Synopsis of the Thesis on

CHARACTERIZATION OF DENITRIFYING MOVING BED BIOFILM REACTOR DEVELOPED WITH A SPECIAL BACTERIAL SEED

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By

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INTRODUCTION

Biological denitrification process

Nitrate is a widely spreaded pollutant. Sources of nitrate contamination in water include the use of fertilizers, industrial wastewater, septic tank emissions, household wastewater etc.(Zhang et al. 2014). Excessive nitrate concentration in water may cause blue baby syndrome due to methemoglobinemia (Sadeq et al. 2008, Skold et al. 2011), central nervous system birth defect (Brender et al. 2013) and eutrophication in groundwater due to harmful algal bloom, fish hypoxia and toxin production (Wilkinson 2017). Various physicochemical processes used for nitrate removal generally produced toxic residual waste, high in cost, showed limited efficiency and operational difficulty. Alternative to that biological denitrification process is low-cost, environmentally friendly, sustainable and highly suitable process. In biological denitrification, nitrate reduce nitrates into nitrites then produce nitric oxide, nitrous oxide, and nitrogen gas.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

It is mediated by heterotrophic denitrifying bacteria which uses organic matter as carbon and energy source. Among denitrifying bacteria *Pseudomonas*, *Paracoccus*, *Flavobacterium*, *Alcaligenes*, and *Bacillus* spp. are widely used denitrifiers. Recently, a large number of denitrifying bacteria used for denitrification studies are *Vibrio* spp. AD2 (Ren et al. 2021), *Cupriavidus* sp. HY129 (Bai et al. 2021), *Pseudomonas sihuiensis* LK-618 (Hong et al. 2020), *Pseudomonas* sp. DM02(Deng et al. 2021), *Pseudomonas* sp. H117 (Su et al. 2020), *Streptomyces* sp. XD-11-6-2 (Zhang et al. 2021) and denitrifier with biofilm-formation and nitrogen removal capacities (Hong et al. 2020) for the removal of nitrate and nitrogen removal from different wastewaters.

Biofilm in wastewater treatment

Biological treatment is cost-effective and efficient process for the elimination of inorganic nutrients like nitrates from wastewater. It is classified into planktonic assemblages (floc-based systems) or surface-associated biofilms (attached growth systems). However, usage of the biofilm reactors has been increased due to its advantages such as less space requirement, flexible in operation, short HRT, flexibility to changes in the environment, high biomass, resistance to dehydration, enhanced ability to degrade recalcitrant and low sludge production (Bassin and Dezotti 2008; Wilderer and McSwain 2004). In biofilm reactors, bacteria are immobilized on the surfaces and it is widely used to keep helpful bacteria in water treatment systems. Bacteria in

biofilm bioreactors are attached to surfaces via extracellular polymeric substances (EPS) that result in the formation of a biofilm (Flemming et al. 2007). EPS is composed of proteins, extracellular DNA, lipids and polysaccharides secreted by bacteria (Flemming et al. 2007). It functions as cementation agents ("glue"), aiding the fixation of the microorganisms to the support medium and each other. Biofilm is metaphorically called as 'city of microbes' (Watnick and Kolter 2000).

Moving bed biofilm reactor (MBBR)

Limited land area near cities was an important factor that led to the development of a compact and more efficient treatment process, which became known as Moving Bed Biofilm Reactor (MBBR) systems. MBBR was first developed in Norway in the late 1980s and early 1990s. They are small scale sewage treatment plants but with large capacity, based on biological and chemical processes. The innovation of this technology is nonclogging suspended (moving-bed) carriers which provide a high specific biofilm surface area that ultimately increases treatment capacity (Odegaard 2006). Nowadays, the use of MBBR has been increased due to its cost-effectiveness, less space requirement and operational flexibility (Casas et al. 2015). The experimental setup of MBBR is shown in Fig. 1. It has many salient features: Robust, Efficient, Flexible (Customizable reactor shapes, Utilization of existing tanks, Upgradation of existing plants, No media clogging, No sludge recycling (Rusten et al. 2006) etc. Carriers used for MBBR are of high-density polyethylene (Dupla et al. 2006), polypropylene (Dupla et al. 2006), polyurethane foams (Chu and Wang 2011) on which the microbial biofilm is formed.

