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RESEARCH PAPER

# *Thauera* sp. for efficient nitrate removal in continuous denitrifying moving bed biofilm reactor

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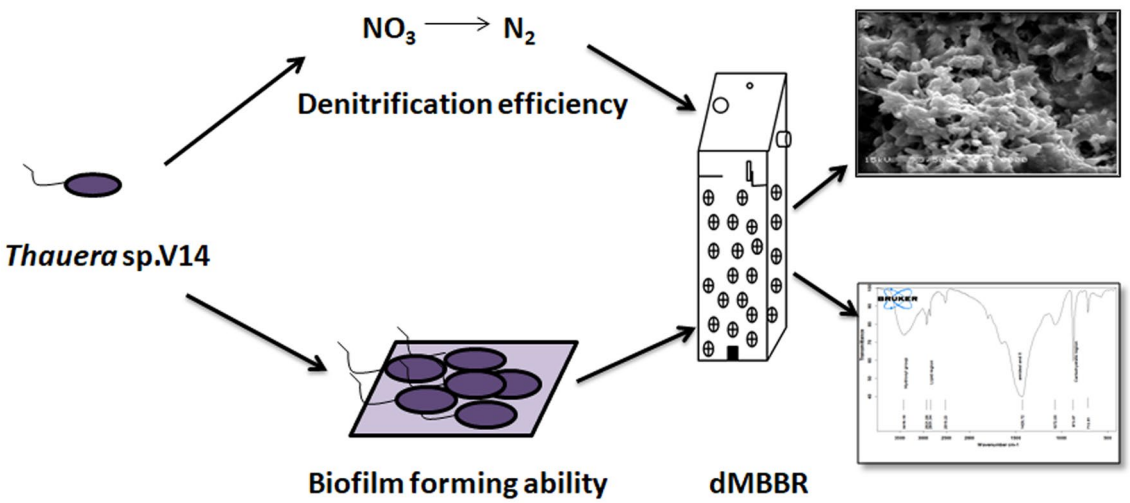
## Abstract

*Thauera* is the most widely found dominant denitrifying genus in wastewater. In earlier study, MBBR augmented with a specially developed denitrifying five-membered bacterial consortium (DC5) where *Thauera* was found to be the most abundant and persistent genus. Therefore, to check the functional potential of *Thauera* in the removal of nitrate-containing wastewater in the present study *Thauera* sp.V14 one of the member of the consortium DC5 was used as the model organism. *Thauera* sp.V14 exhibited strong hydrophobicity, auto-aggregation ability, biofilm formation and denitrification ability, which indicated its robust adaptability short colonization and nitrate removal efficiency. Continuous reactor studies with *Thauera* sp.V14 in 10 L dMBBR showed 91% of denitrification efficiency with an initial nitrate concentration of 620 mg $L^{-1}$ within 3 h of HRT. Thus, it revealed that *Thauera* can be employed as an effective microorganism for nitrate removal from wastewater based on its performance in the present studies.

### Graphical abstract




**Keywords** Denitrification · Denitrifying moving bed biofilm reactor (dMBBR) · Bioaugmentation

## Introduction

Moving bed biofilm reactor (MBBR) has been considered the most efficient and widely used biofilm-based wastewater treatment process [1]. It has attracted greater attention due to its simple operation, cost effectiveness and minimal waste generation. However, due to the increased emphasis

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on reducing the cost of treatment and minimizing organic carbon demand in wastewater treatment, the usage of MBBR has increased worldwide [2]. Moreover, MBBR has been widely used reactors to enable the growth of higher biomass. Bioaugmentation of denitrifying bacterial culture is necessary when the native bacterial community does not remove sufficient nitrate from wastewater. Bioaugmentation of microorganisms is one of the cost-effective and environmentally friendly approaches for the removal of nitrate from wastewater. Bioaugmentation of specific bacterial strains with high adaptability and specialized function can be added into the biologic treatment process to enhance specific functional species, alter the composition of the microbial community, and increase the effectiveness of the removal of contaminants [3–5]. Various denitrifying bacteria have been isolated and shown to reduce nitrate in wastewater. Additionally, few recent reports on bioaugmentation have also suggested that strain of *Zobellella* B307, *Acinetobacter* sp. TAC-1 *Pseudomonas stutzeri* strain HK13, *Hydrogenophaga* sp. H7 and the special microbial seed of biofilm forming denitrifying bacteria enhanced nitrate and nitrogen removal of wastewater [6–10]. Among many denitrifying bacteria, *Thauera* was the most commonly found organism in wastewater treatment plants, polluted freshwater, wet soil, and water contaminated with aromatic or aliphatic organic compounds [11]. *Thauera* is a Gram-negative bacterium that can denitrify nitrogenous oxides under anoxic conditions [12]. It contributes to waste treatment by targeting recalcitrant compounds, humic acids, metal ions and detoxifying them into more easily utilizable compounds and serves as nutrients for other microorganisms [4, 5]. Its degradative abilities toward nitrate and other recalcitrant pollutants make them potential organism for bioremediation. It has been the most widely used organism for wastewater treatment applications due to its adaptable metabolism and ability to grow in a variety of environmental conditions. *Thauera* is well-known for denitrification [13], aerobic denitrification [14], partial denitrification [15], hydrogen-oxidizing autotrophic denitrification [16] and Hydrogen-based denitrification [17]. In the literature, different species of *Thauera* have been reported to be major contributors in a variety of reactors, such as *Thauera mechernichensis* was the major contributor in the treatment of resin wastewater in the aerobic nitrifying-denitrifying membrane bioreactor [18], *Thauera aminoaromatica* in hypoxic quinoline denitrifying sequencing batch reactor [19], *Thauera* sp. SND5 removed nitrogen and phosphorus from wastewater via simultaneous nitrification and denitrification [20]. It was also dominant source of functional genes in aerobic granular sludge [21]. Moreover, it has diverse functional metabolic pathways like carbon, nitrogen, sulphur and phosphorus etc. [20]. The presence of these diverse metabolic pathways made *Thauera* more competitive and one of the potential microorganism for the treatment of wastewater [22]. Therefore, the study aimed to assess the functional potential of *Thauera* sp. V14, a member of the DC5 consortia, in the removal of nitrate from wastewater. Metagenomic data suggested that MBBR inoculated with DC5 showed *Thauera* spp. as the most dominant and persistent microorganism. Thus, to explore the potential application of *Thauera* spp. in the denitrification process, *Thauera* sp. V14 was chosen as the model organism because of its significant properties such as strong hydrophobicity, auto-aggregation ability, biofilm formation and denitrification competence, implying its adaptability for efficient colonization and nitrate removal. Consequently, the findings collectively highlight *Thauera* sp. V14 as a microorganism with significant promise for novel and efficient nitrate removal from wastewater.

