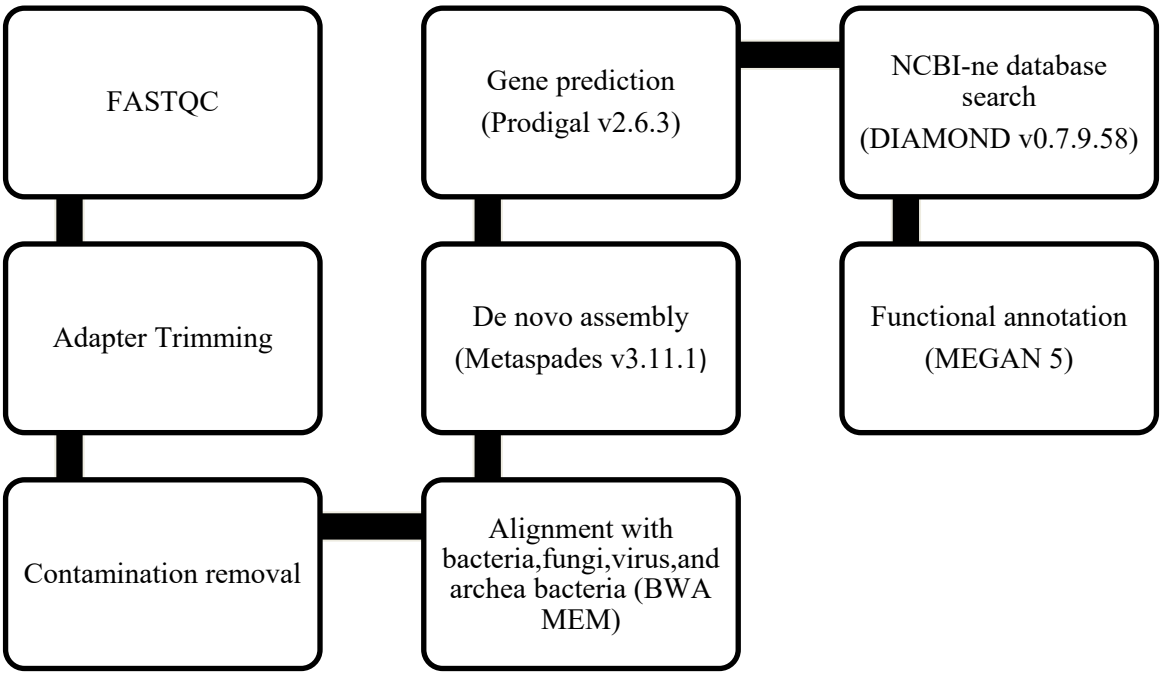
Total three biofilm samples were taken from different parts of the dMBBR. Each biofilm sample contained biofilm pooled from twelve different carriers taken from different parts of the reactor. Genomic DNA from biofilm samples was extracted by using the modified CTAB method (Andreou, 2013). Biofilm developed on the carriers of dMBBR was scrapped and resuspended in 10 ml of PBS. Resuspended biomass was centrifuged at 8000 rpm for 5 min. Pellet was resuspended in 500 µl of Tris-EDTA-Sucrose of buffer (25 mM TrisCl (pH8), 25 mM EDTA (pH8) and 300 mM Sucrose). Then, 8 µl of lysozyme (10 mg/ml) was added to the system and incubated at 37 °C for 1 h. After incubation 10 % SDS was added and samples were mix properly, and kept in water bath at 60 °C for 1 h. Then 200 µl of 5 M NaCl and 80 µl of 10 % CTAB were added and incubated at 65 °C for 10 min. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added and 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. Then double volume of absolute alcohol was added and incubated at chilled temperature for 24 h. After incubation centrifugation was done at 10,000 rpm for 10 min, the supernatant was discarded and the pellet was air dried and resuspended in sterile milliQ water. Resuspended DNA was treated with 3 µl of 0.01 % RNase was added to it and incubated at 65°C for 10 min and stored at 4 °C which was preserved in cold condition. Presence of genomic DNA was detected by 0.8 % agarose gel. The extracted metagenomic DNA was purified using a Geneipure ^{TM} Quick PCR Purification Kit as per the manufacturer's instructions. The quality and quantity of extracted DNA were assessed using agarose gel electrophoresis and Nanospectrophotometer, respectively.

4.2.1.2 Whole metagenome sequencing

Extracted metagenomic DNA from biofilm was outsourced to MEDGENOME Bangalore; India. Sequencing was done by using the Illumina HiSeq X system to generate 2X150 bp sequence reads.

4.2.1.3 Post-sequencing analysis

The work flow for post sequencing analysis was carried out as the following flow chart.



Organism's abundance was predicted up to species level by using the Kraken2 tool. Principle component analysis (PCA) was performed in PAST v 3.21 (Hammer et al., 2001) to determine the correlation (association) between isolates. The assembled metagenomic sequences of biofilm samples were also uploaded to the MG-RAST server to check its functional potential annotations (Wilke et al., 2017).

4.3 Results and Discussion

Whole genome metagenomics of carrier associated biofilm developed inside continuous dMBBR

Whole genome metagenomic approach of biofilm samples gave a comprehensive characterization of microbial taxa and metabolic pathways present in the microhabitat, which facilitated an opportunity to understand the function of dMBBR in the present case. Whole genome metagenome of carrier associated biofilm generated around 7-9 Gb data for three samples. The data obtained from three samples termed as Biofilm1, Biofilm 2 and Biofilm 3 generated 7.74, 8.17 and 8.23 Gb data respectively. Phred quality score Q30 % was above 85 % for all samples and average GC % was around 63 %.

4.3.1 Abundance of phyla, genus and species in carrier associated denitrifying biofilm

As depicted in Fig. 4.1, Proteobacteria was the most abundant phylum in all the three biofilm samples collected from different locations of the dMBBR. Results of this study were consistent with the cited literature where MBBR developed with activated sludge had Proteobacteria as the dominant phylum (Guo et al., 2019). Phylum Proteobacteria contained large number of Gram-negative denitrifying bacteria. Heterotrophic denitrifying bacteria were found to be important contributors to the COD and nitrogen removal processes that played an important role in treating high nitrate wastewater (Su et al., 2017a, b). Therefore, it can be said that higher abundance of denitrifying Proteobacteria in dMBBR was beneficial for better denitrification performance. While the other phyla present in dMBBR in minor proportions were Actinobacteria, Firmicutes, Bacteroidetes, Planctomycetes, Cyanobacteria, Chloroflexi, Euryarchaeota, Deinococcus, Thermus and Verrucomicrobia.

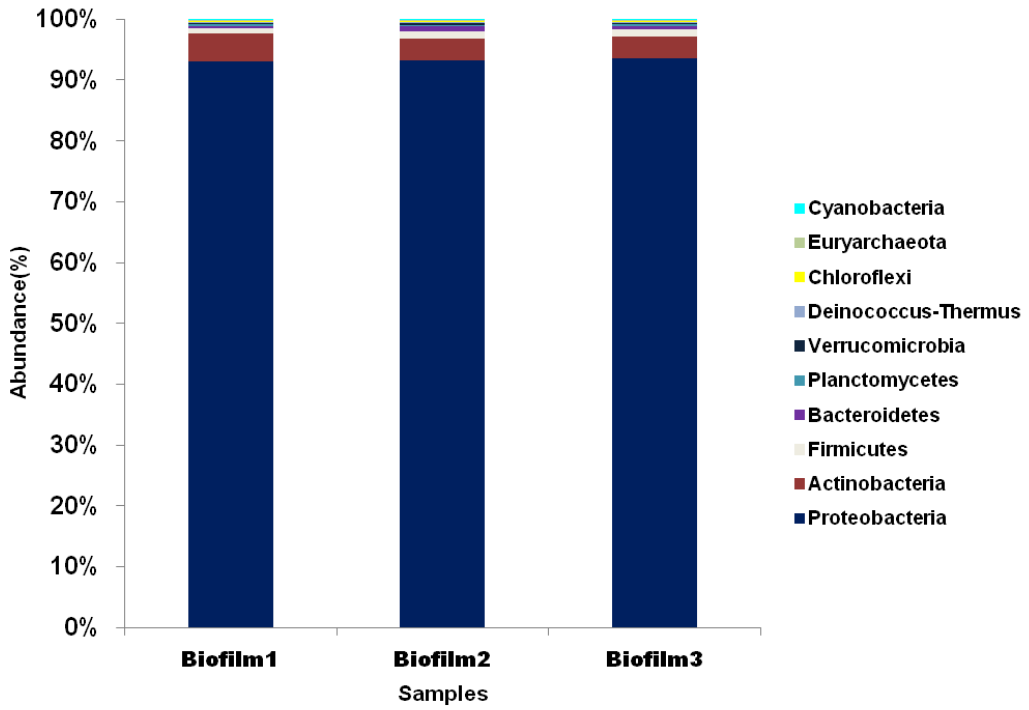


Figure 4.1 Abundance of different phyla in denitrifying biofilm developed with consortium DC5