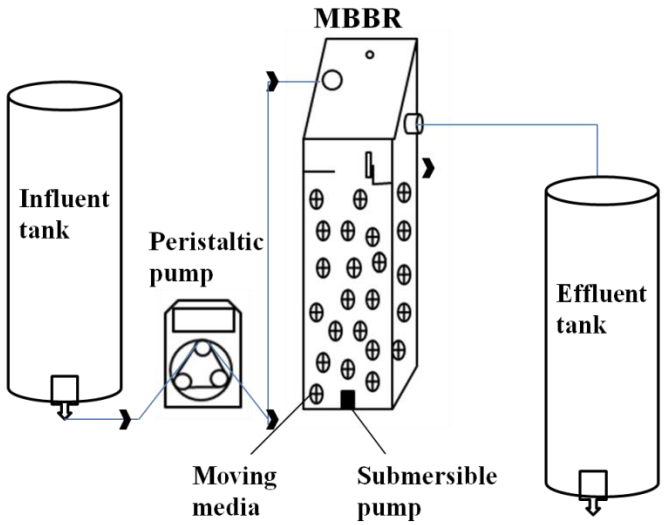

Figure 1: MBBR experimental setup

Special bacterial seed

MBBR seeded by activated sludges rich in microorganisms often shows poor performance due to lack of functionally active microorganisms and that can negatively affect removal of nitrate and overall performance of the MBBR. Therefore, inoculation of specific contaminant-degrading and biofilm-forming bacteria into biofilm reactor is a well developed bioaugmentation method for the removal of various pollutant from waste water (Li et al. 2013). Direct mixing of bacteria with biofilm forming ability and bacteria with contaminant degradation may inhibit the growth and biofilm forming ability of each other(Li et al. 2016). Therefore, bacterial cultures with the biofilm forming ability and pollutant degradation are required for wastewater treatment.

RATIONALE

Biofilm-based technology for effluent treatment like Moving bed biofilm reactor (MBBR) has benefits afforded by both biofilm (attached) and activated sludge (suspended growth) systems. It is useful for various wastewaters and thus it is highly effective in the removal of carbon and nitrate, but negligible studies are available that are paying attention on the bacteria and their biofilm developed inside MBBR. Therefore,denitrifying MBBR with a special bacterial seed of biofilm forming denitrifying bacteria (named Consortium DC5) was developed as a model system, and different parameters affecting nitrate removal were optimized. Further, biofilm community structures and functional potential of the denitrifying MBBR were evaluated by next-generation sequencing. The overall study carried out here provided a promising prospect for the bioaugmentation of a specially prepared consortium of denitrifying bacteria in bench-scale

MBBR for the treatment of nitrate containing wastewater.

OBJECTIVES

1. Selection of special biofilm forming denitrifying bacteria from activated sludge.

2. Performance of bench scale moving bed biofilm reactor (MBBR) with the consortium of the selected biofilm forming denitrifying bacteria and evaluation of its performance.

3. Characterization of the biofilm produced by the selected bacterial isolates and its potential in treatment of different effluents.

4. Studies on most persistent and dominant denitrifying bacterium in continuously operated MBBR.

RESULTS

1. Selection of special biofilm forming denitrifying bacteria from activated sludge.

Enrichment and Isolation of biofilm forming denitrifying bacteria

For enrichment of denitrifying bacteria, Winogradsky columns were set up with denitrifying conditions by providing particular source of nutrients and incubation conditions as a result it allowed enrichment of desired microorganisms. Sodium acetate as carbon source and potassium nitrate as nitrate source were added in the columns to enrich denitrifiers. Activated sludge collected from three different wastewater plants was the source of inoculum for the columns. To enumerate the denitrifying bacteria aliquots from the column were taken from the interface of the soil and media (anaerobic) region every 3 days. MPN(Most Probable Number) index/ml was obtained from the combination of positive tubes of PNB with bubble formation. It showed that as the number of days increased number of denitrifiers were also increased. It showed that on 28^{th} day, MPN index of 1100 i.e. maximum was achieved, which indicated that large number of denitrifiers were enriched within Winogradsky columns.

