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Isolation and Identification of Dibenzothiophene Desulfurizing Bacteria Occurring in Oil Contaminated Soils of Mechanical Workshops

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Abstract

When fossil fuels are oxidized, a wide range of hazardous gases reach the atmosphere. Among these gases, sulfur oxides which are released because of oxidation of organosulfur compounds occurring in fuels become the sources of environmental pollution and acid rain. Hydrodesulfurization, a traditional practice employed for the removal of sulfur content from petroleum products during refining process is not eco-friendly and effective in removal of sulfur content, especially, recalcitrant organosulfur compounds like dibenzothiophene. Biodesulfurization, which employs microbes for the removal of sulfur from fossil fuels, is an eco-friendly method and a better alternative to hydrodesulfurization. The dibenzothiophene is treated as a model organosulfur compound for biodesulfurization studies. The present paper deals with the isolation of bacteria which exhibit dibenzothiophene biodesulfurization via 4S pathway from oil contaminated soils, detection of intermediates and end product of 4S pathway using Gas chromatography-Mass spectroscopy (GC-MS) in the DBT culture broths of isolates positive for gibb's test, amplification of *dsz* operon genes which regulate 4S pathway in desulfurizing bacteria and identification of DBT desulfurizing bacteria by microscopic examination, biochemical tests and 16S rRNA gene anaylsis. Two DBT desulfurizing bacteria isolated were found positive for Gibb's test and Dibenzothiophene sulfone ($DBTO_2$), one of the intermediates and 2-Hydroxy biphenyl, the end product of 4S pathway were detected in DBT containing culture broths of both the desulfurizing bacteria when subjected to GC-MS. In both, the bacteria *dsz* operon genes were detected. The two bacteria were identified as *Streptomyces* sp. VUR PPR 101 and *Streptomyces* sp. VUR PPR 102.

Keywords: Biodesulfurization, Dibenzothiophene, 4S pathway, *dsz* operon, *Streptomyces* sp. VUR PPR 101, *Streptomyces* sp. VUR PPR 102

1. Introduction

The third most abundant element in fossil fuels is sulfur, and numerous organosulfur and sulfur containing inorganic compounds are present in fuels. When fossil fuels are oxidized, sulfur oxides reach the atmosphere (Bordoloi *et al.,* 2016; Sadare *et al.*, 2017) which are potent air pollutants and major source of acid rains (Li *et al.,* 2003). Sulfur dioxide in the environment causes health problems in humans like irritation of eyes, respiratory problems etc. (Wondyfraw, 2014). Even the plants that are exposed to sulfur dioxide exhibit change in their physiology and become susceptible to microbial infections. Prolonged exposure to sulfur dioxide increases rate of transpiration and dark respiration in plants (Khan and Khan, 2011). The conventional hydrodesulfurization (HDS) process used for the removal of sulfur from fossil fuels (petroleum products) which is operated at high temperature and pressure is not so effective in the elimination of sulfur from organosulfur compounds occurring in fossil fuels. Particularly, HDS is not effective in removal of sulfur from recalcitrant organosulfur compounds like Dibenzothiophene (DBT) (Wang *et al.,* 2017; da Silva and Secchi, 2018). To overcome the sulfur dioxide related problems, a new method involving microbes known as Biodesulfurization (BDS) has been developed to remove sulfur content from fossil fuels. The BDS is a very economical and eco-friendly process which does not make use of high temperature and pressure (Wang *et al.,* 2017). For Biodesulfurization studies, DBT which is highly recalcitrant and hazardous is treated as model compound. The DBT is a very persistent compound as compared to most polyaromatic hydrocarbons (PAHs) and other aromatic hydrocarbons. It can remain for 10 years in crude oil polluted sediments, long after most aromatics have disappeared (Li *et al.,* 2005; Mezcua *et al.,* 2008). Microorganisms employ three major pathways for DBT metabolism *viz.*, Kodama, Van Afferden and 4S pathways of which the 4S pathway is regarded as commercially important one (de Araujo *et al.,* 2012). The microbes which metabolize DBT via 4S pathway will not break the ring structure of DBT, and thus calorific value of fuel is unaffected. Specific interest was paid by researchers in isolating bacteria that specifically remove sulfur from organosulfur compounds without breaking the carbon backbone of the original organosulfur compounds. The 4S pathway is such a metabolic pathway exhibited by certain