## Materials and methods

### Microorganism, media and growth condition

*Thauera* sp. V14, a member of the DC5 consortium was used in dMBBR studies. DC5 consortium was prepared as reported in our earlier published work [10]. *Thauera* sp.V14 was isolated from an activated sludge sample, using CPNA (Congo red peptone nitrate agar) medium (0.5 g peptone, 0.3 g beef extract, 1 g potassium nitrate, 2 g agar, 0.005 g Congo red in 100 mL of distilled water). Synthetic wastewater used in this study was prepared as follows: 0.2 g $MgSO_4$ $0.7H_2O$, 0.2 g $K_2HPO_4$, 0.05 g $FeSO_4.7H_20$, 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 g sodium acetate, 0.5 g yeast extract were added per liter of distilled water in the flask. Total 50 mL MM2 (minimal media) medium was prepared. All the chemicals/media used in the experiments were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India).

### Phylogenetic tree

For phylogenetic analysis of sequences of *Thauera* sp. V14, *Thauera* MZ1T and *Thauera humireducens* (spp. obtained in metagenomic analysis) were done in MEGA 11 using the neighbor joining method in MEGA X.

### Auto-aggregation ability of *Thauera* sp.V14

*Thauera* sp. V14 was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. Harvested cells were washed twice with PBS (phosphate buffer solution) and then resuspended in PBS with absorbance set at 0.6 $OD_{600nm}$. This suspension was inoculated in 50 mL of MM2 medium and allowed to stand for 8 h to allow aggregation and the $OD_{600nm}$ was measured again ($A_t$). The auto-aggregation

index was calculated by Eq. (1) to evaluate the auto-aggregation capacity of *Thauera* sp. V14 [23].

$$\text{Auto aggregation index (\%)} = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (1)$$

where $A_t$ and $A_0$ are $OD_{600nm}$ at time t = 1, 2, 3, 4, 5, 6, 7 and 8 and at time t = 0 time respectively.

## Hydrophobicity of *Thauera* sp.V14

The hydrophobicity of cells was determined by measuring bacterial adhesion. *Thauera* sp.V14 was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. Harvested cells were washed twice with PBS and resuspended in PBS with absorbance set at 0.3 $OD_{546nm}$ and inoculated in 50 mL of MM2 medium. Aliquots were drawn at every 24 h intervals for 120 h. To check the hydrophobicity, 4 mL of cell suspension with 1 mL of hexadecane was vortexed for 2 min and the two phases were allowed to separate for 15 min. The aqueous phase was removed carefully, and its $OD_{546nm}$ was taken. The cell surface hydrophobicity was calculated using Eq. (2) [23].

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{OD_{546nm}}{0.3}\right) \times 100 \quad (2)$$

## Confirmation of denitrifying ability of *Thauera* sp. V14

Cells of the *Thauera* sp.V14 were grown for 24 h in PNB and 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 peptone nitrate broth (PNB) and the tube was sealed with suba-seal rubber stopper and incubated for 48 h. After 48 h, 1 µL gaseous sample collected and was injected into the GC. Dinitrogen ($N_2$) content was determined by gas chromatography (GC) using Sigma Instruments Ltd (India) 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.

## Resting cells kinetics using *Thauera* sp.V14

Nitrate and nitrite reduction rates by resting cell suspension of the *Thauera* sp.V14 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 again resuspended in PBS. Sodium acetate and potassium nitrate were added as electron donors and acceptors respectively and the reduction of nitrate and formation of nitrite were estimated for 48 h after every 12 h time intervals.

## Flask level denitrification efficiency of *Thauera* sp.V14

Denitrification studies were carried out with *Thauera* sp.V14 with an initial nitrate concentration of 765 mg $L^{-1}$ in a 250 mL Erlenmeyer flask. It was grown in PNB for 18–24 h and centrifuged at 8000 rpm for 7 min. The cell pellet was washed twice with PBS and resuspended in PBS with absorbance set at 0.5 $OD_{600nm}$. 1% of this was inoculated in a flask containing 100 mL of MM2 medium. Experimental flasks were incubated at 37 °C for 48 h in static conditions; aliquots were drawn at 24 h intervals and assayed for nitrate, nitrite and ammonia. Denitrification efficiency was calculated by the following formula:

$$\text{Denitrification Efficiency (\%)} = \frac{[NO_3]_{In} - [NO_3]_{Out}}{[NO_3]_{In}} \times 100$$

## Inoculum preparation for continuous dMBBR studies

*Thauera* sp.V14 was grown in PNB for 24 h. The absorbance of 0.5 $OD_{600nm}$ was set. The cell pellet obtained after centrifugation at 8000 rpm for 7 min was washed twice with PBS and resuspended in 80 mL of PBS. 80 mL of this suspension was then added to 800 mL of MM2 medium and incubated at 37 °C under the static condition for 24 h. This was used as an inoculum.

## Bench-scale dMBBR developed with *Thauera* sp.V14

Continuous dMBBR studies were carried out with *Thauera* sp.V14 in a 10 L reactor. For continuous reactor studies, 10 L of synthetic effluent (MM2 medium) was continuously fed from the inlet tank to the reactor with the peristaltic pump (Masterflex®). 8% of inoculum was prepared as mentioned in the above section was added in the 10 L reactor containing carriers and biofilm was allowed to form on the carriers for 10 days. Here, 620, 744, 930, 1116, 1500 and 2400 mg $L^{-1}$ nitrate loading and COD concentrations were 186, 223, 279, 335, 450 and 750 mg $L^{-1}$, respectively. Studies were carried out with C/N ratio 0.3, HRT of 3 h and filling ratio 20% of pall ring carrier [10]. pH-8**,** dissolved oxygen (DO) – 0.1–0.8 mg $L^{-1}$, and turbidity were 15–350 Nephelometric Turbidity Unit (NTU) maintained in the dMBBR.

### Analytical methods

Treated synthetic effluent was collected in the outlet tank and assayed for nitrate, nitrite, ammonia, pH, turbidity, biomass and DO at the temperature of 37 °C. Biomass from the carriers was quantified by drying carrier material at 105 °C for 1 h. Nitrate, nitrite, ammonia and chemical oxygen demand (COD) estimation methods were performed according to APHA1998. DO was measured using a DO probe (Thermo Fisher Scientific, India) and the turbidity was checked using a turbidity meter (Hanna Instruments, India).

### Functional potential of genus *Thauera*

The functional potential of genus *Thauera* was checked using MG RAST software was used to filter genus level data and to check its functional potential [24].

