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C–S Targeted Biodegradation of Dibenzothiophene by *Stenotrophomonas* sp. NISOC-04

Moslem Papizadeh · Mohammad Roayaei Ardakani · Hossein Motamedi · Iraj Rasouli · Mohammad Zarei

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Abstract Crude oil-contaminated soil samples were gathered across Khuzestan oilfields (National Iranian South Oil Company, NISOC) consequently experienced a screening procedure for isolating C–S targeted dibenzothiophene-biodegrading microorganisms with previously optimized techniques. Among the isolates, a bacterial strain was selected due to its capability of biodegrading dibenzothiophene in a C–S targeted manner in aqueous phases and medium mostly consisting of separately biphasic water–gasoline. The 16S rDNA of the isolate was amplified using eubacterial-specific primers and then sequenced. Based on sequence data analysis, the microorganism, designated NISOC-04, clustered most closely with the members of the genus *Stenotrophomonas*. Gas chromatography indicated that *Stenotrophomonas* sp. NISOC-04 utilizes 82% of starting 0.8 mM dibenzothiophene within a 48-h-long exponential growth phase. Growth curve analysis revealed the inability of *Stenotrophomonas* sp. NISOC-04 to utilize dibenzothiophene (DBT) as the exclusive carbon or carbon/sulfur source. Gibbs' assay showed no 2-hydroxy biphenyl accumulation, but HPLC confirmed the presence of 2-hydroxy biphenyl as the final product of DBT desulfurization. Under sulfur starvation, *Stenotrophomonas* sp. NISOC-04 produced a huge biomass with untraceable sulfur and utilized atmospheric insignificant sulfur levels.

Keywords Biodesulfurization · Dibenzothiophene · Mineralization · *Stenotrophomonas* C–S targeted biodegradation

Introduction

Basically, high sulfur content of fossil fuels causes catalyst poisoning in exhaust systems of refineries during the hydrothermal cracking process. Large-scale emissions of sulfur oxides due to the combustion of fossil fuels put environmental and biosphere safety/health at risk [1]. Thanks to the revolutionary coming generation of refining technologies, 0 ppm gasoline will be released and developing countries' sulfur-rich oil and gas oil fractions will be cheaply priced in the oil

M. Papizadeh (✉) · M. R. Ardakani
Biotechnology & Biology Research Center, Shahid Chamran University, Ahvaz, Iran
e-mail: moslempapizadeh@yahoo.com

M. R. Ardakani · H. Motamedi
Department of Biology, Faculty of Sciences, Shahid Chamran University, Ahvaz, Iran

I. Rasouli · M. Zarei
Department of Biology, Faculty of Sciences, Shahed University, Tehran, Iran

market in the next decade [2]. Hydrodesulfurization (HDS) is a common technology, a chemical procedure, requiring high pressure (1–20 MPa) and temperature (290–450 °C) to reduce sulfur moiety into hydrogen sulfide [3]. At present, occasional supplementation of low sulfur-contained light crude oil and increasing demands for low sulfur-contained middle-distillated fractions, as well as considerable funds and operational expenses required to lower sulfur condensations through HDS processing, have made biodesulfurization a HDS complementary attractive technology and a cost-effective procedure for lowering the sulfur contents of petrochemicals [4, 5].

Dibenzothiophene (DBT) family is recalcitrant to HDS processing because of the large amount of energy required to break the C–S bond of thiophenes, hence remaining the main undesulphurized fraction of a sulfur-contained fuel product. Thus, DBT is generally considered as a model compound for biodesulfurization [6]. Various microorganisms have been isolated and screened as capable of cleaving C–C or C–S bonds in benzothiophene and dibenzothiophene and their alkylated derivatives [7, 8]. Various strains of *Rhodococcus* [9–11], *Pseudomonas* [12–14], *Bacillus* [15, 16], *Mycobacterium* [17–20], *Microbacterium* [21, 22], *Gordonia* [2, 9, 23, 24], and other genera [25, 26] have been identified to have benzothiophene and/or dibenzothiophene-desulfurizing capability, most of which have been grown in enriched suspensions to characterize prosperous hydrocarbon-degrading microorganisms [7, 8].