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microbes during which sulfur element is removed as sulfite from DBT without affecting its ring structure (Labana *et al.,* 2005). The DBT metabolism via other pathways may result in cleavage of DBT ring or sulfur is not removed. Hence, microbes which exhibit 4S pathway for the desulfurization of DBT are eco-friendly and economically important. Various microbes which exhibit 4S pathway include *Rhodococcus erythropolis* XP, *Rhodococcus erythropolis* IGTS8, *Gordonia alkanivorans* RIPI90A etc. (de Araujo *et al.,* 2012; Bordoloi *et al.,* 2016). The 4S pathway is a multienzymatic system with four different activities (Gray *et al.,* 1996). First enzyme of the 4S pathway is monooxygenase (DszC) which catalyzes the DBT oxidation to Dibenzothiophene sulfone ($DBTO_2$) in two steps. The second is also a monooxygenase (DszA) which converts $DBTO_2$ to Hydroxyphenyl benzene sulfonate (HPBS), and in the final step DszB (HPBS desulfinase) converts HPBS to 2-hydroxy biphenyl (2-HBP) and sulfite. In this metabolic pathway, FMN-reductase (DszD) has an important role in the activity of the monooxygenases, as it is responsible for the maintenance of reduced flavin levels. The *dsz A, B* and *C* genes of *dsz* operon synthesize DszA, DszB and DszC enzymatic proteins, respectively (Muraka *et al.,* 2019). The microbial cells have been majorly exploited in pharmaceutical and food industries to produce antibiotics, vitamins, amino acids, alcoholic beverages etc., on large scale. Even various microbial strains are employed in reclamation of polluted sites (bioremediation) and treatment of used engine oils. The biocatalytic desulfurization of fuels is an important and useful emerging process in the present-day context in view of curbing the environmental pollution. The biodesulfurizing microbial strains could pave the way for effective desulfurization process and can be employed in future to generate sulfur free fuels. In the present work, an attempt was made to isolate bacterial strains exhibiting DBT biodesulfurization via 4S pathway using Gibb's assay and by detecting the intermediates and end product of 4S pathway in DBT containing culture broth using Gas chromatography-Mass spectroscopy (GC-MS), identification of *dsz* operon genes responsible for 4S pathway using specific primers and identification of the positive DBT desulfurizing bacteria based on morphological and microscopic examination, biochemical tests and 16S rRNA gene sequencing.

2. Materials and Methods

2.1. Isolation of bacteria growing in DBT supplemented medium

To isolate Dibenzothiophene desulfurizing bacteria, different mechanical workshop sites in Karimnagar town, Telangana, India were selected and designated as A, B, C, D, E and F. Heavy motor vehicles in workshops A and B, light motor vehicles in workshops C and D, and two wheelers in workshops E and F are repaired and serviced. From these sites, oil contaminated soil samples were collected bimonthly for a period of one year. For the isolation of DBT-desulfurizing bacteria, ten grams of each soil sample was suspended in 90 ml of distilled water and 5 ml of the suspension was inoculated into 250 ml flask containing 45 ml of basal salt medium (BSM) supplemented with glucose (5 g/l) as carbon source and 5 mM per liter DBT as sole sulfur source. Cultivation was performed at 30^{o} C in rotary shaker for 5 days. After five sub cultivations, the culture broth medium was spread onto BSM agar medium supplemented with glucose and DBT (Khedkar and Shanker, 2014). All the viable and dominantly growing representative bacterial cultures were isolated from samples of Workshops.

2.2. Gibb's assay

The recovered cultures were grown in BSM broth supplemented with glucose and DBT and tested for the occurrence of 4S pathway within them by detecting 2-HBP, the end product of 4S pathway by the Gibb's assay. The test was used to detect the 2-HBP produced by the bacteria after growing them in the medium containing DBT. Gibb's reagent reacts with aromatic hydroxyl groups such as 2-hydroxy phenyl to form blue colored complex. The pH of bacterial culture broths was adjusted to 8.0 using 10% sodium bicarbonate or sodium carbonate and 100 µl of Gibb's reagent (10 mg of 2,6-dichlorquinone-4-chlorimide prepared in 1 ml of ethanol) was added to 5 ml of each bacterial culture broth and incubated for 30 minutes. Formation of blue color complex indicates the positive result and brown color indicates negative result for Gibb's assay. The cultures positive for Gibb's assay were expected to produce 2-HBP via 4S pathway from DBT (Kayser *et al.,* 1993).