Further, at genera level *Thauera* and at species level *Thauera humireducens* and *Thauera* sp. MZ1T were the most abundant in all the biofilm samples (Fig.4.2 & Fig.4.3). Abundance of *Thauera* in all the biofilm samples suggested that the conditions prevalent in the developed dMBBR were most suitable for *Thauera* spp. *Thauera* represents a metabolically heterogeneous group with a broad range of capabilities, such as obligate autotrophic, facultative autotrophic and heterotrophic denitrification (Xing et al., 2018). It has been reported that *Thauera* sp. plays an important role in anaerobic/anoxic/oxic reactor (Zhao et al., 2018) and denitrifying biofilter (Cui et al., 2017). *Thauera* has emerged as an important genus in the investigation of metabolic versatility and remediation of different environmental pollutants (Liu et al., 2013). This result emphasized that *Thauera* spp. played a key role in dMBBR since it showed from 45 % to 60 % abundance. The next abundant genus amounting to about 10 % was *Paracoccus* species that are good denitrifiers. Among the different species of *Thauera, T.* sp. MZ1T, *T.humireducens, T. aromatic, T.* sp. K11 and *T.chlorobenzoica* were shown to be present in the biofilm while, among *Paracoccus, P. denitrificans* and *P. aminovorans* species were shown to be present which are potential organism to remove high nitrates from

wastewaters without accumulation of intermediates like nitrite (Srinandan et al., 2011) (Fig 4.3).

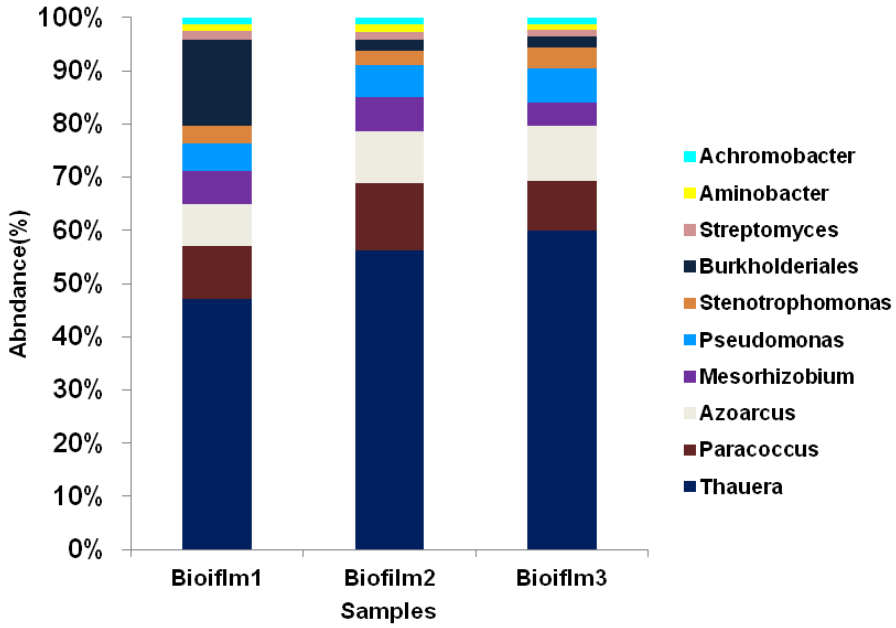


Figure 4.2 Abundance of different genera in denitrifying biofilm developed with consortium DC5

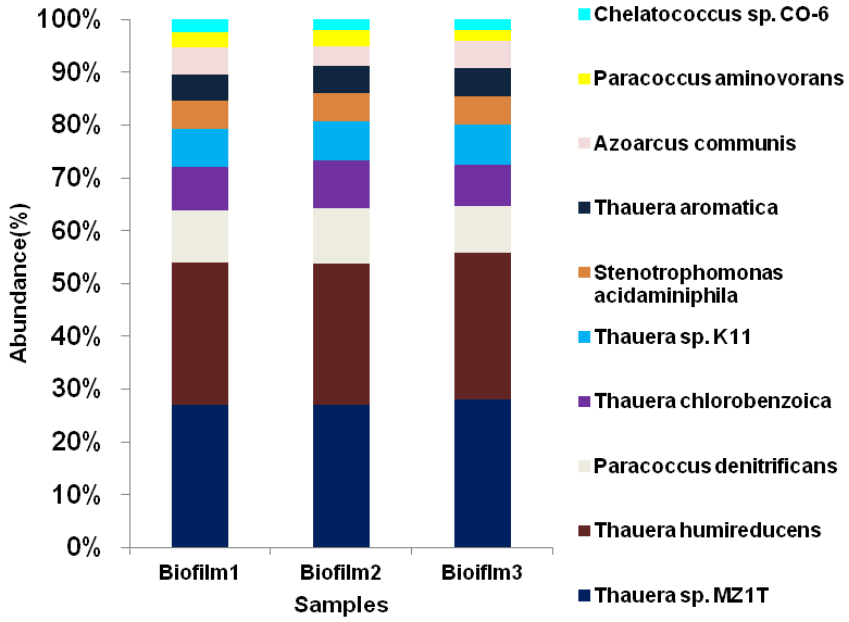
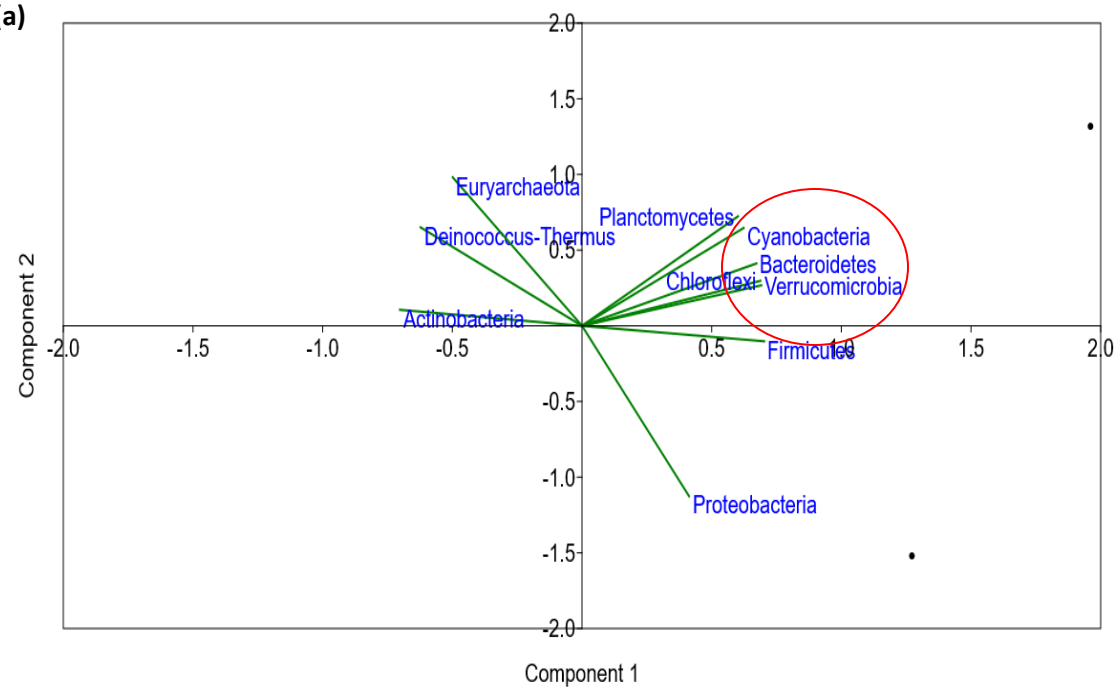


Figure 4.3 Abundance of different *Thauera* spp. and *Paracoccus* spp. in denitrifying biofilm developed with consortium DC5

Principle component analysis (PCA) revealed that at phylum level *Cynobacteria, Chloroflexi, Bacteroidetes, Verrucomicrobia* and *Firmicutes* were positively correlated with each other (Fig.4.4a). At the genera level *Pseudomonas*, *Thauera* and *Azoarcus* were positively correlated with each other and negatively with *Paracoccus* (Fig.4.4b).This might be due to their preference for different carbon sources during denitrification. *Azoarcus* and *Thauera,* primarily consume acetate and butyrate, respectively whereas *Paracoccus* consumes butyrate, valerate and propionate over acetate (Albuquerque et al., 2013). Since the carbon source provided was acetate the predominance of *Thauera* and *Azoarcus* over *Paracoccus* was noted. Also, positive correlation between these genera might be due to similar biochemical pathways of denitrification in genus *Pseudomonas, Azoarcus* and *Thauera* (Heider and Fuchs, 2015). Among the five members of the consortium DC5 used as seed, *Thauera* spp. and *Pseudomonas* were found functionally active in long term operated dMBBR. In the PCA analysis, at the species level, *Thauera* sp. MZ1T*, Thauera humireducens, Thauera aromatic* and *Thauera* sp. K11 were positively correlated with each other (Fig.4.4c). Results of PCA analysis suggested that bioaugmentation of consortium DC5 increased relative abundance of other known denitrifying bacteria such as *Paracoccus*, *Azoarcus*, *Stenotrophomonas*, *Chelatococcus* etc.