We hereby used modified techniques of isolation and screening to introduce *Stenotrophomonas* sp. NISOC-04, a novel dibenzothiophene-desulfurizing bacterium, and represent its delightful sulfur utilization capabilities.

Material and Methods

Material

Gibbs' reagent (2,6-dichloroquinone 4-chloroimide), DBT (99%), biphenyl, 2-hydroxy biphenyl (2-HBP; sold as 2-phenyl phenol, 99%), ethyl acetate, and ingredients of mineral salt medium were obtained from Merck. Liquid paraffin was purchased from Acros organics Co. Gasoline and crude oil samples were courtesy of the National Iranian South Oil Company (NISOC).

Culture Medium

Mineral salt medium (MSM) was prepared as: KH_2PO_4, 2 g; Na_2HPO_4, 0.25 g; NH_4Cl, 2.3 g; $MgCl_2$, 0.6 g; $FeCl_3$, 0.001 g; and $CaCl_2$, 0.001 g and dissolved in double-distilled deionized water (DDW) set to 1 L. Desirable pH 6.8 was set by 50% NaOH titration. After autoclaving (121 °C, 20 min), filter-sterilized aqueous ethanol was added to a final concentration of 4% as the exclusive carbon source. Sulfur sources were added to a final concentration of 0.8 mM. DBT was added from a stock solution of ethanol or acetone (0.05 M) as the source of sulfur to prepare the MSM+DBT medium. $MgSO_4$ was added as the sole source of sulfur in the MSM+sulfate medium.

Enrichment and Isolation of DBT-Desulfurizing Microorganism

Glass slides were immersed in crude oil, gasoline, and liquid paraffin containing 20 mM DBT, buried in soil samples, and incubated at 30 °C. After 5 days, each slide was inoculated into a 250-ml Erlenmeyer flask containing 100 ml of MSM+DBT and incubated in an orbital shaker for 10 days (150 rpm, 30 °C). Of the second transfer, 80 μl was serially streaked on 1.5% agar–agar solidified sulfur-free MSM+Glucose and MSM+Glucose+$MgSO_4$. After 10 days of

incubation at 30 °C, representative single colonies were obtained. Colonies were purified and single colonies were inoculated into three separate flasks containing 100 ml of MSM+Glucose+$MgSO_4$ (A), MSM+Glucose+DBT (B), and sulfur-free MSM+Glucose (C) to detect DBT metabolization (starting cell concentration of 12×10^4 cells/ml).

Analytical Procedures

Growth

Three milliliters of each flask was withdrawn, centrifuged (4,000 rpm, 12 min), and incubated at 4 °C every 24 h. The t_0 sample of each medium was used as the blank for measuring biomass (OD_{600}). Then, the samples were used for metabolite extraction.

Gibbs' Assay

A: Of the culture, 3 ml was withdrawn, pH was calibrated at nearly 8, and 20 μl of Gibbs' reagent (5 mM in ethanol) was added to 1 ml of the sample. After single hour incubation at 30 °C, the reaction tubes were centrifuged (5,000 rpm, 10 min) and A_{610} was measured.

B: Biomass of 3 ml culture samples was precipitated by centrifugation at 5,000 rpm for 10 min, pH was adjusted to about 8, and 10 μl of Gibbs' reagent (10 mM in ethanol) was added to 1 ml of the supernatant. Cell-free MSM+DBT+Gibbs' reagent was the blank solution. After single hour incubation at 30 °C, A_{610} was measured.

C: Ethyl acetate extraction was performed for 3-ml culture samples three times by 3-min vortex-shaking, 5-min phase separation, and ethyl acetate removal. Twenty microliters of Gibbs' reagent (5 mM ethanol solution) was added to 1 ml of the sample. After single hour incubation at 30 °C, A_{610} was measured.

Metabolite Extraction

Medium aliquots (0.5 ml) were acidified at pH≤2.0 by titration with 50% HCl and extracted by ethyl acetate, a universal solvent, as previously described [22].