2.3. GC-MS analysis of DBT culture broths of bacteria positive for Gibb's test

The culture broth was centrifuged and the supernatant was acidified with 6N hydrochloric acid to pH 2.0. The acidified supernatant was mixed with equal volume of ethyl acetate, and the extracted samples were subjected to GC-MS analysis. The GC-MS instrument employed was an Agilent 7890A model in which GC was coupled with a Pegasus HT TOFMS. The specifications of GC column include 29.8 m x 0.2 µm and 0.2 m x 320 µm. The initial and final temperatures employed for sample analysis were 60ºC and 320ºC, respectively. The rate of increase of temperature during sample analysis was 15ºC per minute. The temperature maintained during the entire sample run was 280ºC. Helium was used as carrier gas with a flow rate of 1.2 ml per minute to run 1 µl of DBT culture broth sample.

2.4. Identification of dsz operon genes responsible for 4S pathway

The forward and reverse primers used for the amplification of *dszA* gene were 5'-GCGCGGCAAGTTCGATCTGT-3' and 5'-TCCCGCAGGATGTCCTTGATC-3', respectively. The primers used for the amplification of *dszB* gene were 5'-ATCGAACTCGACGTCCTCAG-3' (forward) and 5'-TCAGGACCACAGCTACAAGG-3' (reverse). For the amplification of *dszC* gene, the forward and reverse primers employed were 5'-CTGTTCGGATACCACCTCAC-3' and 5'-GTGCCTGAAGGTGTTGCA-3', respectively (Duarte *et al.,* 2001; Li *et al.,* 2007).

2.5. Identification of bacterial isolates

2.5.1. Colony characteristics

Colony characteristics of the bacterial isolates positive for DBT desulfurization via 4S pathway were observed on DBT containing basal salt medium. The color, configuration, elevation, margin and size of the colonies were recorded.

2.5.2. Microscopic examination and Biochemical tests

Gram staining and spore staining of the DBT desulfurizing bacteria were performed and the morphological shapes were also noticed. The biochemical tests *viz*., starch hydrolysis, casein hydrolysis, sucrose test, catalase test, oxidase test, hydrogen sulfide production, lipid activity, indole test, methyl red test, Voges-Proskaeur test, citrate utilization test, urea hydrolysis test and nitrate reduction test were performed as per the standard procedures (Kim *et al.,* 2001; Reddy *et al.,* 2011; Bennett *et al.,* 2018).

2.5.3. 16S rRNA sequencing and phylogenetic analysis of the bacterial isolates

The 16s rRNA sequencing and phylogenetic analysis of the bacterial isolates positive for DBT desulfurization were carried out at Bioaxis DNA Research Centre (BDRC), Hyderabad for the identification. The primers used for the amplification of 16S rRNA gene were 5'-GCAATAACAGGTCTGTGATGCCC-3' (forward) and 5'-GCATCACAGACCTGTTATTGC-3' (reverse) (Frank *et al.,* 2008).

2.6. Isolation of chromosomal DNA

The cultures of B39 and B40 were transferred separately into 50 ml BSM broth supplemented with glucose and dibenzothiophene taken in 250 ml flasks and incubated in a shaker at 30^{o} C at 180 rpm for 4 days. After incubation period, the culture broths were subjected to centrifugation at 10,000 rpm for ten minutes. Then 0.1 grams of mycelium of each isolate was crushed with liquid nitrogen in a clean porcelain dish. Then crushed mycelium of each isolate was transferred to a tube consisting of 500 µl TE buffer containing lysozyme enzyme and incubated for half an hour at 37^{o} C. Then, 20 µl SDS (10%) and 20 µl proteinase K were added, and the tube was incubated for thirty minutes at 55^{o} C. After incubation period, the mixture was cooled and centrifuged at 10,000 rpm for five minutes and extracted with phenol-chloroform (1:1) solution. The aqueous phase of the mixture was taken in clean tube and DNA precipitate was obtained by using 90% ethyl alcohol at -20^{o} C. Then DNA pellet was recovered after centrifuging for ten minutes at 10,000 rpm. The pellet was dissolved in TE buffer and 20 µl of RNase enzyme was added and incubated at 37^{o} C for one hour to get RNA free DNA. The DNA is again precipitated by using 90% ethyl alcohol at -20^{o} C. Then, pure DNA pellet was obtained by centrifugation (at 10,000 rpm for 10 minutes) (Kumar *et al.,* 2010), and the purity of DNA was determined by using UV spectrophotometer.