### Measurement of the abundance of *Thauera* sp.V14

Biofilm samples were taken from different parts of the dMBBR. Genomic DNA from biofilm samples was extracted using the modified CTAB (cetyltrimethylammonium bromide) method. 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. The pellet was resuspended in 500 µL of Tris–EDTA-Sucrose of buffer (25 mM TrisCl (pH 8), 25 mM EDTA (pH 8) 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 mixed properly, and kept in a 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. An 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. The upper aqueous phase was collected and an equal volume of chloroform:isoamylalcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. Aqueous phase was extracted and 1/10th 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 Milli-Q water. Resuspended DNA was treated with 3 µL of 0.01% RNase added to it incubated at 65 °C for 10 min and stored at 4 °C which was preserved in cold conditions. The quality and quantity of extracted DNA were assessed using agarose gel electrophoresis and nanospectrophotometer, respectively. From this isolated gDNA abundance of *Thauera* in the biofilm developed inside dMBBR was quantified using Real-Time PCR (Applied Biosystems StepOne™ Real-Time PCR System, India). For this *Thauera* specific primers were used Forward: TGCATTGCTGCTCCGAAC and Reverse: CGCTCGTTG CGGGACTTAACC and its abundance was quantified as per [25].

### Observation of carrier-associated biofilm

SEM was used to characterize biofilm morphology on carriers in dMBBR. Carriers before biofilm development and continuous nitrate removal studies (after biofilm development) were collected from the reactor without disruption. After collection carriers with biofilm were allowed to dry in the hot air oven at 100 °C for 1 h then carriers were cut into small pieces. Further for SEM analysis they were coated with gold and examined under JEOL JSM-6380 LV SEM, accelerating voltage of 15 kV.

### Qualitative biochemical analysis of *Thauera* sp. V14 biofilm

FTIR analysis was used to characterize the major components of the *Thauera* sp. V14 biofilm. Carriers with developed biofilm were collected after continuous denitrification studies from the dMBBR and dried at 70 °C for 1 h, and then biomass of biofilm was scrapped off from the carriers and it was analyzed using FTIR Spectrometer (FTIR Spectrometer: ALPHA, India).

## Results and discussions

In our earlier investigations, *Thauera* spp. emerged as the most dominant and persistent microorganisms in the MBBR augmented with consortium DC5, comprising *Diaphorobacter* sp. R4, *Pannonibacter* sp. V5, *Thauera* sp. V9, *Pseudomonas* sp. V11 and *Thauera* sp. V14. After 300 days of continuous operation, whole-genome metagenomic studies confirmed *Thauera* spp.'s dominance as the key contributor to the denitrification of nitrate-containing synthetic wastewater [10]. These findings led to an in-depth investigation into the functional potential of *Thauera* spp. specifically investigating the metabolic pathways present in the genus. To investigate the practical application of *Thauera* spp. in denitrification processes, *Thauera* sp. V14 was chosen as the model organism due to its distinctive attributes.

### Metabolic potential analysis of genus *Thauera* in dMBBR bioaugmented with consortium DC5

Metabolic potential of the microorganisms is directly related to the bioremediation process [26]. Functional metabolic potential of microorganism is generally categorized 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.

*Thauera* contains high abundance of metabolic functions (57.44%) followed by genetic information processing (17.96%), environmental information processing (18.24%), cellular processes (6.11%) and organismal systems (0.26%) were identified at KO level 1 (KEGG Orthology level 1) (Fig. 1a). Genes responsible for cell growth and death (1.58%), cell motility (3.56%), transport and catabolism (1.10%), membrane transport (9.12%), signal transduction (8.30%), folding, sorting and degradation (3.31%), replication and repair (5.72%), transcription (1.64%), translation (7.61%), amino acid metabolism (20.49%), biosynthesis of other secondary metabolites (0.18%), carbohydrate metabolism (11.30%), energy metabolism (6.52%), glycan biosynthesis and metabolism (2.65%), lipid metabolism (2.45%), metabolism of cofactors and vitamins (6.68%), metabolism of other amino acids (1.06%), metabolism of terpenoids and polyketides (1.13%), nucleotide metabolism (4.67%), xenobiotics and biodegradation and metabolism (0.65%), environmental adaptation (0.25%) were identified at KO level 2 in genus *Thauera* (Fig. 1b). In genus *Thauera*, high abundance of genes were involved in amino acid metabolism i.e., 20.49% which might be providing carbon and energy source for bacterial metabolism [27]. High abundance of genes was also involved in carbohydrate metabolism i.e. 11.30% were attributed to a decomposition of complex organics into more easily degradable matter [28]. High abundance of amino