Gas Chromatography

All ethyl acetate-extracted samples were analyzed to determine DBT concentration and its sulfurous metabolites with a GC equipped with a sulfur chemiluminescence detector (SCD). One microliter of the sample was injected into a 0.32-mm interior diameter and 30-m column with a 0.015-mm film CP-SIL 5CB for the sulfur column operated at a 1.7-ml/min volume velocity of carrier helium. The injector and detector temperatures were maintained at 275 °C and 800 °C, respectively. The column temperature was retained at 200 °C according to the different organic sulfur compounds until the character peak appeared.

Also, samples of MSM+sulfate, cell-free blanks and sulfur-free MSM were analyzed using GC to relate the organic sulfur compounds to the DBT metabolism as previously described [22].

High-performance Liquid Chromatography

Detection and quantification of DBT, phenol, 2,2′-dihydroxy biphenyl, and 2-HBP were carried out using a HPLC on a Water instrument (model pomp 600E), with a Novapack C18 column (300×3.9 mm, 4 μm) and Shimadzu UV model 490 Waters detector ($\ddot{e}$=240 nm, AUFS=0.1)

and Rheodyne injector (fitted with a 20-μl loop). The mobile phase was acetonitrile/water (70:30, *v/v*) with a flow rate of 1 ml/min. The column was maintained at 40 °C. Standard concentrations of DBT, phenol, 2,2′-dihydroxy biphenyl, and 2-HBP were analyzed by HPLC, and the HPLC chromatograms of culture samples were analyzed according to standards.

UV Spectrophotometry

Metabolite extraction samples were also used for UV spectrophotometry. λ_{285} and λ_{323} were used to detect a decrease in the value of DBT concentration, and λ_{392} (3-hydroxy-2-formyl-benzothiophene), λ_{248} (biphenyl), and λ_{266} (2-hydroxy biphenyl) were used to detect any increase in the concentration of the DBT desulfurization metabolites [22, 27].

Energy Dispersive Spectroscopy

One milliliter of the sulfur-free MSM medium was concentrated by drying at 50 °C and room pressure on an aluminum foil. The dried salt fraction was analyzed using an electronic scanning microscope equipped with an energy-dispersive spectroscope.

16S rDNA Sequence-Based Identification

Primers

The 16S rDNA was amplified using eubacterial-specific primers FD1 (5′-CCGAATTCGTC GACAACAGAGTTTGATCCTGGCTCAG) and RP1 (5′-CCCGGGA TCCAAGCT TACGGTTACCTTGTTACGACTT) to obtain 1,500-bp product, approximately a full length of the 16S rDNA of the isolate. Proficiency of primers FD1 and RP1 was analyzed by CLC main workbench and Genious software and BLASTN 2.2.18+ program of the National Center of Biotechnology Information (NCBI) [22].

DNA Extraction

A 3-ml aliquot of an overnight culture was centrifuged at 5,000 rpm for 15 min, washed with Tris-EDTA (TE) buffer, and centrifuged again; the pellet was resuspended in 600 μl of RSB buffer and 65 μl of 10% sodium dodecyl sulfate. After gentle shaking, 500 μl of the Tris-buffered phenol (pH 8) was added. After gentle shaking, the mixture was centrifuged for 10 min in a centrifuge tube (Eppendorf, Germany) at 10,000 rpm, and the aqueous phase was extracted with phenol/chloroform/isoamyl alcohol (25:24:1). Finally, DNA was precipitated with 24 μl of 2.5 M NaCl and 600 μl of −20 °C absolute ethanol, centrifuged at 13,000 rpm for 30 min, and washed with 70% ethanol. The pellet was air-dried and resuspended in 50 ml of the TE buffer [22].

Polymerase Chain Reaction and Sequence Analysis

All PCR amplifications were carried out using a Bio-Rad thermal cycler (USA). The 50 μl PCR reactions were prepared with 1 μl of template DNA, 5 μl of PCR buffer (Fermentas, France), 10 mmol of each deoxyribonucleoside triphosphate, 2.5 mM $MgSO_4$, and 10 pmol of each of the primers, 5 U of *PFU* DNA polymerase, and an appropriate volume of DDW. A hot-start procedure (94 °C for 4 min) was applied before the enzyme was added to prevent nonspecific annealing of the primers. Negative controls (PCR mixture without

added target DNA) were included. PCR entailed 30 cycles (94 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min, plus one additional cycle with a final 20-min chain elongation). Agarose gel electrophoresis (1%) of the PCR products indicated a sharp band in 1,500 bp subsequently purified and sequenced by Genvanavaran Biotech Corp. [22].