2.7. PCR amplifications

The PCR mixture containing 200 µM dNTPs, 100 ng of genomic DNA, 6 mM magnesium chloride, 10% dimethyl sulfoxide, each primer of 30 pmol and 2.5 units of Taq Pol. in 50µl buffer was prepared. The amplification was performed by initially increasing the temperature of mixture to 94^{o} C for 1 minute (denaturation), then decreased to 57^{o} C for 60 seconds (annealing) and increased to 72^{o} C for 1 minute (extension of hybridized primers), followed by final extension at 72^{o} C for 5 minutes in thirty-five cycles. Then the PCR reaction mixture was subjected to agarose gel electrophoresis by employing a size marker of 1 kb DNA ladder. The dideoxy chain termination procedure was used for the sequencing of rRNA genes (Kurnijasanti *et al.,* 2017). The 16S rRNA gene sequences of B39 and B40 isolates were submitted to NCBI Genbank. Further, neighbor joining method was employed for the construction of phylogenetic trees of the isolates.

3. Results and Discussion

3.1. Isolation of predominantly growing bacteria on DBT containing medium and screening for bacterial strains positive for Gibb's test

In the present work, a total of 46 predominantly growing bacterial representative types on BSM medium supplemented with DBT were obtained from oil contaminated sites of different mechanical workshops. On screening, all these isolates for the presence of 4S pathway metabolic activity through Gibbs assay, only two bacteria (designated as B39 and B40) isolated from the soil of mechanical workshop B were found positive for 4S pathway. This was confirmed by the development of blue coloration during Gibbs assay indicating the production of 2-HBP which is the end product of 4S pathway (Rahpeyma *et al.,* 2017). The Gibb's assay was used to identify the desulfurization activity of bacteria through 4S pathway by several earlier workers (Sadare *et al.,* 2017; Shahaby and El-din, 2017; Li *et al.,* 2019). The other isolates of our study which were negative for Gibb's assay may be utilizing the other pathways *viz*., Kodama pathway and Van Afferden pathway for metabolizing DBT as they were also grown abundantly on basal salt medium supplemented with glucose and DBT as sole sulfur source.

3.2. Detection of intermediates and the end product of 4S pathway by GC-MS

The two Gibb's test positive bacterial isolates (B39 and B40) were subjected to GC-MS analysis for the detection of metabolites of 4S pathway. In GC-MS analysis of B39 isolate culture broth, the compounds with retention time of 13.6117 and 9.22667 minutes were identified as DBT sulfone and the 2-HBP, respectively (Figure 1). Similarly, GC-MS of B40 culture broth showed the presence of the same compounds with retention time of 13.61 and 9.23167 minutes that were identified as DBT sulfone and 2-HBP, respectively (Figure 2). Li *et al.* (2003) have detected DBT sulfone and 2-HBP by GC-MS in the DBT culture broth of *Mycobacterium* sp. X7B. Similarly, Rhee *et al.* (1998) during their DBT desulfurizing studies with newly isolated *Gordona* strain CYKS1, observed DBT sulfone and 2-HBP in the GC chromatogram of DBT culture broth. In the mass spectrum of DBT sulfone pertaining to B39 isolate, a molecular [M^{+}] ion peak at *m/z* 216 was found corresponding to the molecular mass of DBT sulfone and the major fragmentation ions were seen at *m/z* 139 and 63 (Figure 3). Similarly, in the mass spectrum of 2-HBP of B39, a molecular [M^{+}] ion peak at *m/z* 170 was observed corresponding to the molecular mass of 2-HBP and the major fragmentation ions were seen at *m/z* 141,115 and 63

(Figure 4). In the mass spectrum of DBT sulfone of B40 isolate, a molecular [M^+] ion peak at *m/z* 216 was noticed corresponding to the molecular mass of DBT sulfone and the major fragmentation ions were seen at *m/z* 187, 136 and 63 (Figure 5). Similarly, in the mass spectrum of 2-HBP of B40, a molecular [M^+] ion peak at *m/z* 170 was found corresponding to the molecular mass of 2-HBP and the major fragmentation ions were seen at *m/z* 141,115, 89 and 63 (Figure 6). This observation is in great concurrence with several earlier reports in this aspect (Mohebali *et al.*, 2008; Mohammed *et al.*, 2015; Ismail *et al.*, 2016). Even, Akhtar *et al.* (2009) also detected similar molecular ion peaks of DBT sulfone and 2-HBP in the mass spectra of DBT culture broths of *Rhodococcus* species, when subjected to GC-MS.