(a)



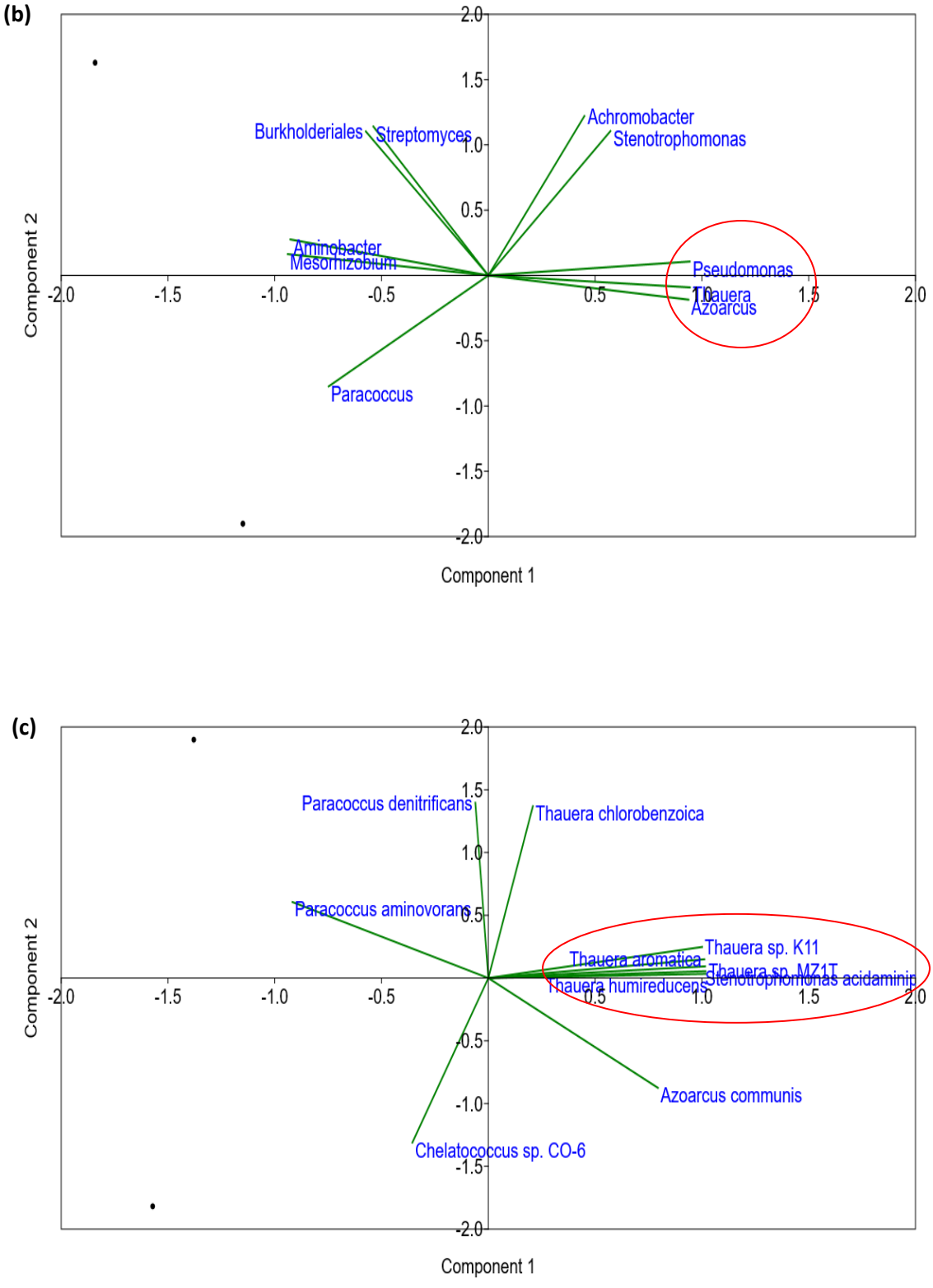


Figure 4.4 PCA component analysis (a) Phyla level (b) Genera level (c) Species level

Functional metabolic pathways in carrier associated biofilm of consortium DC5

4.3.2 Functional potential evaluation KEGG database

Functional profile of microbes can be categorized mainly into two groups. (i) The housekeeping genes that are included in the first group encode essential enzymes involved in the metabolism of carbohydrates, proteins, amino acids, lipids, fatty acids, DNA and RNA, cofactors and vitamins, cell walls and capsules, respiration, membrane transport, cell division and cell cycle, cell signaling, motility and chemotaxis (ii) Second group of genes are those that are involved in specific function such as stress response, virulence, toxic compound resistance, xenobiotic substance metabolism and degradation.

In this study genes assigned to the metabolism of carbohydrates, amino acids and energy metabolisms were numerously found in the biofilm since they are related to the housekeeping functions of all living organisms. Biofilm developed inside dMBBR possesses high abundance of metabolic functions (57 %) followed by genetic information processing (16 %), environmental information processing (20 %) and cellular processes (7 %) at KO (Kegg Orthology) level 1(Fig.4.5a). KO level 2 included genes responsible for cell growth and death (2 %), cell motility (4 %), transport and catabolism (1 %), membrane transport (11 %), signal transduction (7 %), folding, sorting and degradation (3 %), replication and repair (5 %), transcription (2 %), translation (7 %), amino acid metabolism (20 %), carbohydrate metabolism (11 %) at the respective percentages mentioned in the bracket. High abundance of genes involved in amino acid metabolism indicates that amino acids provide carbon and energy source for bacterial metabolism (López-González et al., 2015). High abundance of carbohydrate metabolism genes implies that the biofilm bacteria can decompose complex organics into more easily degradable matter (Wei et al., 2018). The amino acids and carbohydrates also act as electron donors and carbon sources for denitrification in the developed biofilm (Cui et al., 2019). Carbohydrate metabolism, mainly including central carbon metabolism (glycolysis, citrate cycle and pentose phosphate pathway), produces the ATP for energy or NADPH for reducing power, which is necessary for cell metabolism. Genes involved in energy metabolism (7 %) mainly included those of oxidative phosphorylation and

nitrogen metabolism. They were mostly related to the degradation of phosphate and nitrogen containing pollutants (Yan et al., 2021). Genes involved in xenobiotics biodegradation (1 %) and genes of biosynthesis of secondary metabolites (6 %) are playing important role in defense against other microorganisms and against harmful stresses such as toxins or UV exposure, were also identified in the developed biofilm (Fig.4.5b).

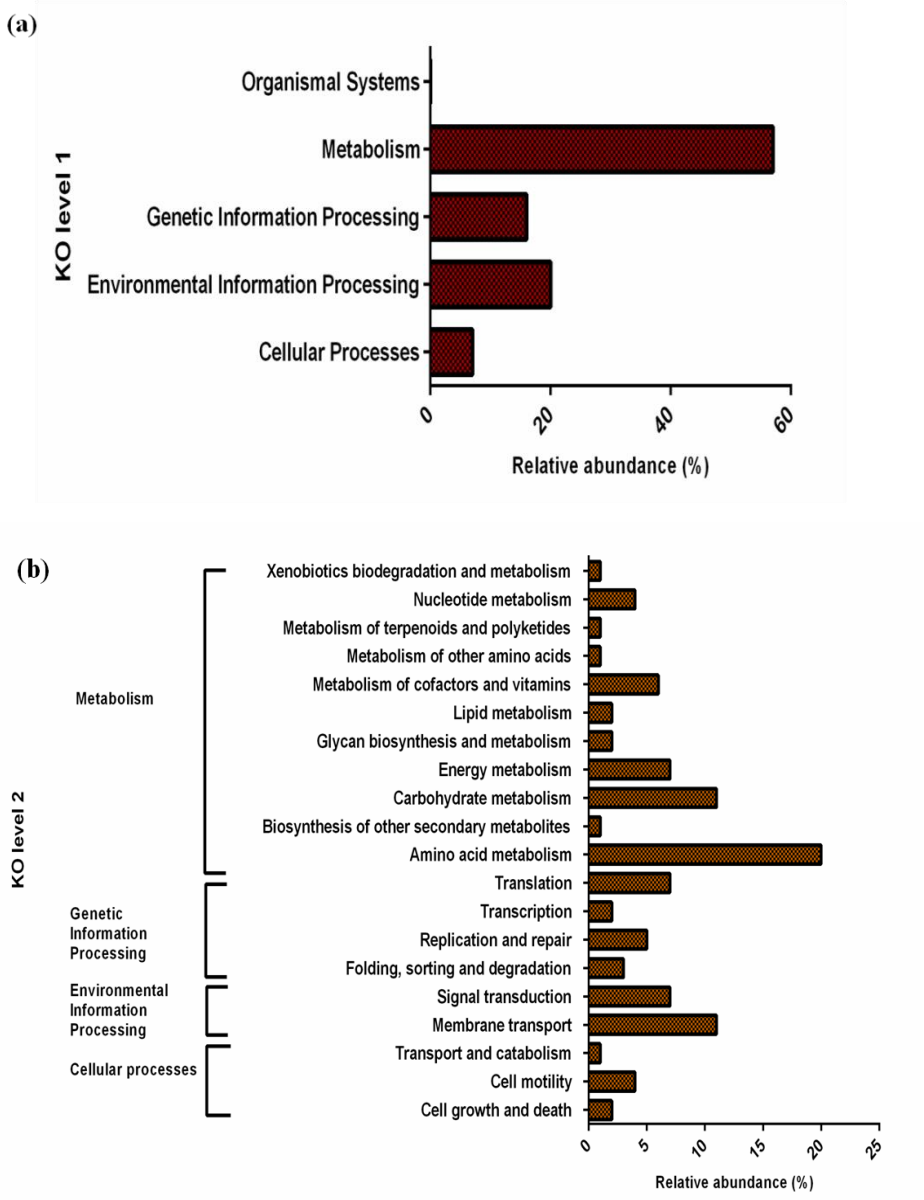
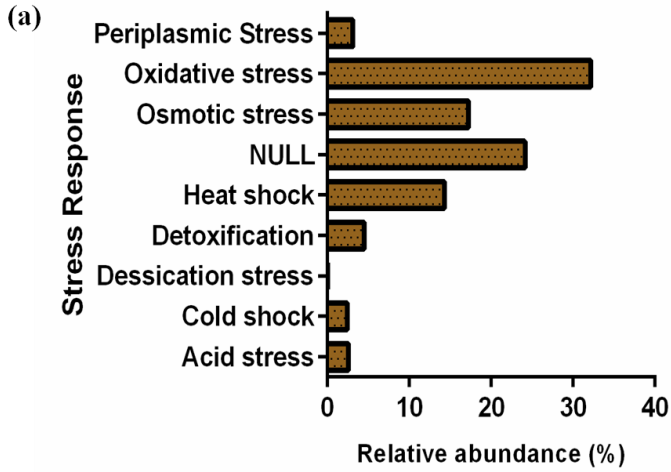