Primers FD1 and RP1 were used to sequence nearly 1,500-bp PCR products from beginning to end; the 1,500-bp sequence was prepared by Bioedit and CLC combined workbench softwares, and the sequence analysis for 16S rRNA phylogenetic identification was performed by BLASTn 2.2.18+ utilities.

Results

Isolation and Screening

There were four bacterial isolates capable of utilizing DBT as the sulfur source. The growth curve of MSM+Glucose+DBT shown in Fig. 1 indicates that DBT utilization increased biomass in comparison to the sulfur-free flask.

Biomass Production in Sulfur-Free MSM

GC-SCD analysis of the sulfur-free MSM and DDW ethyl acetate-extracted samples and energy-dispersive spectroscopy (EDS) analysis results revealed that trace amounts of organosulfurs are possibly introduced into the culture medium, hence enhancing biomass accumulation. Given that 1- to 2-min retention times of these organosulfurs and their variable amount were found, it was assumed that they were volatile small organosulfurs originated from laboratorial environment introduced into the medium. UV spectrophotometric studies indicated 57.8% and 91.6% decreases in absorptions at 285 and 323 nm, respectively (Fig. 2). Gibbs' assay showed no 2-HBP or another phenolic compound(s) accumulation, although we have used three different techniques to perform Gibbs' assay; however, HPLC studies revealed that approximately 0.35 mM of 2-HBP was produced. Standard concentrations of the purchased 2-HBP were studied to measure the de novo 2-HBP. De novo 2-HBP was diluted into the medium during the stationary phase of growth, as shown by HPLC.

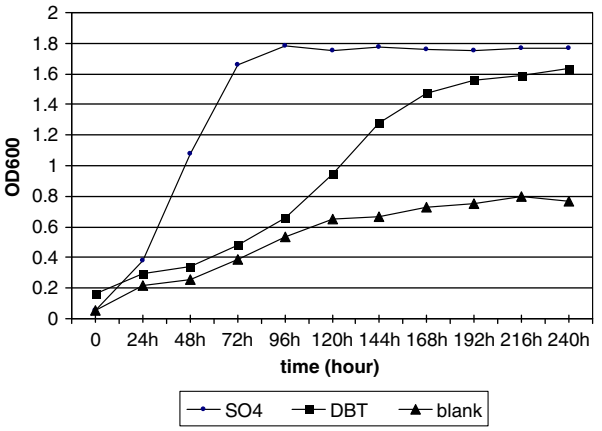

Fig. 1 Growth curve of *Stenotrophomonas* sp. NISOC-04 in media: MSM+$MgSO_4$ (*small square*), MSM+DBT (*black square*) and sulfur-free MSM (blank, *triangle*)

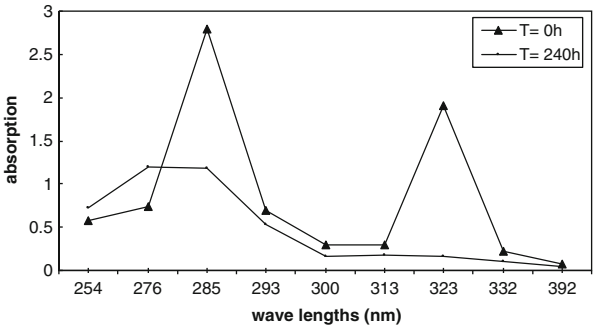

Fig. 2 UV absorption pattern of extraction samples of t_0 (*trianlge*) and t_{240} (*square*)

GC-SCD studies indicated that there was no accumulated sulfurous by-product(s) (the end product(s) were desulfurized). Regarding DBT concentration and the area of DBT peak in the chromatogram, there was a linear correlation between 0.04 and 0.8 mM DBT, and quantitative analyses of peaks showed a 97.36% decrease in DBT concentration during the incubation (Fig. 3).