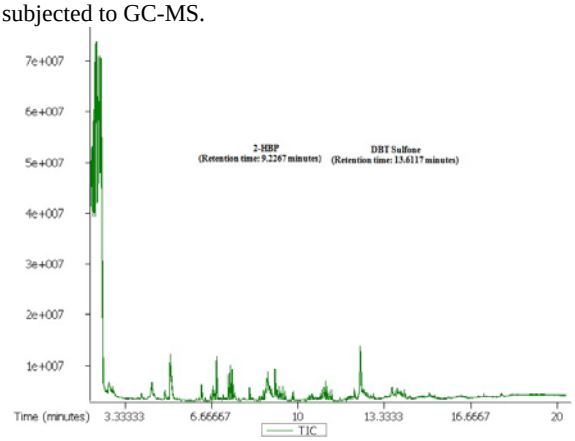

Figure 1. Gas Chromatogram of DBT broth extract of the bacterial isolate B39

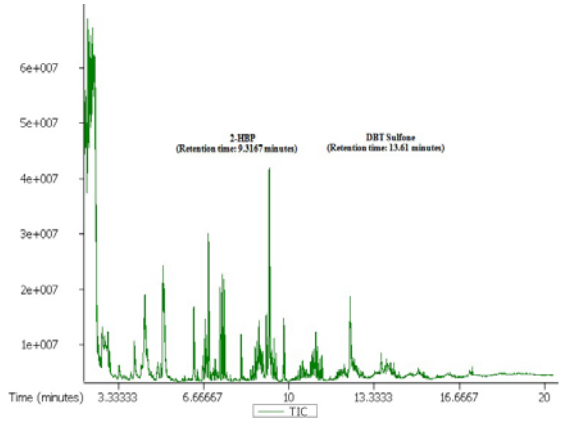

Figure 2. Gas Chromatogram of DBT broth extract of bacterial isolate B40

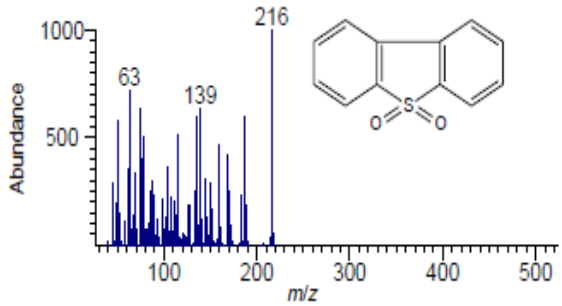

Figure 3. Mass spectrum of DBT sulfone of the bacterial isolate B39

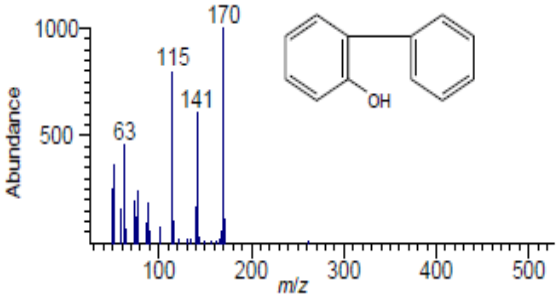

Figure 4. Mass spectrum of 2-HBP of the bacterial isolate B39

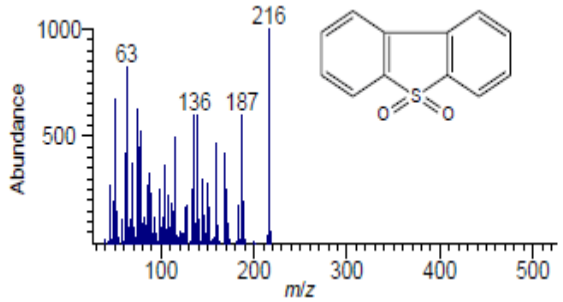

Figure 5. Mass spectrum of DBT sulfone produced by bacterial isolate B40

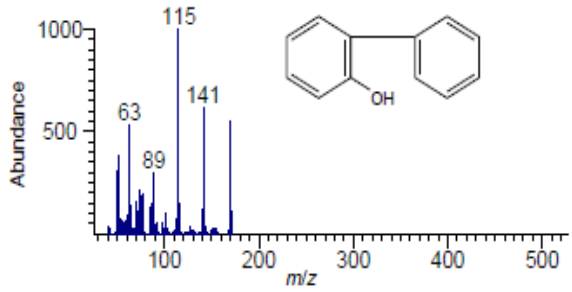

Figure 6. Mass spectrum of 2-HBP produced by bacterial isolate B40

3.3. Amplification of dsz operon genes in DBT desulfurizing bacteria

In both the bacterial isolates, *dsz ABC* operon genes were amplified (Figure 7) indicating their presence. Interestingly, the *dsz* operon in both the isolates was located on genomic DNA, which has good concurrence with earlier report of Shavandi *et al.* (2010) who identified the presence of *dsz* operon genes responsible for DBT desulfurization on chromosome in *Gordonia alkanivorans* RIPI90A. However, in *Rhodococcus erythropolis* IGTS8 strain, which is a model strain for biodesulfurization studies, the *dsz* operon was reported to be present on plasmid.

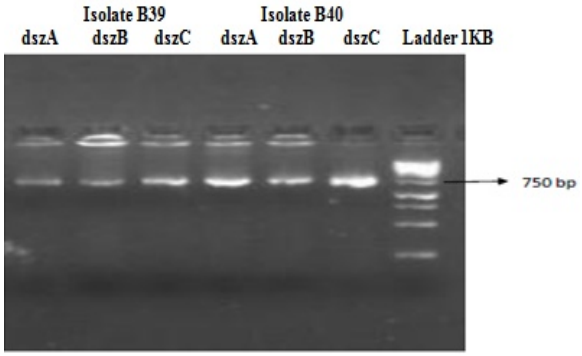

Figure 7. PCR gel picture of amplified *dsz* operon genes of the B39 and B40 bacterial isolates

3.4. Identification of bacteria positive for DBT desulfurization via 4S pathway