After enrichment of denitrifying bacteria, isolation was carried out from developed Winogradsky columns and direct activated sludge samples. Total 33 isolates were isolated on CPNA (Congored peptone nitrate agar) plates. CPNA allows the growth of amyloid producing

bacteria.Congo red specifically binds to the β-sheets of the amyloid proteins imparting red color to the colonies of amyloid producers.Amyloids are important components of bacterial biofilms and are widely present in wastewater sludges (Larsen et al.2007).Therefore it was envisaged that amyloid producing bacteria could be good biofilm forming bacteria. The isolates obtained on CPNA medium were further tested for their denitrification ability. Out of 33 isolates obtained on CPNA, 24 isolates showed gas production in PNB (peptone nitrate broth) which is indicative of denitrification activity.

Screening of biofilm forming denitrifying bacteria

Screening of biofilm forming denitrifying bacteria was done based on their biofilm forming ability, denitrification efficiency and lack of nitrite accumulation. It revealed that out of 24 isolates 9 isolates showed denitrification efficiency above 80%; 12 isolates showed good biofilm forming ability. Out of 24 isolates, 5 isolates showed denitrification efficiency above 80%, high biofilm forming ability, and did not accumulate nitrite. Production of amyloid protein on the surface of the 5 isolates was checked by Thioflavin T staining where all the isolates showed green fluorescence.

Finally, a denitrifying consortium DC5 of the selected five isolates R4, V5, V9, V11, and V14 with biofilm forming ability, high denitrification efficiency, lack of nitrite accumulation and amyloid production was prepared and used for further studies. Identification of the selected five isolates was done based on the 16srRNA sequencing. Isolates R4, V5, V9, V11, and V14 showed 99%, 99%, 100%, 100%, and 99% sequence similarity with *Diaphorobacter* sp., *Pannonibacter* sp., *Thauera* sp., *Pseudomonas* sp., and *Thauera* sp., respectively.Sequences of these isolates were deposited in the GenBank database with the accession number MN880203, MN880206, MN880207, MN880204 and, MN880205, respectively.

Flask level denitrification studies with consortium DC5

Flask containing consortium DC5 showed 100% nitrate reduction within 10h with initial nitrate concentration of 200 mg L^{-1} and no nitrite accunulation. Flask containing individual isolates of *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. Individual 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 *Thauera* sp.V9, *Thauera* sp.V14, and DC5 showed no nitrite accumulation from the beginning itself. No ammonia was

detected in the case of all the isolates.

Preparation of consortium DC5

Growth of all the selected isolates was similar to each other , which is an important attribute of individual isolates for the formation of consortium.

Consortium DC5 was developed by growing all the selected isolates in PNB for 24 h. 0.5 OD600nm was set and 400 µL of each isolate was 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. 1 mL of this suspension of the consortium DC5 was then added in the 100 mL of MM2 medium and incubated at 37 °C under the static condition for 24 h to be used as inoculum.

2. Development of laboratory-scale moving bed biofilm reactor (MBBR) with the consortium of the selected denitrifying bacteria and evaluation of its performance.

To carry out continuous reactor studies with consortium DC5, MBBR was constructed from the polyacrylic material of 45 cm height and 16 cm width with a working volume of 10 L. A submersible pump was fixed at the center of the reactor which facilitated the movement of the carrier inside the reactor. Synthetic effluent (MM2 medium) was continuously fed from the inlet tank to the reactor with the peristaltic pump.

Assessment of MBBR performance

C/N ratio

At C/N ratio 0.7, 0.4 and 0.3 nitrate removal was above 95% with an initial nitrate concentration of 620 mg L^{-1}. At higher C/N ratio 0.7, COD was above permissible range (i.e. 250 mg L^{-1}), whereas at C/N ratio 0.4, 0.3, 0.2 showed 96%, 100%, and 78% nitrate removal, respectively, and COD below permissible range (i.e. 250 mg L^{-1}). High C/N ratio, i.e 0.7 increased COD in wastewater can lead to secondary pollution in wastewater, whereas low 0.2 C/N ratio decreased nitrate removal efficiency inside MBBR. Hence, 0.3 C/N ratio was selected for further studies.

HRT

At HRT 8, 6, and 3 h nitrate removal was 100% with initial nitrate loading of 620 mg L^{-1} and COD was below permissible range. At HRT 2 h nitrate removal efficiency decreased from 100 to 70%, because of less contact time to complete nitrate removal. HRT 3 h is the short time with maximum nitrate removal efficiency and increasing HRT above 3 h did not influence the nitrate

removal. Hence, 3 h was selected as an optimum HRT for the DC5.