DBT metabolism was restricted to the log phase and its trigger was in the middle of diauxic growth (Fig. 4). Regarding the growth curve in MSM+Glucose+DBT, after a lag phase, a 48-h-long exponential growth begins, and during this phase, DBT concentration reduced by 82%. After this accelerated DBT utilization (t_{96}–t_{144}), a decreasing growth rate and DBT utilization occurred (t_{144}–t_{240}) to reach a stationary phase of growth. A 13.5% DBT concentration occurred within this period (t_{144}–t_{240}). Regarding the 1.36% decrease in DBT concentration in the cell-free MSM+DBT flask, 96% of DBT reduction was associated with bacterial DBT (sulfur source) assimilation.

Neither growth trend was found with DBT treatments as the exclusive carbon or carbon/sulfur source, nor was a significant change detected at 285 and 323 nm. The data were

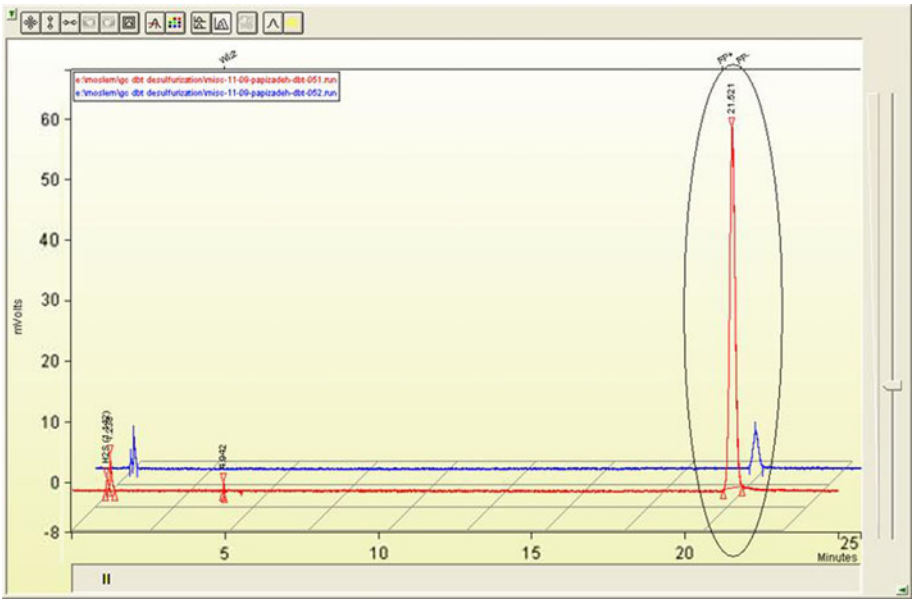

Fig. 3 GC-SCD chromatograms of extraction samples of t_0 and t_{240}

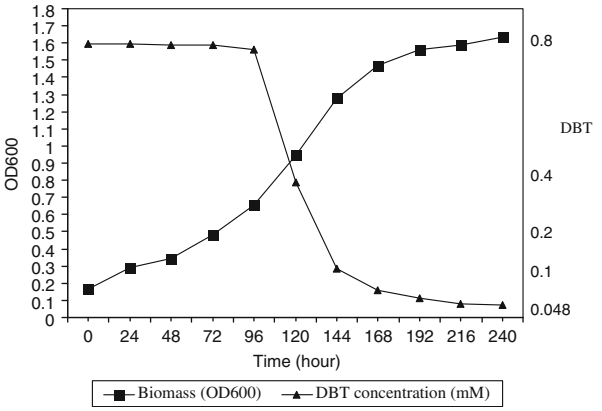

Fig. 4 Biomass production (*square*) and reduction of DBT concentration (*triangle*) in a 240-h period

analyzed to conclude that *Stenotrophomonas* sp. NISOC-04 is a new sulfur-selective DBT-desulfurizing bacterium. *Stenotrophomonas* sp. NISOC_04 was unable to utilize 2-HBP as a carbon source, leading to the conclusion that detoxification reactions modified the effects of elevating 2-HBP condensation.