Based on colony characteristics (Table 1), morphological (shape), staining (Gram's and Spore) and biochemical studies *viz*., starch hydrolysis, casein hydrolysis, sucrose test, catalase test, oxidase test, hydrogen sulfide production, lipid activity, indole test, methyl red, Voges-Proskaeur test, citrate utilization test, urea hydrolysis and nitrate reduction test (Table 2), the two desulfurizing bacteria are identified as Gram positive, filamentous and tentatively as *Streptomyces* species. Taddei *et al.* (2006) based on the similar morphological and biochemical studies, identified bacteria isolated from Venezuelan soils as *Streptomyces* species.

Table 1. Colony characteristics of the bacterial isolates

Colony character	B39 isolate	B40 isolate
Color	White	Light grey
Configuration	Round	Round
Elevation	Convex	Convex
Margin	Ciliate	Ciliate
Size (Diameter)	4.2 mm	3.5 mm

Table 2. Morphological, Gram staining, Spore shape and Biochemical tests.

Characteristics	Bacterial isolates	
	B39	B40
Microscopic observation		
Morphological shape	Filamentous	Filamentous
Gram staining	Gram positive	Gram positive
Spore shape	Oval to circular	Oval
Biochemical tests		
Starch hydrolysis	Positive	Positive
Casein hydrolysis	Positive	Positive
Sucrose test	Positive	Positive
Catalase test	Positive	Positive
Oxidase test	Positive	Positive
H_2S Production test	Positive	Positive
Citrate utilization test	Positive	Positive
Lipid activity	Positive	Positive
Indole test	Negative	Negative
Methyl red test	Positive	Positive
Voges-Proskaeur test	Negative	Negative
Urea hydrolysis	Positive	Positive
Nitrate reduction test	Positive	Positive