Nitrate loading

Effect of different nitrate concentrations was checked by increasing the nitrate concentration in synthetic wastewater from 620 to 2400 mg L^{-1}. Denitrification efficiency in the developed MBBR was 100%, 92.25%, 93.02%, 80.43%, 72.23%, 70.45% at 620, 744, 930, 1116, 1500, and 2400 mg L^{-1} of nitrate concentration respectively with COD reduction below permissible range i.e. 250 mg L^{-1}.Overall results suggest that the consortium DC5 was able to reduce nitrate up to 2400 mg L^{-1} at optimized C/N ratio 0.3 and HRT of 3 h.

Carriers of different designs

Three different types of carrier designs were checked having surface area 275 m^2/ m^3 (Pall ring), 500 m^2/m^3 (Kaldnes K1), and 400 m^2/ m^3 (Fluidized biomedia). Among three carriers pall ring carriers showed highest nitrate removal efficiency from 620 to 2400 mg L^{-1} and COD reduction was below permissible limit. Biomass quantified from Pall ring, Kaldnes K1, and Fluidized biomedia was 35, 11.6, and 12 mg/carrier, respectively. EPS component analysis from different carriers showed protein as a major component in the biofilms obtained from all the carriers and pall ring carriers contain highest EPS components and biomass. Therefore, pall ring carriers were used for further studies.

Filling ratio

Filling fraction of carrier is also an important factor for performance of MBBR. The filling fraction should be below 70% for the carriers to move freely in the reactor (Odegaard 2006). At 20% filling ratio DC5 showed highest nitrate removal efficiency in the MBBR. However, different filling ratio did not affect COD reduction in the reactor. It was below permissible range for all the filling ratios.As filling ratio increased from 20 to 40% denitrification efficiency and biomass on the surface of the carriers was decreased from 35 to 12 mg/carrier, respectively.

Comparative studies:

Suspended reactor and MBBR developed with consortium DC5

Comparative nitrate removal studies between biofilm reactor (i.e. MBBR) developed with consortium DC5 and suspended reactor developed with consortium DC5 showed that MBBR showed 70-100% over the nitrate loading of 2400-620 mg L^{-1} whereas reactor developed with suspended reactor showed nitrate removal of 60-65% at nitrate loading from 620-2400 mg L^{-1}.

MBBR developed with activated sludge and MBBR developed with consortium DC5

Comparative studies between reactor developed (inoculated) with activated sludge sample and with consortium DC5. Reactor developed with consortium DC5 showed denitrification efficiency of 70-100% over the nitrate loading of 2400-620 mg L^{-1} whereas reactor developed with activated sludge showed nitrate removal of 40-60% at nitrate loading from 620-2400 mg L^{-1}. COD was below permissible range in both reactors. This suggests that reactor developed with consortium DC5 showed higher nitrate removal.

Control reactor (without inoculum) and biofilm reactor

Continuous reactor studies carried out without adding any inoculum under unsterile condition was set up as a control reactor. Control reactor was able to reduce nitrate from 30-60% whereas reactor developed with consortium DC5 reduced nitrate from 70-100 % nitrate over 2400-620 mg L^{-1}.COD level was below permissible range for both the reactors.

3. Characterization of the biofilm produced by the selected bacterial isolates and its potential in treatment of different effluents.

Effect of different factors on biofilm formation and denitrifying efficiency of consortium DC5:

Metal ions

No significant effect was observed on denitrification efficiency in presence of Mg^{2+}, K^{+}, Ca^{2+}, Fe^{2+} metal ions. Whereas 5 mM Mg^{2+}, 5 mM Ca^{2+} and 0.5 mM K^{+} supplementation showed increased biofilm formation by 85 %, 60 % and 80 % , respectively.

pH

Alkaline pH was preferred over acidic pH for higher denitrification efficiency (78 %) and biofilm formation. pH 3 and pH 5 showed around 70 % less denitrification efficiency and low biofilm formation than alkaline pH.

Inoculum size

No significant difference was observed in denitrification efficiency and biofilm forming ability of consortium DC5 at different inoculum %.

Carbon sources

Except glucose, all the carbon sources (Ethanol, Methanol, Molasses, Sodium acetate, Glycerol) showed around 90 to 100% denitrification efficiency and in the case of biofilm, ethanol showed less biofilm forming ability compared to other carbon sources.