Identification

16S rDNA (1,500 bp) was sequenced. Taxonomic report and neighbor joining distance tree of the results of the BLASTN 2.2.18+ program in NCBI were analyzed using Genious software alignment tools. Most hits of the BLASTN program belonged to the Genus *Stenotrophomonas*, showing 98% similarity to *Stenotrophomonas maltophilia*. Comparing the 16S rRNA gene sequences of 64 species of Xanthomonadaceae which are available at Ribosomal Database Project (RDP) and tree designation revealed a close relationship between *Stenotrophomonas* sp. NISOC-04 and *Stenotrophomonas acidaminiphila* (accession no. AF273080). The biochemical and morphological characteristics of *Stenotrophomonas* sp. NISOC-04 are shown in Table 1.

Discussion

A highly narrow sense enrichment procedure was implemented for the selection of microorganisms assimilating gasoline/paraffin-resolved DBT. The colonies that appeared on solidified MSM+Glucose were hydrocarbon-tolerant DBT-utilizing bacterial/fungal species, most of which totally biodegraded DBT or cleaved C–C bonds. The microorganisms utilized gasoline and crude oil as carbon or carbon/sulfur sources. Indeed, some DBT C–S-degrading bacteria were detected, among which *Stenotrophomonas* NISOC-04 and *Microbacterium* NISOC-06 [22] were the most important considering the rate at which they utilized DBT. High extent of gasoline tolerance and desulfurization supported the strains as catalyst candidates for biphasic systems [28].

Stenotrophomonas NISOC-04 produced a significant biomass in the absence of chemically detectable sulfur source as determined by biomass measurement, EDS analysis, and GC-SCD (Fig. 4). The capability was bounded by the strain's ability to utilize trace atmospheric organosulfurs which triggers an approximately 24-h growth time course followed by a lag phase and enzymatic system reorganization for replacing trace

Table 1 Biochemical and morphological characteristics of *Stenotrophomonas* sp. NISOC-04

Phenotypic tests	*Stenotrophomonas* sp. NISOC-04	*S. acidaminiphila*
Gram stain	Negative	Negative
Shape of cell	Rod	Rod
Colony morphology	Round, flat	Round, flat
O/F metabolism	Highly oxidative	Highly oxidative
Oxidase	Negative	Negative
Catalase	Positive	Positive
Gelatinase	Negative	Positive
Hydrocarbon utilization	Positive	Positive
Motility	Negative	Two or more flagella
Pigmentation	Yellow	Yellow or green
Amino acid requirement	Negative	Positive
Growth on:		
Ethanol	Positive	Positive
Glycerol	Positive	Negative
Sucrose	Negative	Positive
Glucose	Positive	Positive
Paraffin	Positive	Not reported
Phenol	Positive	Not reported
Toluene	Positive	Not reported
Naphthalene	Negative	Not reported
Anthracene	Negative	Not reported

organosulfurs by DBT; such growth pattern is known as a diauxic trend (Fig. 4). Additionally, imposed sulfur starvation triggers the synthesis of scavenging proteins for the uptake of trace sulfur moiety which could be assimilated [29].

Previous studies showed that Gibbs' assay itself cannot be used as a trustworthy test to make a decision on confirming an isolate's ability to produce 2-HBP from DBT. Because of the toxic nature of 2-HBP and the high probability of false negative results of Gibbs' assay, UV spectrophotometry was performed as a preliminary screening approach. False negative results of Gibbs' assay occurred probably due to the presence of phenolic compounds produced by microorganisms or microbial 2-HBP detoxification and transformation. Gibbs' assay and UV spectrophotometry were used for the primary screening of DBT-desulfurizing and DBT-degrading microorganisms. GC-SCD and HPLC were secondary screening tools to investigate DBT metabolism carried out by the primarily screened microorganisms. UV spectrophotometry analysis of t_0 and t_{240} samples displays any sign of accumulation of phenolic and/or aromatic biphenyl compounds and its differentiation from DBT mineralization. It was applied to relate the decreased DBT concentration (decrease of absorption at 285 and 323 nm) and increased specific phenyl-based compound(s) concentration. Considering Fig. 2, there is a different absorption reduction at various wavelengths, particularly at 276–285 and 323 nm, and the accumulation of aromatic compounds whose λ_{max} is restricted to 240–285 was the most possible reason that was not explained by Etemadifar et al. [27]. λ_{246} is the λ_{max} of biphenyl. Other chemically similar metabolites probably have their λ_{max} at 240–285 nm, and their concentrations increase the absorption at 250–285 nm. In fact, C–C bond degradation and DBT mineralization notably