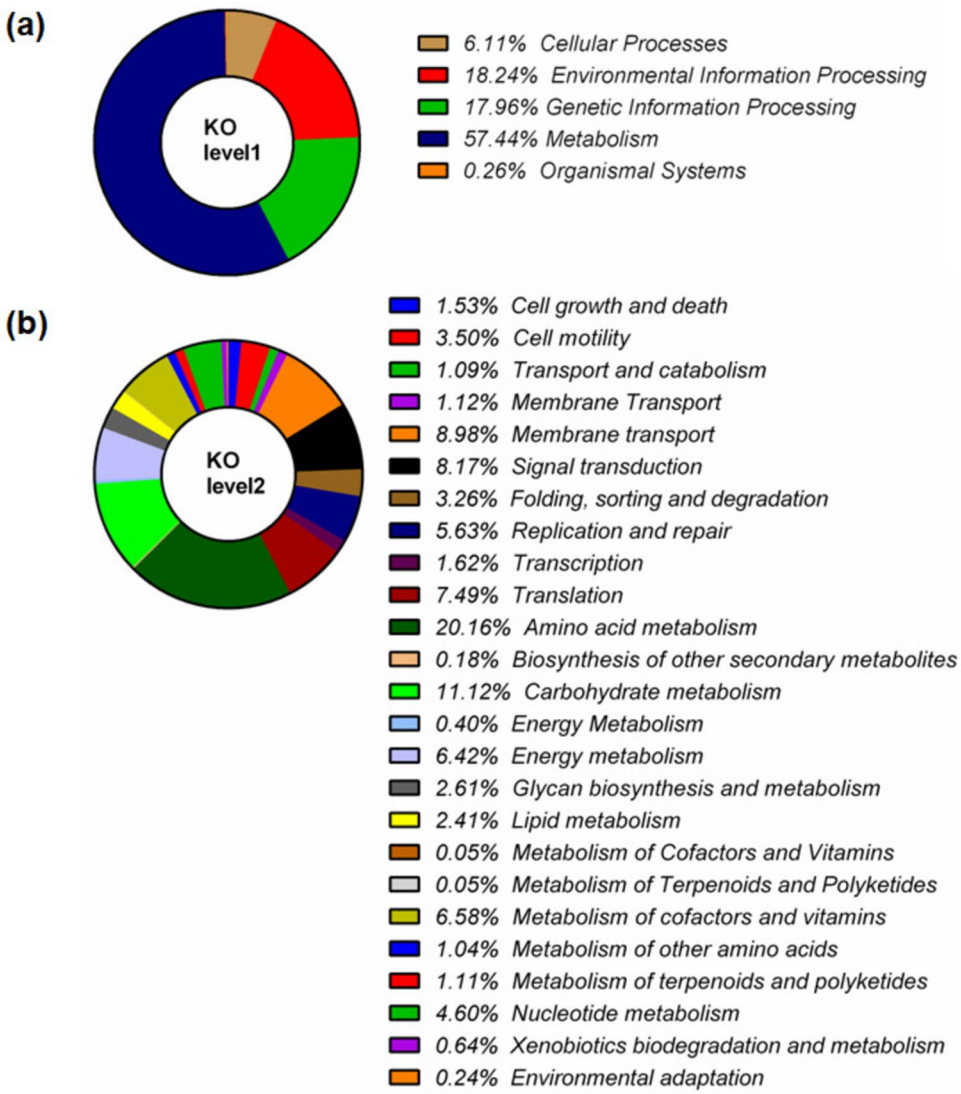



**Fig. 1** Relative abundance of different pathways involved in metabolisms **a** KO level 1 and **b** KO level 2

acid metabolism and carbohydrate metabolism in *Thauera* may act as electron donors and carbon sources for denitrification [27, 29]. These carbohydrate metabolism genes might be involved in the central carbon metabolism (glycolysis, citrate cycle, and pentose phosphate pathway), producing ATP for energy or NADPH for reducing power, which is necessary for cell metabolism. Moreover, it also can decompose complex organics into more easily degradable matter [28]. Genes involved in energy metabolism (6.52%) mainly include oxidative phosphorylation and nitrogen metabolism. Genes involved in oxidative phosphorylation and nitrogen metabolism might be related to the degradation of phosphate and nitrogen containing pollutants [30]. Genes involved in xenobiotics biodegradation (0.65%) and genes of biosynthesis of secondary metabolites (0.18%) play important role in defense against other microorganisms and harmful stresses such as toxins or UV exposure, were also identified in the genus *Thauera*.

## Genes and functions associated with organic contaminant degradation in *Thauera* spp. developed inside dMBBR

Denitrification process is often associated with the degradation of organic compounds under anaerobic or anoxic conditions. These organic compounds provide electrons that 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 conditions [31]. Therefore, the presence of genes associated with organic contaminant degradation and denitrification was also checked in the genus *Thauera.*

## Relative abundance of genes involved in nitrogen metabolism

The biologic process of nitrogen removal in wastewater takes a specific path, depending on the microbial communities involved and the substrates available [40, 41]. 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 as well as it could also be linked to COD removal [32]. According to KO annotation level-2 in genus *Thauera,* genes involved in the denitrification process were most abundant than other enzymes involved the nitrogen metabolism. In nitrogen metabolism pathways 42.06% of genes nitrate reductase catalytic subunit [EC: 1.7.99.4], NapA [EC: 1.7.99.4], NapB, NapC, NapD, NapF, NapG, NapH were involved in the conversion of nitrate to nitrite.6.2% of nirB; [EC: 1.7.1.4] and nirD; [EC: 1.7.1.4] genes were involved in the conversion of nitrite to nitric oxide. 11.8% of norB [EC: 1.7.2.5], norC [EC: 1.7.99.7], norD [EC: 1.7.99.7] and norF [EC: 1.7.99.7] of genes were responsible for the conversion of nitric oxide to nitrous oxide and 1.18% nosZ [EC: 1.7.2.4] involved in the conversion of nitrous oxide to nitrogen gas. Figure 2a shows the relative abundance of genes involved in nitrogen metabolism.

## Relative abundance of genes involved in sulfur metabolism in genus *Thauera*

Sulfur metabolism plays an important role in central biochemistry as a carbon carrier and stable redox center [33]. In the absence of molecular oxygen and nitrate microbes often prefer sulfur compounds as electron donors or acceptors for energy transformation and metabolism [17–19]. Among different metabolic pathways present in the genus *Thauera* genes involved in Assimilatory sulfate Reduction (ASR) pathway were most abundantly present. These genes involved in ASR have been found to contribute to sulfate removal in various wastewater treatments [19]. In sulfur metabolism 32.25% of cys [EC:2.7.7.4] genes convert sulfate to adenylylsulfate, 5.49% cysH [EC:1.8.4.8] genes for the conversion of 3 phosphoadenylylsulfate (PAPS) to sulfite, 4.52% cysI gene converts sulfite to $H_2S$ and total 57.73% of cysN/cysNC [EC:2.7.7.4;2.7.1.25] converts adenylsulfate(APS) to 3 phosphoadenylsulfate (PAPS) (Fig. 2b).

## Relative abundance of genes involved in methane metabolism

As reported by [34] in biofilm processes under anaerobic conditions methanogenesis and denitrification process co-exist. The anaerobic methane-driven denitrification process directly utilizes methane as electron donor that is abundant in biogas produced from the anaerobic digestion of organic waste. In anoxic environments in the presence of inorganic electron acceptors (e.g., sulfate or nitrate), methanogenesis plays an important role in the biodegradation of organic matter. Figure 2c shows the relative abundance of genes involved in methane metabolism in the genus *Thauera.* In methane metabolism, the hydrogenotrophic methanogenic pathway was present that reduces hydrogen gas to formate or $CO_2$ to methane. In this pathway, 72.84% of genes were hydrogen dehydrogenase [EC: 1.12.1.2] which reversibly interconvert protons and electrons to molecular hydrogen, 24.43% of metF gene [EC: 1.5.1.20] catalyzes the reversible conversion of 5, 10- methylenetetrahydrofolate to 5-methyltetrahydrofolate and 2.73% of frmB, ESD fghA [EC: 3.1.2.12] hydrolyses to formate which can be converted to carbon dioxide.