Figure 4.5 Relative abundance of genes of metabolic pathways in denitrifying biofilm developed with consortium DC5 (a) KO level 1 (b) KO level 2

4.3.3 Functional profiles for heavy metal resistance, antibiotic resistance and toxic compounds at subsystem level using MG-RAST

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 (Fig.4.6a). Genes involved in antibiotic resistance involved six main mechanisms: antibiotic efflux, antibiotic cell permeability reduction, antibiotic inactivation, antibiotic degradation inside cell, target modification and target bypass and protection (Boolchandani et al., 2019). Here, out of the known six mechanisms of antibiotic resistance, the major mechanism in developed biofilm must be antibiotic efflux pumps (Fig.4.6b). Various genes related to antibiotic resistance such as multidrug resistance efflux, fluoroquinolones, fosfomycin, vancomycin, strptothricin, erythromycin, methicillin and multidrug resistance clusters annotated in developed biofilm of dMBBR suggests that wide resistance to various antibiotics was present in the microorganisms contained in the carrier biofilm. Genes related to toxic compounds such as copper homeostasis, mercury resistance, arsenic resistance, cobalt-Zinc-Cadmium resistance, chromium, etc. were also annotated in denitrifying biofilm (Fig.4.6b). Presence of genes related to heavy metal resistance, antibiotic resistance and toxic compound metabolism suggested that denitrifying biofilm developed with consortium DC5 can tolerate various antibiotic and toxic compounds present in wastewaters and can be used as a potential microbial seed for the treatment of nitrate containing effluents.



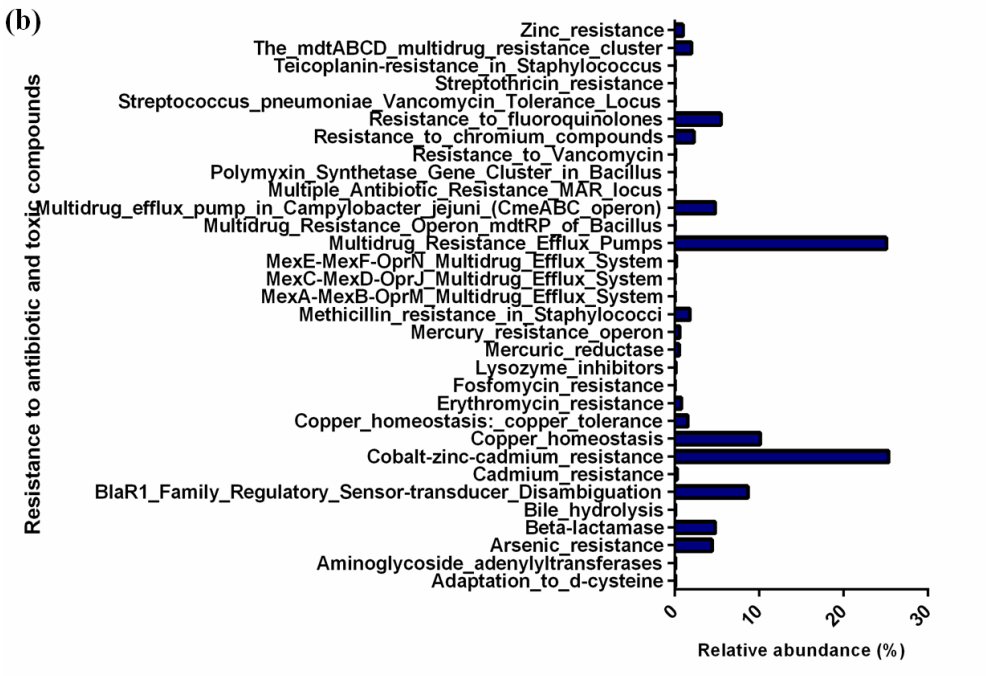


Figure 4.6 Relative abundance of genes resistance to heavy metal, antibiotic and toxic compounds in biofilm developed by consortium DC5

(a) Relative abundance of genes involved in stress response by using SEED subsystem level 2 analysis using MG-RAST server (b) Relative abundance of genes involved in antibiotic and toxic compounds resistance

Genes and functions associated with organic contaminant degradation in biofilm developed inside dMBBR

Denitrification process is often associated with the degradation of organic compounds under anaerobic or anoxic conditions. Organic compounds provide electrons which are subsequently used by different electron acceptors (such as sulfate, nitrate and CO_2) which are further converted into harmless gases such as sulfide, nitrogen gas and biomethane gas under anaerobic or anoxic condition (Nzila, 2018) (Fig.4.7). Therefore, presence of genes associated with organic contaminant degradation and denitrification were also checked in developed biofilm with consortium DC5.

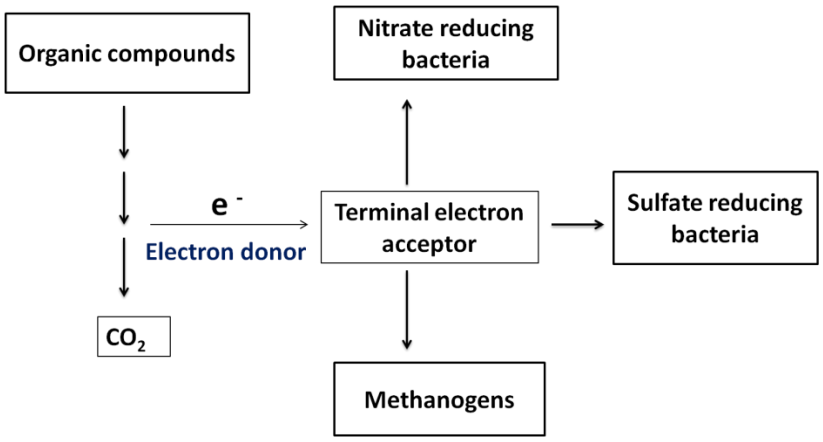


Figure 4.7 Different strategies of anaerobic/anoxic biodegradation of organic compounds in bacteria

4.3.4 Relative abundance of genes involved in nitrogen metabolism inside dMBBR biofilm

The metagenomic approach has been widely used to monitor the activities of nitrogen metabolism in wastewater treatment systems (Wang et al., 2017; Fang et al., 2018). From wastewater treatment to bioremediation, microbial nitrogen metabolism has industrial applications (Ye et al., 2001). In different environmental conditions, the biological process of nitrogen removal in wastewater takes a specific path, depending on the microbial communities involved and the substrates available (Shchegolkova et al., 2016; Ye and Zhang, 2013). It is significant in ecosystems with low levels of available oxygen, where bacteria prefer nitrate compounds as electron donors or acceptors for energy transformation and metabolism conservation (Erisman et al., 2007). Because resources are generally limited in perturbed ecosystems due to an imbalanced C: N ratio, nitrogen cycling is a crucial function in the polluted environment (Yergeau et al., 2014). The major pathway in nitrogen metabolism is the denitrification process which could be linked to carbon catabolic pathways including amino acid, fatty acid and carbohydrate degradation and could provide electron donors for nitrate reduction and efficient nitrogen removal could be linked to COD removal (Sul et al., 2016). Table 4.1 shows relative abundance of genes involved in nitrogen metabolism in biofilm associated with carriers in dMBBR.