Supplementation of purified amyloid proteins

Different concentration (5, 15, 25, and 50 mg L^{-1}) of purified amyloid supplemented in MM2 was inoculated with consortium DC5 in microtiter plate. Biofilm and denitrification efficiency of DC5 was seen to increase maximally at 50 mg L^{-1} purified amyloid.

Whole genome metagenomics of carrier associated biofilm developed inside continuous denitrifying MBBR:

Whole genome metagenomic data analysis of biofilm developed inside MBBR gave a complete characterization of microbial community, and functional potential of biofilm developed inside denitrifying MBBR. It allowed understanding of community structure and function of the biofilm developed inside denitrifying MBBR. Domain bacteria (99.27 %) was most abundantly present in all the biofilms developed inside denitrifying MBBR. Proteobacteria (87.68 %) was the most abundantly present phylum followed by Bacteroidetes, Firmicutes, Actinobacteria, Chloroflexi, Planctomycetes, Verrucomicrobia, Acidobacteria, and Chlorobi. PCA component analysis of between phyla showed that all the phyla are positively correlated with each other. *Thuaera* was the most abundantly present genus followed by *Azoarcus, Aromatoleum, Mesorhizobium, Pseudomonas, Paracoccus, Sinorhizobium, Burkholderia, Chelativorans, Dechloromonas.* PCA analysis between different genus showed that genus *Thauera, Pseudomonas,* and *Burkholderi*a were positively correlated with each other. *Thauera* MZ1T was the most abundant species followed by *Azoarcus* sp. BH72, *Aromatoleum aromaticum, Mesorhizobium loti, Paracoccus denitrificans*, *Chelativorans* sp. BNC1, *Dechloromonas aromatic, Mesorhizobium opportunistum*, *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*. Results of PCA analysis showed that abundance of all the species was positively correlated with each other.

Functional potential of biofilm developed inside denitrifying MBBR

Biofilm developed inside denitrifying MBBR contains a high abundance of metabolic functions such as Amino Acid, Biosynthesis of other secondary metabolites, Carbohydrate, Energy, Glycan, Lipid, Cofactors and vitamins, Terpenoids, Polyketides, Nucleotide, Xenobiotics biodegradation. Genes encoding for metabolism were followed by genetic information

processing (Folding, sorting and degradation, Replication and repair, Transcription, Translation), Environmental information processing (Membrane transport, Signaling molecules and interaction,Signal Transduction), Cellular processes (Cell communication, Cell growth and death, Cell motility, Transport and catabolism), and organismal systems (Circulatory system, Digestive system, Endocrine system, Environmental adaptation, Excretory system, Sensory System).

Nitrogen metabolism

Nitrogen metabolism involved genes such as nitronate monooxygenase, nitrite reductase (NO-forming), nitrate reductase catalytic subunit, cah, cynT, napA, napB, napC, napG, napH, nirB, norB, norC, norD, norF, nosZ were abundantly present in the biofilm developed inside denitrifying MBBR. PCA analysis of genes involved in nitrogen metabolism showed that all the genes involved in denitrification process were positively correlated with each other, and are highly abundant in the denitrifying MBBR.

Nitrate removal studies by consortium DC5 from different effluents

i] Dye industry effluent

Nitrate removal studies were carried out with dye industry effluent in continuous MBBR developed with consortium DC5. It showed 75% nitrate reduction and 60% COD reduction within 3h of HRT. DO was below 1.5 and pH 8.5 was maintained inside developed denitrifying MBBR. Nitrite accumulated in the MBBR was 0.5 mg/L - 0.8 mg/L (below permissible range) and no ammonia was detected inside the reactor.

ii] Pharma industry effluent

Treatability of pharma industry effluent with consortium DC5 showed 85% reduction in nitrate and 60 % COD reduction within 3h of HRT. No nitrite and ammonia were accumulated inside the reactor and proper denitrification conditions (DO below 1.5 and pH 8.5) were maintained in the reactor.

iii] Healthcare pharma industry effluent

Treatability of healthcare pharma industry effluent with consortium DC5 showed 100 % reduction in nitrate and 60 % COD reduction within 3h of HRT. No nitrite and ammonia were accumulated inside the reactor and denitrification conditions (DO below 1.5 and pH 8.5) were maintained in the reactor.

4. Studies on most persistent and dominant denitrifying bacterium in continuously operated MBBR

In whole genome metagenome analysis, *Thauera* was the most dominant and key contributor in the denitrification of nitrate containing wastewater in MBBR developed with consortium DC5. Therefore, further studies were undertaken to characterize biofilm forming denitrifying organism *Thauera* sp.V14 isolated from activated sludge of domestic sewage treatment plant.