reduce total UV absorption, although in the case of C–S targeted desulfurization, the end product(s) showed higher UV absorption values at particular wavelengths.

In the presence of sulfate, thiosulfate, and sulfite in separate flasks containing MSM+DBT+ethanol, DBT concentration was not changed as indicated by GC-SCD and UV spectrophotometry studies, suggesting the relationship between DBT utilization and UV absorption differences at definite wavelengths. Also, *Stenotrophomonas* sp. NISOC-04 could not utilize DBT as the carbon or carbon/sulfur sources, and no significant change were found in UV absorption. Consequently, the changes in UV absorption were exclusive to DBT metabolization as a sulfur source, but not a carbon one. In accordance with GC-SCD studies during the incubation, no sulfurous metabolite concentration was observed. Thus, metabolites of DBT desulfurization might be metabolized too rapidly to be detected in culture samples. Li et al. suggested that negative Gibbs' assay is a result of 2-HBP methylation and detoxification; however, other metabolic pathways can be used to decrease the concentration and toxic effects of 2-HBP as well [21, 22]. Detection of 2-HBP traces with HPLC, the results of UV spectrophotometry, and the strain's incapability of growing on DBT and 2-HBP as carbon sources proposed a novel or modified biocatalytic desulfurization pathways as indicated in *Microbacterium* sp. NISOC-06 and *Microbacterium* ZD-M2. Gibbs' assay results were different from HPLC, probably due to the presence of interfering metabolites inhibiting Gibbs' assay or 2-HBP transformation and detoxification. There were some controversies over using biochemical and morphological characterization tests; thus, sequencing and investigating the full-length 16S rRNA gene was introduced as a method for verifying the isolate as the member of the genus *Stenotrophomonas* [24]. In comparison to *Microbacterium* ZD-M2 which utilized 100% of 0.2 mM DBT within 58 h, NISOC_04 desulfurized 0.656 mM of 0.8 mM DBT within 48 h of exponential growth (under the same incubation conditions). DBT-desulfurizing Gram-negative bacteria have been rarely reported; the species' unique DBT metabolism encouraged the authors to describe its desulfurization capability and study the DBT desulfurization of other isolated Gram-negative strains. Dibenzothiophene biodesulfurization efficiency of NISOC_04 is compared with recently reported strains in Table 2. Due to its desulfurization rate, it can be useful in the removal of sulfur from gasoline and other petroleum products.

The authors named the isolate *Stenotrophomonas* sp. NISOC-04 regarding its locality (NISOC oilfields). Comparing *Stenotrophomonas* sp. NISOC-04 with *S. acidaminiphila* revealed significant morphological and biochemical differences, proposing the isolate a novel bacterium. Further studies on dsz operon analysis and optimization of desulfurization process are going on.

Table 2 Efficiency of dibenzothiophene utilization by recently reported strains

Strain	Efficiency of DBT utilization	Time of incubation (h)	Reference	Type of utilization
Gordonia alkanivorans Strain 1B	64.8% of 0.5 mM	120	[29]	S source
Arthrobacter sp. P1-1	82% of 0.22 mM	336	[30]	C source
Microbacterium ZD-M2	100% of 0.2 mM	58	[21]	S source
Microbacterium sp. NISOC_06	90.6% of 1 mM	120	[22]	S source
Rhodococcus erythropolis R1	100% of 0.3 mM	72	[31]	S source
Stenotrophomonas sp. NISOC_04	82% of 0.8 mM	48	Current report	S source

S source sulfur source, *C source* carbon source

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