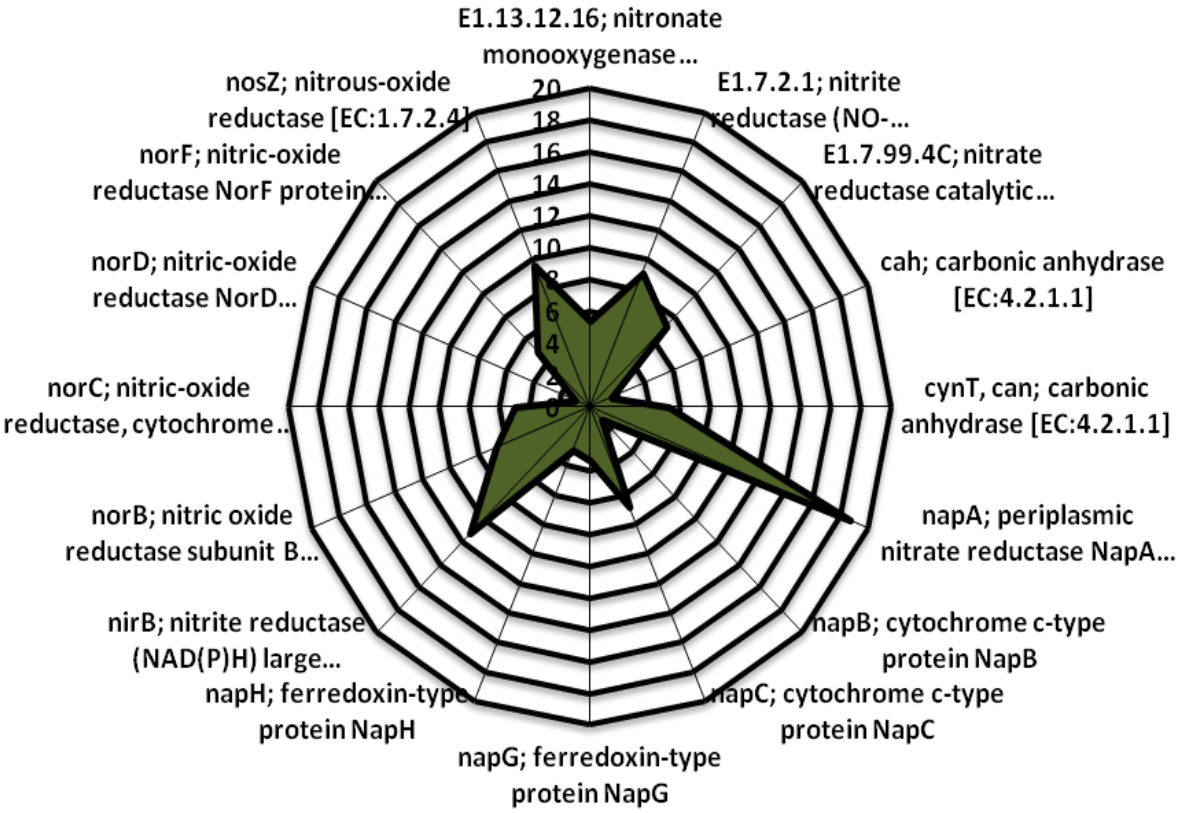
The relative abundance of genes for nitrogen metabolism were involved mainly in two pathways.

1) **Nitroalkane catabolism pathway -** In this pathway genes involved in nitronate monooxygenases (NMO) enzyme were abundantly present which are important for bioremediation of nitroalkanes compounds that are extensively found in the chemical industry.
2) **Denitrification pathway**- Presence of genes involved in nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase in the biofilm suggested complete dentrification pathway was most abundantly present in the dMBBR. Presence of genes coding for nitrous oxide reductase (EC 1.7.2.4) reducing the green house gas (N_2O) emitted further to N_2 is an important attribute of the dMBBR, as it is not contributing to green house gas emission and thus is beneficial for the environment. Fig.4.8b shows nitrogen metabolism pathways in detail.

Table 4.1 Relative abundance of genes involved in nitrogen metabolism

Genes	Relative abundance (%)	Function
Nitronate monooxygenase [EC: 1.13.12.16]	5.43	Oxidizes alkyl nitronates to aldehydes and nitrite. Nitronate monooxygenases (NMO)
Periplasmic nitrate reductase NapA [EC: 1.7.99.4]	18.63	Conversion of nitrate to nitrite
NapB	1.1	
NapC	6.82	
NapG	3.37	
NapH	2.94	
Nitrite reductase (nir) [EC:1.7.1.4]	20.38	Conversion of nitrite to nitric oxide
nitric oxide reductase (nor) [EC:1.7.2.5]	29.49	Conversion of nitric oxide to nitrous oxide
Nitrous-oxide reductase (nos) [EC: 1.7.2.4]	9.67	Conversion of nitrous oxide to nitrogen gas

(a)


E1.13.12.16; nitronate monooxygenase...
E1.7.2.1; nitrite reductase (NO-...
E1.7.99.4C; nitrate reductase catalytic...
cah; carbonic anhydrase [EC:4.2.1.1]
cynT, can; carbonic anhydrase [EC:4.2.1.1]
napA; periplasmic nitrate reductase NapA...
napB; cytochrome c-type protein NapB
napC; cytochrome c-type protein NapC
napG; ferredoxin-type protein NapG
napH; ferredoxin-type protein NapH
nirB; nitrite reductase (NAD(P)H) large...
norB; nitric oxide reductase subunit B...
norC; nitric-oxide reductase, cytochrome...
norD; nitric-oxide reductase NorD...
norF; nitric-oxide reductase NorF protein...
nosZ; nitrous-oxide reductase [EC:1.7.2.4]
20
18
16
14
12
10
8
6
4
2
0

(b)

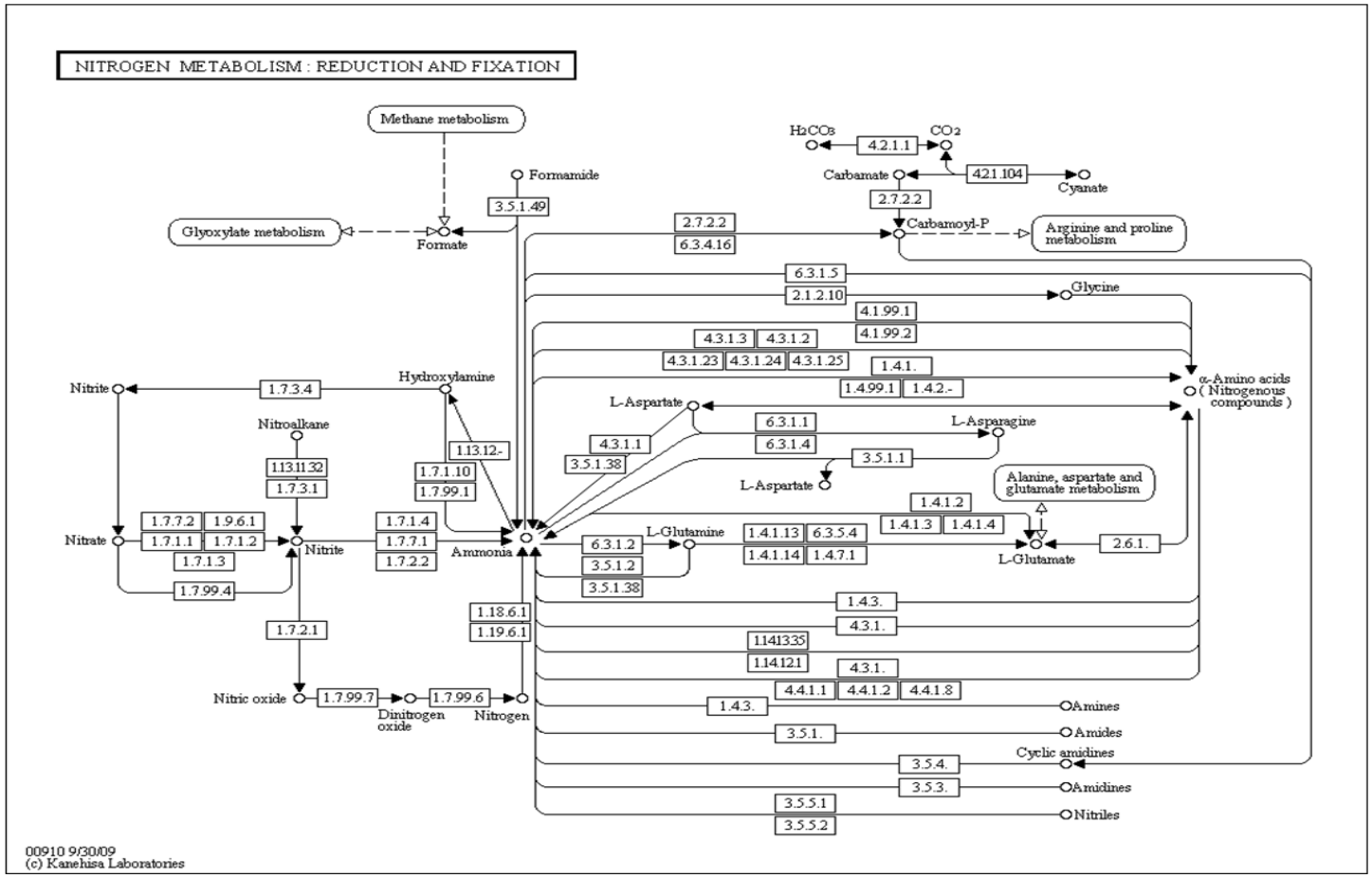


Figure 4.8 (a) Relative abundance of gene involved in nitrogen metabolism (b) Nitrogen metabolism pathways by KEGG mapper in biofilm associated with carriers in dMBBR