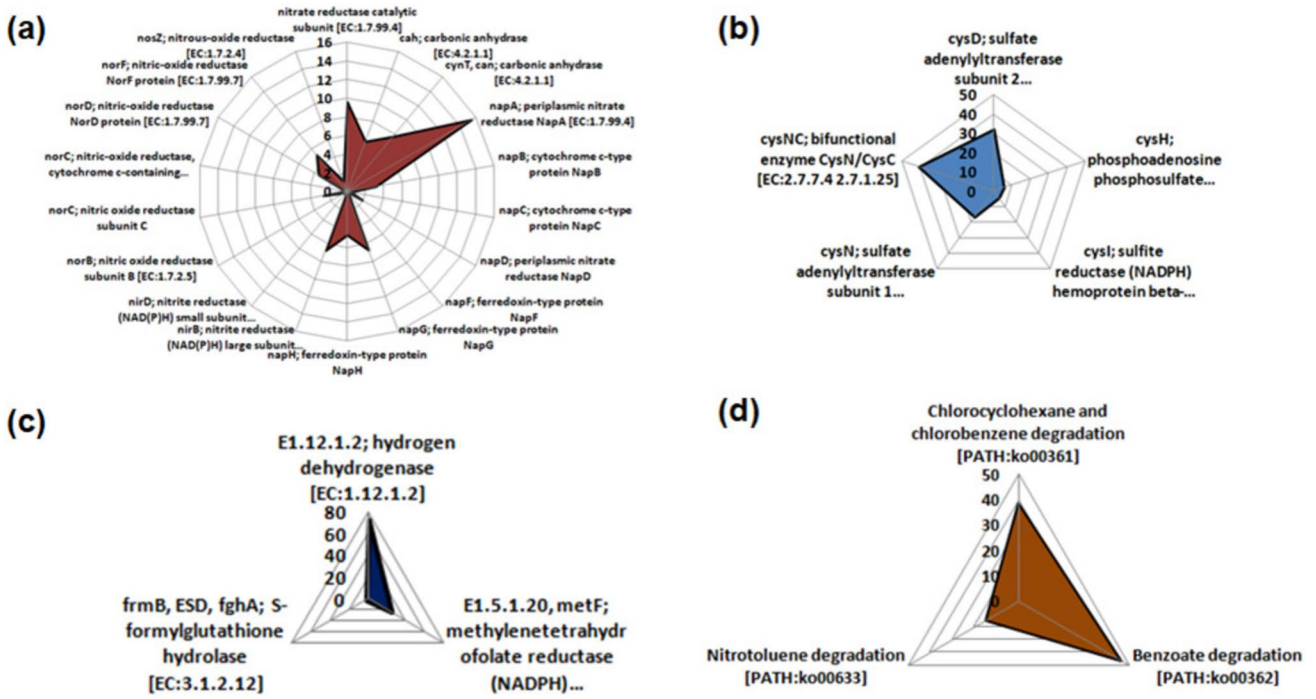



**Fig. 2** Relative abundance of genes and its functions associated with organic contaminant degradation **a** Nitrogen metabolism **b** Sulfur metabolism **c** Methane metabolism **d** Xenobiotic biodegradation

## Xenobiotic biodegradation and metabolism genes in genus *Thauera*

Presence of genes related to xenobiotics biodegradation and metabolism in *Thauera* suggest that it has a well-developed mechanism to neutralize the harmful effect of xenobiotic compounds, which is also important for the removal of nitrate containing organic pollutants. 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. Chlorocyclohexane and chlorobenzene degradation, Benzoate degradation and Nitrotoluene degradation were the most abundantly present pathways in the genus *Thauera* that were involved in the xenobiotic biodegradation and metabolic pathways. 46.55% of the genes were involved in the benzoate degradation [PATH: ko00362] (Fig. 2d) followed by 38.62% chlorocyclohexane and chlorobenzene degradation [PATH: ko00361] and 14.83% of genes that were involved in the nitrotoluene degradation [PATH: ko00633]. Benzoate pathways are the common intermediate compound of all aerobic and anaerobic metabolic pathways of aromatic compounds (phenolics, polycyclic aromatic hydrocarbons). Ubiquitous degradation pathways for benzoate and interrelated compounds are the central pathways for xenobiotics mineralization and detoxification by microbial communities [35].

## Phylogenetic analysis of *Thauera* spp.

Phylogenetic analysis of *Thauera* sp. V14, *Thauera* MZ1T and *Thauera humireducens* were performed using 16S rRNA gene sequences. 16s rRNA sequences of *Thauera* MZ1T and *Thauera humireducens* were obtained from NCBI. Our strain *Thauera* sp.V14 showed 99.38% sequence similarity with *Thauera mechernichensis* which was closely related to *Thauera humireducens* and *Thauera* sp. MZ1T which are similar in genetic makeup [10]. Therefore, our further reactor studies were carried out with *Thauera* sp. V14 as a model organism to check its potential role in the treatment of nitrate containing wastewater (Fig. 3).

## Characterization of *Thauera* sp.V14

### Denitrification efficiency, auto-aggregation and hydrophobicity

Biofilm forming denitrifying *Thauera* sp.V14 showed 99% sequence similarity with *Thauera* sp. [10]. Figure 4a showed that *Thauera* sp. V14 showed 100% denitrification efficiency within 72 h with an initial nitrate concentration of 765.89 mg $L^{-1}$. No nitrite and ammonia were detected in the medium. It