The 16S rRNA genes of both the bacterial isolates were amplified using the standard primers and sequenced. The partial length of rRNA gene sequences of bacterial isolates, B39 and B40 were 1,393 and 1,395, respectively. The phylogenetic analysis revealed that B39 and B40 were closely related to *Streptomyces* sp. SPMA113 (Accession No. HQ340163.1) and *Streptomyces* sp. Antag6 (Accession No. JQ417273.1), respectively (Figure 8). The percentage of identity between the *Streptomyces* species was determined using NCBI BLASTn tool. The percentage of identity between B39 and *Streptomyces* sp. SPMA113 was found to be 100%. Similarly, the percentage of identity between B40 and *Streptomyces* sp. Antag6 was also 100%. There was 98.91% of identity between B39 and B40 isolates. The rRNA gene proved to be a universal tool for the phylogenetic analysis and interrelation among the organisms as it is ancient, universally distributed and most conserved region in the genome of the microorganisms. Although there are three different ribosomal RNAs i.e., 5S, 16S and 23S in prokaryotes, only 16S rRNA sequence is used because the nucleotides in 16S rRNA are neither less nor more in length and easy to sequence. The rRNA sequence is used to construct phylogenetic tree by applying distance-matrix method. The evolutionary distance is determined by recording differences in the sequences of two or more organisms by software computer analysis. A statistical correction factor is applied due to the reason that some changes might have taken place in the genome which would lead back to the same sequence. After measuring the evolutionary distance, the phylogenetic tree is constructed. The different evolutionary distances of the two microorganisms are directly proportional to the total length of the branches separating them. Depending on software/computer program and the number of microorganisms, different formats of phylogenetic trees are possibly constructed (Chapus *et al.*, 2005). The gene sequences were submitted to NCBI Genbank designating B39 and B40 isolates as *Streptomyces* sp. VUR PPR101 and *Streptomyces* sp. VUR PPR 102, respectively. The Genbank had accepted and given the accession numbers, KF551242.1 and KF551243.1 to *Streptomyces* sp. VUR PPR 101 and *Streptomyces* sp. VUR PPR 102, respectively.

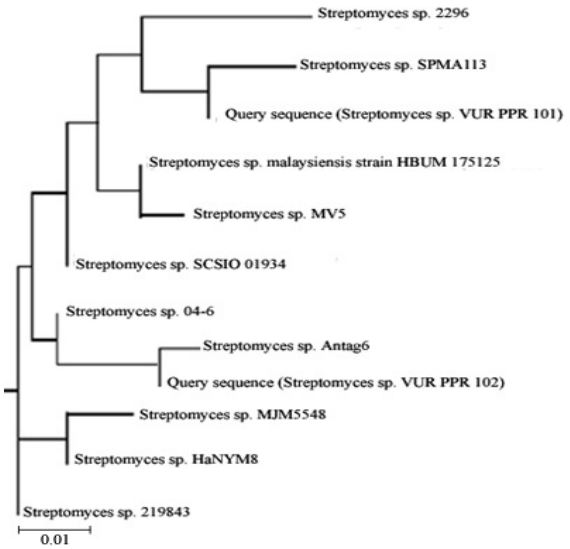

Figure 8. Phylogenetic analysis of *Streptomyces* sp. VUR PPR 101and *Streptomyces* sp. VUR PPR 102.

The scale bar infers 1% of the estimated nucleotide difference.

The *Streptomyces* species isolated in the present work are aerobic in nature and obviously exhibiting the aerobic DBT desulfurization (4S) pathway. Therefore, they will be commercially and economically important when compared to microbial strains exhibiting anaerobic biodesulfurization pathways. When microbial strains exhibiting oxidative DBT desulfurization pathway are employed during oil refining process, they form water soluble sulfite from DBT which can be easily disposed by employing an aqueous phase. On the other hand, anaerobic desulfurization occurs by a reductive pathway during which sulfur from DBT is removed as hydrogen sulfide which later releases sulfur atom. Though the anaerobic strains can be employed for desulfurization, it is a difficult and costly affair to maintain anaerobic conditions throughout the process, moreover it is time consuming and undesirable products may form from organic constituents of fuels (Sadare *et al.,* 2017). Further, there is a scope to genetically modify these *Streptomyces* species for enhanced DBT biodesulfurization activity. Such genetically improved biodesulfurizing *Streptomyces* strains can be commercially important and potent to employ in desulfurization of fuels during refining process to produce sulfur free fuel products.

4. Conclusion

In the present study, two potential DBT desulfurizing *Streptomyces* species were isolated from oil contaminated soils of mechanical workshops. Both the desulfurizing species are commercially important as they showed the ability to desulfurize the DBT occurring in fuels, the model compound for biocatalytic desulfurization studies by 4S pathway without breaking the ring structure of DBT and not leading to the reduction of calorific value of fuel (mileage). The *dsz* operon genes (*A*, *B* and *C*) responsible for 4S pathway were identified in both the *Streptomyces* species. These two DBT desulfurizing *Streptomyces* species gain ecological and commercial importance in Biodesulfurization of fuels.

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Conflict of interests

The authors declare that they have no conflict of interests.

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