4.3.5 Relative abundance of genes involved in sulfur metabolism in dMBBR biofilm

Sulfide and nitrogen-containing contaminants are the primary toxic pollutants in municipal and industrial wastewater (Wang et al., 2019a). Sulfur metabolism plays an important role in central biochemistry as a carbon carrier and stable redox center (Klotz et al., 2011). In the absence of molecular oxygen and nitrate, microbes prefer sulfur compounds as electron donors or acceptors for energy transformation and metabolism (Purcell et al., 2011; Plugge et al., 2011; Yamamoto et al., 2011; Klotz et al., 2011). In denitrifying biofilm genes involved in Assimilatory Sulfate Reduction (ASR) pathway were abundantly present. ASR has been found to contribute to the sulfate removal in wastewater treatment (Wu et al., 2013; Yu et al., 2018). The assimilatory pathway produces reduced sulfur compounds for the biosynthesis of S-containing amino acids and does not lead to direct excretion of sulfide.

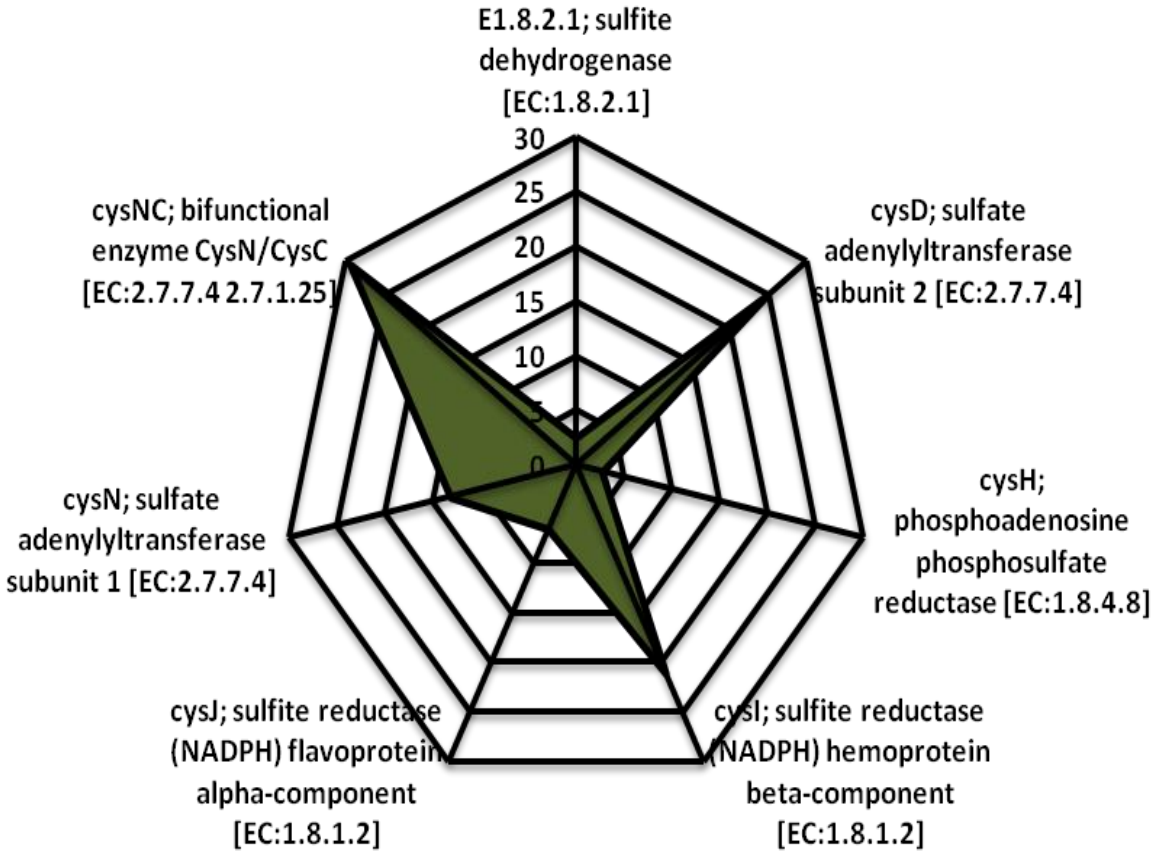
Table 4.2 shows abundance of genes involved in ASR pathway in biofilm associated with carriers in dMBBR. In ASR pathway sulfate is reduced to sulfide and transformed into cysteine, from which biological sulfur-holding molecules are formed (Grein et al., 2013; Günal et al., 2019). In the treatment of high-sulfate-containing wastewater, ASR is a more active sulfate-reducing pathway than Dissimilatory sulfate reduction pathway (DSR) (Li et al., 2020). Presence of genes associated with sulfate removal in biofilm indicated its potential for treating high sulfate containing wastewater (Fig.4.9a). It can be also suggested that biofilm developed with consortium DC5 can be used for the removal of simultaneous nitrate and sulfate that are generated from different effluents such as petroleum, pulp production and pharma industries effluents. Fig.4.9b shows sulfur metabolism pathways in detail.

Table 4.2 Relative abundance of genes involved in sulfur metabolism pathway

Gene	Relative abundance (%)	Function of genes
Sulfite dehydrogenase [EC:1.8.2.1]	2.53	Converts sulfite to sulfate
cysD [EC:2.7.7.4]	24.78	Sulfate to adenylylsulfate (APS)
cysH [EC:1.8.4.8]	2.73	Conversion of 3 phosphoadenylylsulfate

		(PAPS) to sulfite
cysI/cysJ [EC:1.8.1.2],	21.03	Converts sulfite to H_2S
cysJ [EC:1.8.1.2]	6.46	
cysN [EC:2.7.7.4]	13.09	Converts adenylylsulfate(APS) to 3 phosphoadenylylsulfate (PAPS)
cysNC [EC:2.7.7.4;2.7.1.25]	29.39	

(a)



(b)