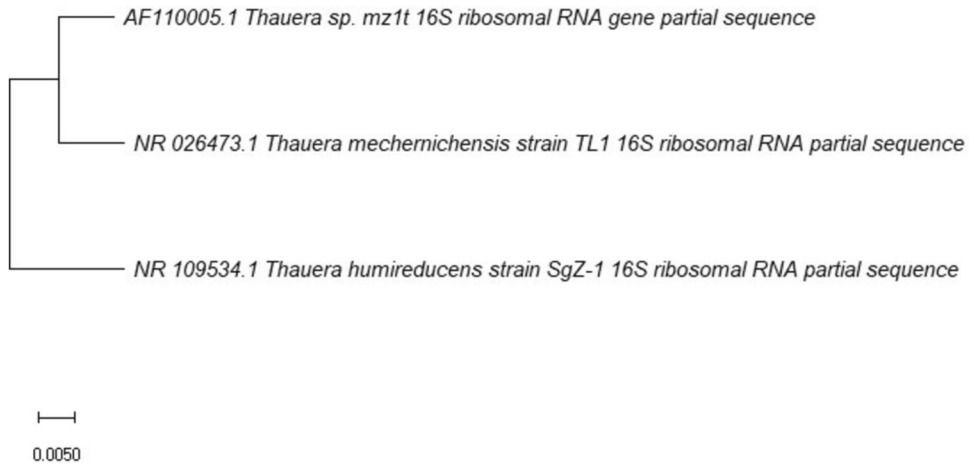



**Fig. 3** Phylogenetic tree of *Thauera* Spp

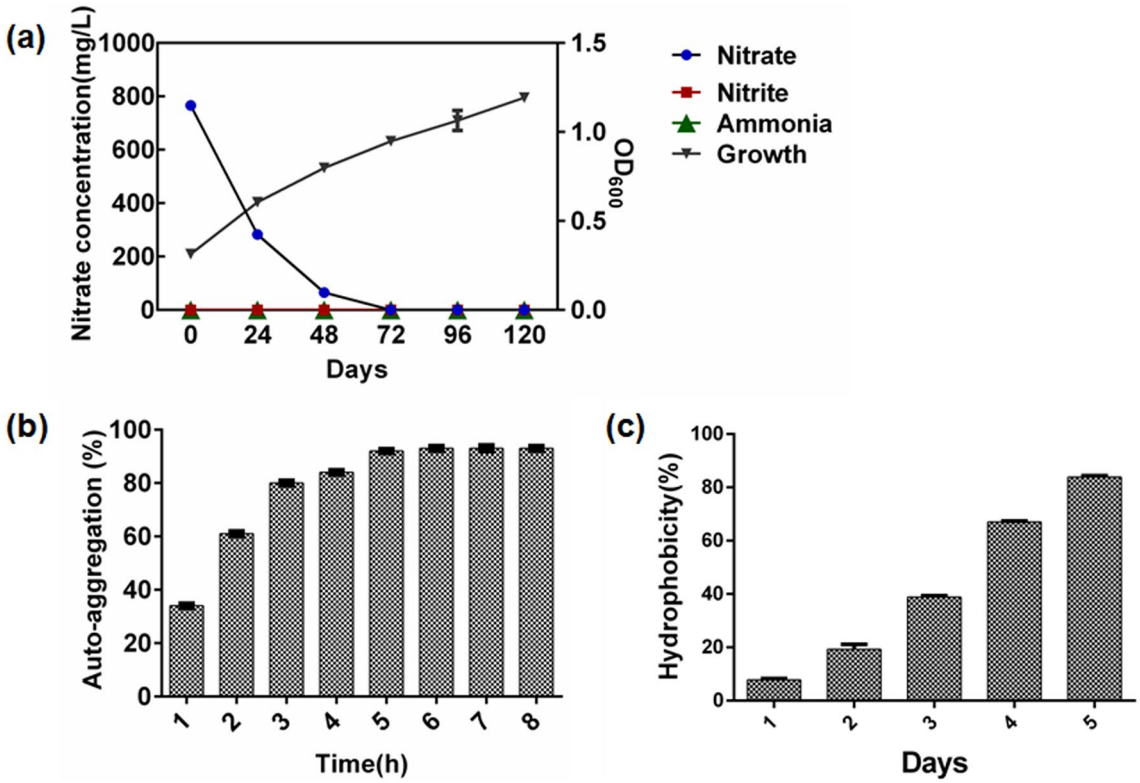



**Fig. 4** **a** Flask level denitrification studies with *Thauera* sp.V14, **b** Auto-aggregation ability, **c** Hydrophobicity of *Thauera* sp.V14

indicates that *Thauera* sp.V14 exhibited complete denitrification efficiency without accumulation of intermediates like nitrite and ammonia that have harmful effects on human and environment and potential to contribute to water pollution.

Denitrification efficiency of *Thauera* sp.V14 showed 100% denitrification efficiency with 765.89 mg $L^{-1}$ of nitrate which was higher compared to other organisms reported in the literature such as *Acinetobacter haemolyticus* ZYL removed 100% at 36 h with the initial concentration of 443 mg $L^{-1}$ [36]. In addition, *Pseudomonas stutzeri* strain XL-2 removed 97.9% of nitrate within 24 h with an initial concentration of 443 mg $L^{-1}$ [37]. *Pannonibacter phragmitetus* B1 reduced 98.77% nitrate within 18 h with an initial nitrate concentration of 65.16 mg $L^{-1}$ [38]. *Pseudomonas* sp. JQ-H3 was able to reduce 438.6 mg $L^{-1}$ of nitrate within 72 h [39]. *Zoogloea* sp. N299 reduced 75.42% nitrate within 72 h [40]. It also suggested that it was also one of the most efficient denitrifying microorganism that was reported in the literature.

A high auto-aggregation (93%) index of *Thauera* indicates a strong tendency of cells to cluster and it is a primary step in the development of biofilm [41]. Auto-aggregation increased gradually from 34% at 1 h to 93% at 8 h (Fig. 4b) and hydrophobicity from 7.7% on day 1 to 83.8% on day 5 (Fig. 4c). High denitrification efficiency, auto-aggregation and hydrophobicity suggest *Thauera* as a suitable organism for denitrification in biofilm reactor.

*Thauera* sp.V14 also showed 93% auto-aggregation capacity and 83.8% hydrophobicity, which is favorable for the formation of biofilm in dMBBR. A high auto-aggregation index of *Thauera* sp.V14 indicates a strong tendency for cells to cluster and hydrophobicity of isolate leads to better auto-aggregation ability. Auto-aggregation ability and hydrophobicity of bacteria are closely related to the aggregation and adhesion between the bacterial surface and between other bacteria [23]. Bioaugmentation of bacteria with these properties reduces the loss of bacteria from the wastewater treatment system [42]. Auto-aggregation ability and hydrophobicity of *Thauera* sp.V14 were higher than *Enterobacter* sp. strain FL which showed 54.3% at 48 h [42] and *Methylobacterium gregans* DC-1, showed 38.7% at 72 h [43] suggested *Thauera* as a suitable microorganism for biofilm reactor i.e., dMBBR.