Flask level denitrification studies with denitrifying *Thauera* sp.V14

At flask level denitrification studies *Thauera* sp.V14 showed 100% denitrification efficiency within 72h with an initial nitrate concentration of 765.89 mg L^{-1} and no nitrite and ammonia were accumulated in the medium.

Auto-aggregation and Hydrophobicity of *Thauera* sp.V14

Auto-aggregation ability and hydrophobicity of *Thauera* sp.V14 increased with time. Auto-aggregation increased gradually from 34% at 1h to 93% at 8h and hydrophobicity from 7.7% at day 1 to 83.8% at day 5.

Continuos MBBR studies with *Thauera* sp .V14

Denitrification ability of the *Thauera* sp.V14 in the denitrifying MBBR was checked by using different concentration of nitrate 620, 744, 930, 1500, and 2400 mg L^{-1} in the influent of synthetic wastewater. Denitrification efficiency in the MBBR was 91%, 90%, 76%, 66%, and 60% at 620, 744, 930, 1500, and 2400 mg L^{-1} of initial nitrate concentration respectively and COD reduction was below permissible range i.e. 250 mg L^{-1}. *Thauera* sp. V14 was also able to denitrify nitrate and COD below permissible range up to initial nitrate concentration of 744 mg L^{-1} inside continuous MBBR.

Characterization of carrier associated *Thauera* biofilm developed inside MBBR

SEM of biofilm developed inside denitrifying MBBR developed with *Thauera* sp.V14 showed the presence of rod shaped bacteria on the carrier surface. Further, the presence of *Thauera* in the carrier associated biofilm was confirmed by Real time PCR, which showed 2×10^8 copy number /µl of *Thauera*. EPS components of the biofilm developed inside MBBR showed a broad absorption region between 3418 cm^{-1} assigned to the O–H bond in hydroxyl functional groups. It confirmed the presence of the hydrogen bond of amines and alcohols (or phenols) in the biofilm. Small peaks of 2851, 2921cm^{-1} were due to fatty acids and esters. Broad peak at 1426 cm^{-1} was due to the presence of amides I and II. Peak at 1082 cm^{-1} showed the presence of carbohydrates.

Results of FTIR analysis indicate that EPS of *Thauera* showed the presence of Carbohydrates, Proteins, and Lipid components in the carrier-associated biofilm developed in the denitrifying MBBR.

Data extracted from whole genome metagenomic analysis of denitrifying biofilms

To check the functional potential of genus *Thauera* data were extracted from the earlier whole genome metagenomics data. It showed that genes encoding for different metabolic functions were the most dominant followed by genetic information processing (Folding, sorting and degradation, Replication and repair, Transcription,Translation), Environmental information processing (Membrane transport,Signal transduction), Cellular processes (Cell growth and death, Cell motility, Transport and catabolism) and Organismal systems.

Functional potential of genus Thauera

Highest genes involved in different metabolism were Amino acid, Carbohydrate followed by, Cofactors and vitamins, Energy, Nucleotide, Glycan, Lipid, Terpenoids and polyketides and other amino acids. PCA analysis of different metabolic pathways showed positive correlation between Aminoacid, Energy, Carbohydrate, Lipid, Xenobiotics. Presence of genes involved in biodegradation of xenobiotic compounds such as Benzoate, Nitrotoluene, Chlorocyclohexane, Chlorobenzene suggests that it has a well-developed mechanism to neutralize the harmful effect of xenobiotic compounds. *Thauera* genus showed presence of different genes involved in nitrogen metabolism such as cah, cynT, can, napA, napB, napC, napD, napF, napG, napH, nirB, nirD, norB, norC, norD, norF, nosZ.Genes involved in denitrification pathways such as nitrate reductase (NapA, NapB, NapC, NapD, NapF, NapG and NapH), nitrite reductase (NIR) (nirB, nirD), nitric oxide reductase (NOR) (norC, norB, norD, norF) to nitrous oxide (N2O) NosZ which reduce N2O to N2 were present in the *Thauera*. Genes involved in inorganic sulfur metabolism such as cysD, cysN ,cysI, cysH, cysNC were also present. Genes involved in methane metabolism such as metF, frmB, fghA were present in genus *Thauera*.

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