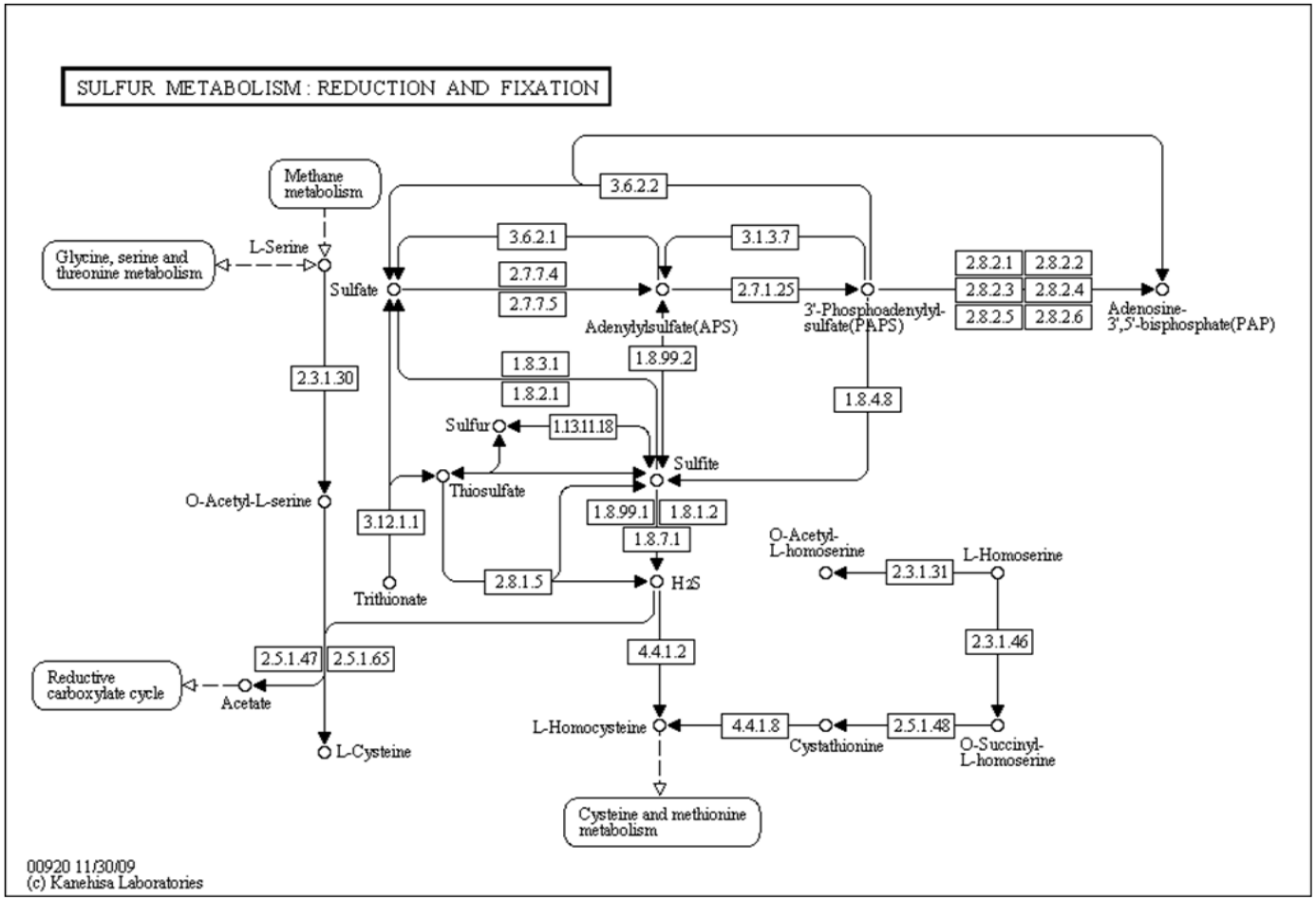


Figure 4.9 (a) Relative abundance of genes involved in sulfur metabolism (b) Sulfur metabolism pathways by KEGG mapper

4.3.6 Relative abundance of genes involved in methane metabolism in dMBBR biofilm

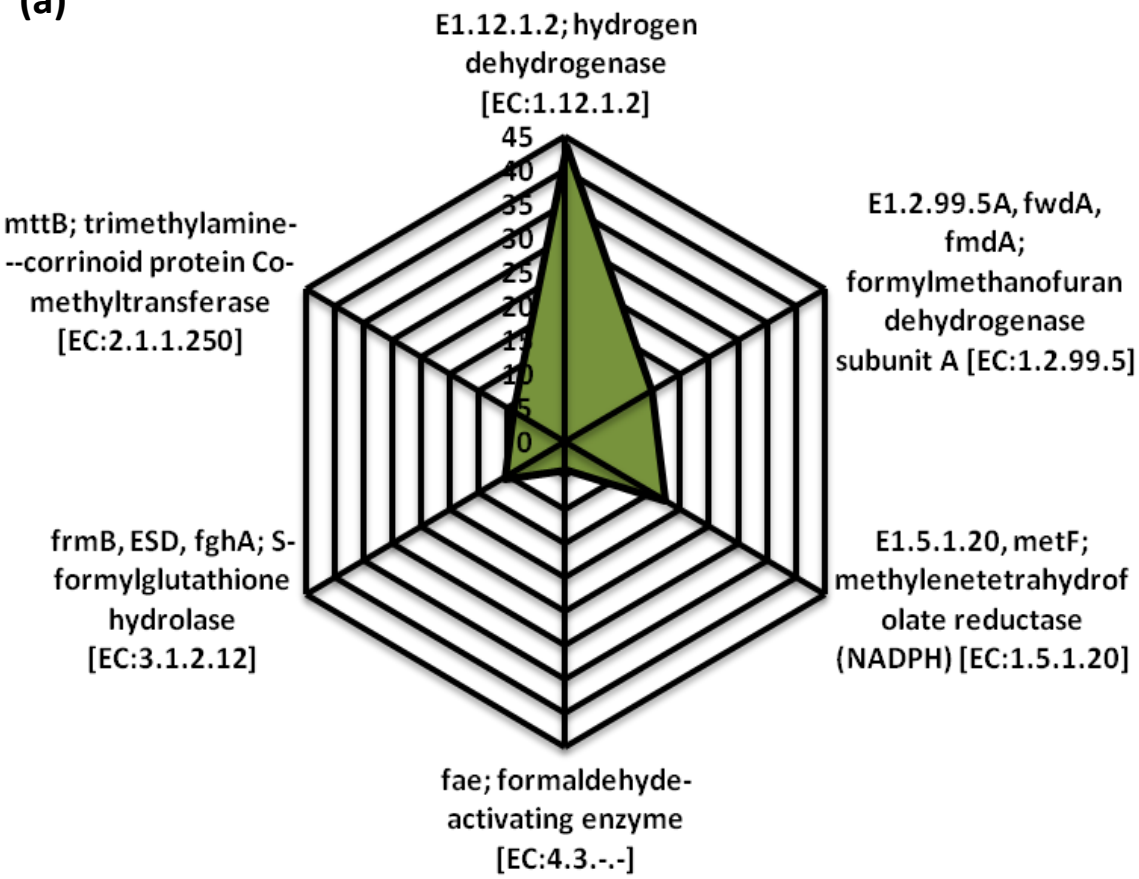
Anaerobic methane-driven denitrification process directly utilizes methane as electron donor that is abundant in biogas produced from anaerobic digestion of organic waste. In anoxic environments in presence of inorganic electron acceptors (e.g., sulfate or nitrate), methanogenesis plays important role in the biodegradation of organic matter. Table 4.3 shows relative abundance of genes involved in methane metabolism in biofilm associated with carriers in dMBBR. It showed the hydrogenotrophic methanogenic pathway was most abundant in denitrifying biofilm of MBBR that reduces hydrogen gas to formate or CO_2 to methane. Fig.4.10b shows methane metabolism pathways in detail. Andalib et al., (2011) have also reported that in biofilm processes under proper anaerobic conditions methanogenesis and denitrification process co-exist.

Presence of methane metabolism pathways also indicates that biofilm developed with consortium DC5 can effectively breaking down organic pollutant present in wastewater.

Table 4.3 Relative abundance of genes involved in methane metabolism

Gene	Relative abundance (%)	Function of genes
Hydrogen dehydrogenase [EC:1.12.1.2]	43.68	Reversibly interconvert protons and electrons to molecular hydrogen)
fwdA, fmdA,[EC:1.2.99.5]	14.92	Catalyzes the reversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate
metF genes[EC:1.5.1.20]	17.22	
frmB, ESD fghA[EC:3.1.2.12]	10.81	Hydrolses to formate and can be converted to carbon dioxide

(a)


E1.12.1.2; hydrogen dehydrogenase [EC:1.12.1.2]
45
40
35
30
25
20
15
10
5
0
E1.2.99.5A, fwdA, fmdA; formylmethanofuran dehydrogenase subunit A [EC:1.2.99.5]
E1.5.1.20, metF; methylenetetrahydrofolate reductase (NADPH) [EC:1.5.1.20]
fae; formaldehyde-activating enzyme [EC:4.3.-.-]
frmB, ESD, fghA; S-formylglutathione hydrolase [EC:3.1.2.12]
mttB; trimethylamine--corrinoid protein Co-methyltransferase [EC:2.1.1.250]