### Kinetic analysis of nitrate removal by *Thauera* sp.V14

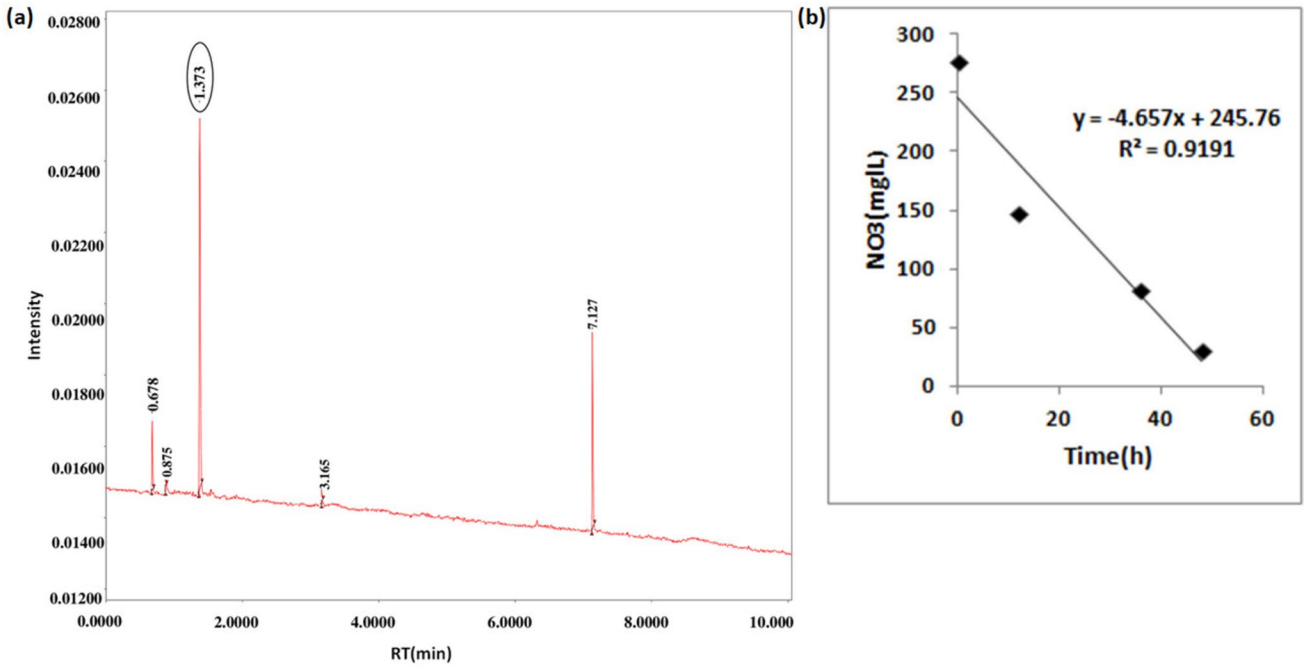
Nitrogen gas production by *Thauera* sp.V14 was confirmed by gas chromatography. Production of nitrogen gas was confirmed by comparing the peak at 1.373 with the standard of nitrogen gas. In PNB media after 48 h of incubation *Thauera* sp.V14 showed 64.68% of nitrogen gas production (Fig. 5a).

The nitrate removal rate by *Thauera* sp.V14 was investigated using the zero-order kinetic model [44]. The model can be described as follows:

$$C_t = C_i - K_0 t \tag{3}$$

where $C_i$ represents the initial $NO_3$ concentration (mg $L^{-1}$), $C_t$ (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. (3), and the regression coefficients ($R^2$). $R^2$ value for *Thauera* sp.V14 was 0.92 respectively suggesting a better fit of zero-order kinetic model (Fig. 5b). Nitrate removal rate constant for *Thauera* sp.V14 was 4.6 mg $L^{-1}$ $h^{-1}$.

Further, relative rates (RR) of nitrate reduction were calculated using the following formula:




| Sr. no. | RT(min) | Area(mV*sec) | Width(sec) | Area% |
|---|---|---|---|---|
| 1 | 0.678 | 1.421 | 1.700 | 8.474 |
| 2 | 0.875 | 0.073 | 0.700 | 0.435 |
| 3 | 1.373 | 10.846 | 2.800 | 64.686 |
| 4 | 3.165 | 0.142 | 0.900 | 0.849 |
| 5 | 7.127 | 4.285 | 2.100 | 25.556 |

**Fig. 5** **a** GC analysis of *Thauera* sp.V14, **b** Resting cell kinetics of *Thauera* sp.V14

$$RR = \frac{KNO_3}{KNO_3\text{-}KNO_2} \quad (4)$$

In the case of no nitrite build up RR would be equal to 1, and $KNO_2 = 0$. Whereas $RR \geq 1$ signifies the buildup of nitrite [44]. Relative rate of *Thauera* sp.V14 was 1 suggesting no nitrite build up by *Thauera* sp.V14. The results of kinetics studies and GC analysis suggested *Thauera* sp.V14 as an efficient denitrifying organism which is able to reduce nitrate without accumulation of nitrite, which is harmful.

## Nitrate removal studies with *Thauera* sp.V14 in continuous 10L dMBBR

To check the denitrification ability of the *Thauera* sp.V14 in the dMBBR different concentrations of nitrate viz. 620, 744, 930, 1500 and 2400 mg $L^{-1}$ were added in the influent of synthetic wastewater. As shown in Fig. 6 denitrification efficiency in the dMBBR was 91%, 90%, 76%, 66% and 60% at 620, 744, 930,1500 and 2400 mg $L^{-1}$ of $NO_3$ concentration and every time COD reduction was below the stipulated permissible range i.e. 250 mg $L^{-1}$. Notably, no nitrite and ammonia were detected inside dMBBR which was important for the complete denitrification process. It was also observed that as the nitrate concentration increases denitrification efficiency in the dMBBR was decreased. This could be due to the saturation of denitrifying enzymes. Because as the denitrifying enzymes saturated their denitrification efficiency decreases. Hence, it can be suggested that *Thauera* sp.V14 was able to reduce nitrate up to 2400 mg $L^{-1}$ with 60% efficiency. In the context of nitrate contamination in India, where concentration in the groundwater and surface water was in the range of 139 to 557 mg $L^{-1}$ in wastewater [45–53], and our isolate *Thauera* sp. V14 showed high denitrification efficiency i.e., 90% till 744 mg $L^{-1}$, suggested that it can be used for the treatment of high nitrate containing wastewater. Moreover, nitrate removal efficiency of *Thauera* in dMBBR was also higher than other reported denitrifying organisms such as *Pseudomonas mendocina* IHB602 in sequencing batch biofilm reactor [23], *Acinetobacter* sp.TAC-1in MBBR [7] and *Diaphorobacter polyhydroxybutyrativorans* in the denitrifying reactor [54]. These findings suggested *Thauera* sp.V14 as the most efficient microorganism and can be used as a potential organism for denitrification of nitrate containing wastewater. Furthermore, abundance of *Thauera* sp.V14 in the biofilm of dMBBR was checked i.e. $2 \times 10^8$ copy number/µL of biomass as per RTPCR suggesting that dMBBR was fully conditioned with *Thauera* and this organism may be contributing majorly in the denitrification. Moreover, High abundance of *Thauera* inside the developed biofilm of dMBBR suggested its successful bioaugmentation in the dMBBR.