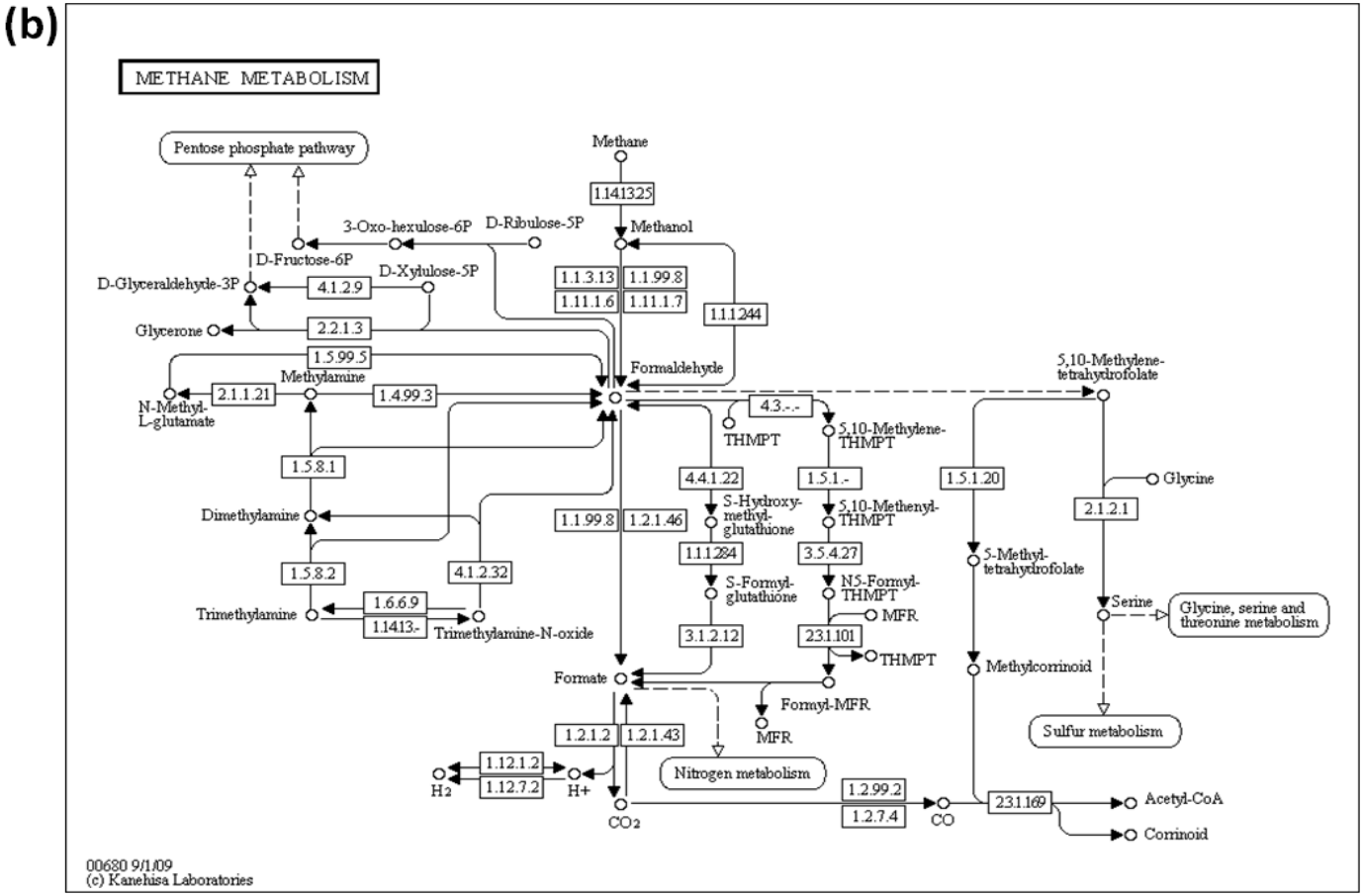


Figure 4.10 (a) Relative abundance of genes involved in methane metabolism (b) Methane metabolism pathways by KEGG mapper in biofilm associated with carriers in dMBBR

4.3.7 Xenobiotic biodegradation and metabolism genes in dMBBR biofilm

Prokaryotes can catabolize aromatic compounds in three ways: (i) peripheral (upper) pathways, (ii) central (lower) aromatic intermediates and (iii) anaerobic degradation of aromatic compounds. Xenobiotic compounds are chemicals that are foreign to microorganisms. Biotransformation is the metabolic modification of the molecular structure of a compound and involves the breakdown of the organic compound into less complex ones. Nineteen types of genes involved in xenobiotic biodegradation and metabolic compounds were identified in the biofilm of dMBBR. Table 4.4 shows abundance of different xenobiotic metabolic pathways present in biofilm. Benzoate degradation pathway was major pathway present in denitrifying biofilm (Fig. 4.11). It is the common intermediate compound of all aerobic and anaerobic metabolic pathways of aromatic compounds (phenolics, polycyclic aromatic hydrocarbons). Ubiquitous degradation pathway for benzoate and interrelated compounds are the central pathways for xenobiotics mineralization and detoxification by microbial communities (Cheng et al., 2013). Presence of these genes indicated that biofilm developed with consortium DC5 has wide potential for the removal of various xenobiotic compounds present in wastewater.

Table 4.4 Relative abundance of xenobiotic degradation pathways present in biofilm

Metabolism Pathways	**Relative abundance (%)**
1,1,1-Trichloro-2,2-bis (4-chlorophenyl)ethane (DDT) degradation [PATH:ko00351]	0.31
Chlorocyclohexane and chlorobenzene degradation [PATH:ko00361],	29.28
Benzoate degradation [PATH:ko00362]	45.44
Bisphenol degradation [PATH:ko00363]	0.35
Fluorobenzoate degradation [PATH:ko00364]	0.12
Dioxin degradation [PATH:ko00621]	0.55
Xylene degradation [PATH:ko00622]	0.16
Toluene degradation [PATH:ko00623]	0.27
Polycyclic aromatic hydrocarbon degradation [PATH:ko00624]	0.06
Chloroalkane and chloroalkene degradation [PATH:ko00625]	0.92

Aminobenzoate degradation [PATH:ko00627]	9.21
Nitrotoluene degradation [PATH:ko00633]	7.78
Styrene degradation [PATH:ko00643]	0.21
Atrazine degradation [PATH:ko00791]	2.15
Caprolactam degradation [PATH:ko00930]	0.96
Drug metabolism - other enzymes [PATH:ko00983]	2.13
Steroid degradation [PATH:ko00984]	0.11

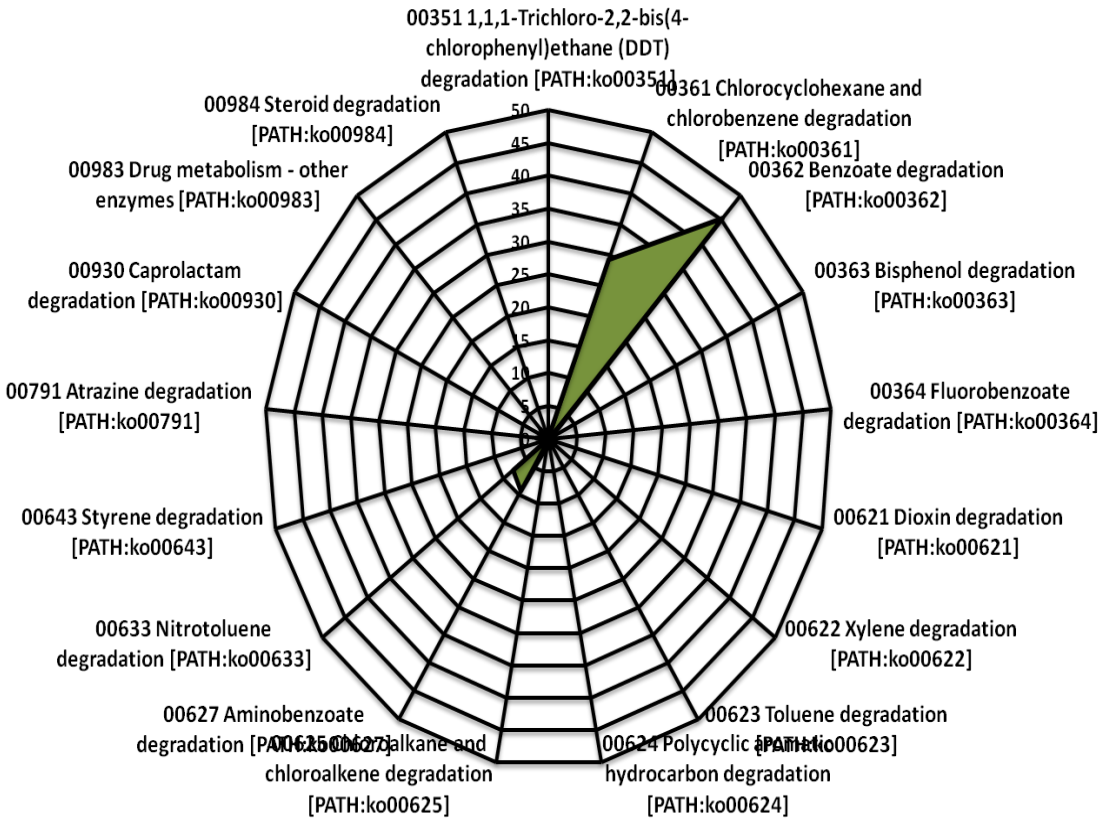


Figure 4.11 Relative abundance of genes involved in xenobiotic biodegradation and metabolism by KO

In this chapter composition and potential functions of the microbial communities in dMBBR bioaugmented with consortium DC5 was unraveled. Long term performance of the developed dMBBR demonstrates that the conditions in it were most suitable for *Thauera* spp. It also emphasized that *Thauera* spp. are playing key role in dMBBR. It also indicates that the denitrification process could be closely related to carbon metabolism, such as the degradation of amino acids, fatty acids and carbohydrates that

generate electrons for nitrate reduction. The presence of genes assigned to functions like nitrogen, sulfur, methane and xenobiotic biodegradation of aromatic compounds suggested that proper anaerobic/anoxic conditions were maintained that are essential for the high performance of dMBBR. This might be due to the feature of the process of denitrification 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.

The analysis of metagenomics studies undertaken in this chapter revealed that genus *Thauera* was most abundant and persistent in the long term operated, acetate fed MBBR. Also the biofilm developed on the carriers in MBBR was robust and well equipped genotypically to ameliorate many types of pollutants and xenobiotics.