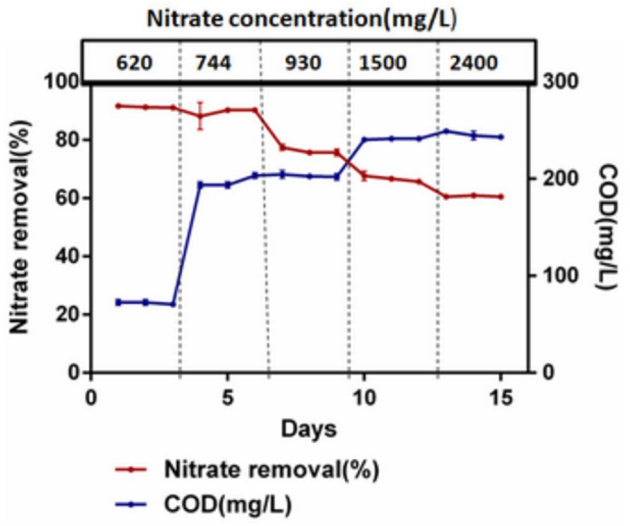



**Fig. 6** Nitrate removal studies in dMBBR developed with *Thauera*sp. V14

## Characterization of *Thauera* biofilm developed inside dMBBR

### Surface topology and EPS composition of the biofilm developed in the dMBBR with *Thauera* sp.V14

The biofilm morphology developed on the pall ring carrier was examined using SEM images. Figure 7a represents the SEM images of the carriers before biofilm formation and after nitrate removal studies and biofilm formation on Pall ring carriers. Biomass on the carrier material was 30 ± 5 mg/carrier. Carriers after biofilm formation showed high bacterial density and biofilm seemed to be dominated by rod shaped bacteria. These results were similar to [55] who also showed rod-shaped bacteria as the most predominant bacteria in the denitrifying bioreactors. Successful colonization of bacteria on the pall ring carriers and the carrier surface provided a good porous structure so that bacteria did not easily wash away in dMBBR.

Further, FTIR spectroscopy was employed to analyze the composition and functional group characteristics present in the developed biofilm of the dMBBR. The FTIR profiles of different functional groups are illustrated in Fig. 7b. Results of the FTIR spectrum (Fig. 7b) revealed that the spectrum in Fig. 7b displayed a broad absorption region between 3418 $cm^{-1}$ assigned to the O–H bond in hydroxyl functional groups [56]. This confirmed the presence of the hydrogen bond of amines and alcohols (or phenols) in the biofilm. Small peaks of 2851 and 2921 $cm^{-1}$ were due to $CH_2$ stretching. The peak at 2519 $cm^{-1}$ was due to O–H and C–N stretching and bending of carboxylic acids [57].

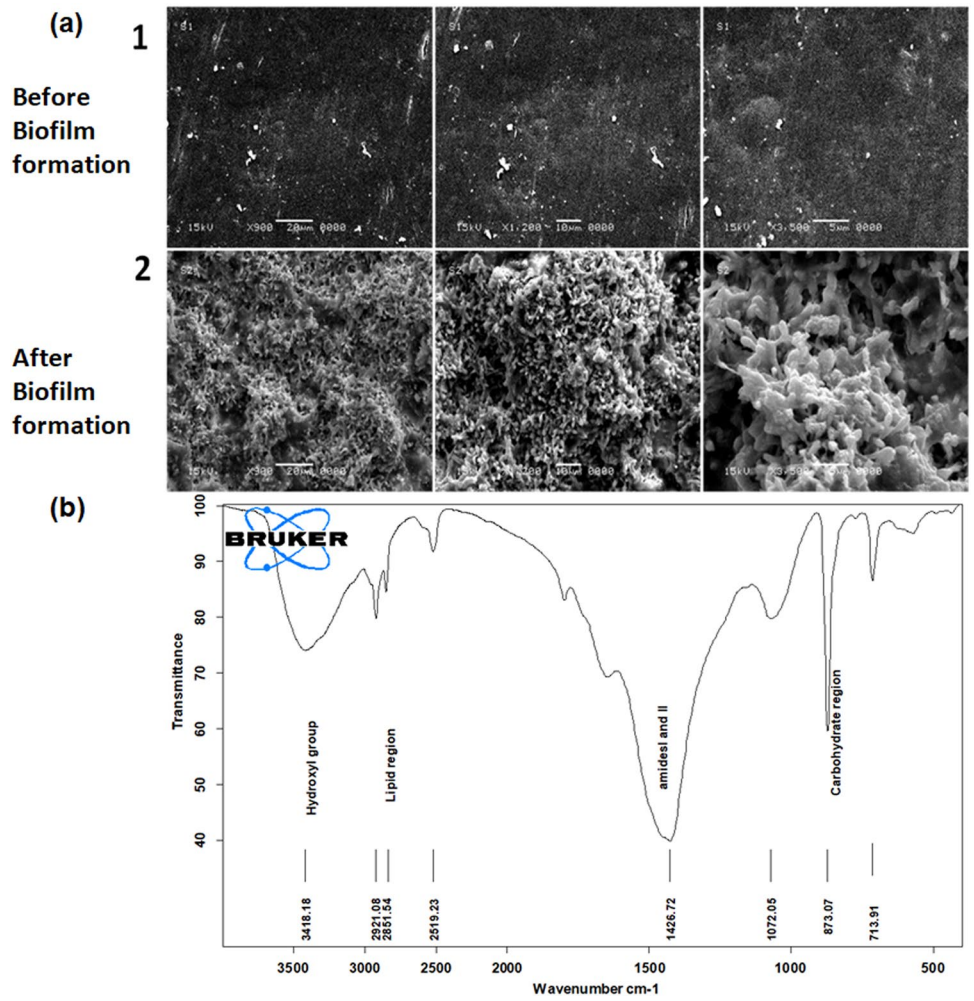



**Fig. 7** **a** SEM image of the surface of the carrier before use and surface of the carrier after biofilm development inside dMBBR developed with *Thauera* sp.V14, **b** FTIR of the biofilm developed on the carrier

The peak at 860 $cm^{-1}$ (aromatic C–H bending vibration) [58] and, peaks in the region of 600–800 $cm^{-1}$ are due to aromatic compounds [59], which could be an indicator of humic substrates. The broad peak at around 1426 $cm^{-1}$ was mainly derived from amides I and II [56]. In addition, a broad peak at 1072 $cm^{-1}$ exhibits the character of carbohydrates or carbohydrate-like substances, which indicates that carbohydrates were present in the EPS [60].

## Conclusion

Overall, the results of this study suggested that the denitrifying bacterium *Thauera* sp.V14 has great potential in the denitrification of nitrate containing wastewaters. Bioaugmentation of dMBBR with *Thauera* sp.V14 successfully enhanced denitrification efficiency as well as biofilm formation in the reactor. *Thauera* sp.V14 could efficiently remove nitrate and COD from wastewater without accumulation of $NO_2$ and $NH_4$ and form biofilms. Its hydrophobicity, auto-aggregation ability, biofilm formation ability, and denitrification competence indicate its adaptability for efficient colonization and nitrate removal. These characteristics emphasize potential of *Thauera* sp. V14 as an effective bacterium for the removal of nitrate from wastewater.

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**Availability of data and materials** Not applicable.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

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