Desulfurization of Dibenzothiophene by a Novel Strain
Brevibacillus invocatus C19 Isolated from Egyptian Coke

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In this study, an effective enrichment technique was applied to isolate different bacterial strains with capabilities to utilize dibenzothiophene (DBT) as a model compound of polyaromatic sulfur heterocyclic compounds (PASHs). Twenty-eight different desulfurizing bacterial strains were isolated from a mineral coke sample with sulfur content of 3.8%. Only nine of them showed the ability to utilize DBT as a sole-sulfur source through the 4S-pathway without altering its hydrocarbon skeleton. From all; a Gram +ve bacterial isolate designated C19 showed a higher biodesulfurization (BDS) efficiency relevant to the well-known biodesulfurizing bacterium strain R. erythropolis IGTS8, recording 66.85% and 50% removal of 1000 ppm DBT with the production of 31.98 and 31.34 ppm 2-hydroxybiphenyl (2-HBP), as a dead end product, respectively. C19 was identified by 16S rDNA gene sequence analysis to be Brevibacillus invocatus (NCBI Gene Bank Accession no. KC999852) with similarity of 99.05%. BDS of different PASHs was also studied and Brevibacillus invocatus C19 showed good BDS capabilities compared to those of IGTS8.

Key words: Biodesulfurization, Dibenzothiophene, 4S-pathway, Thiophenic compounds, 16S rDNA.

Combustion of petroleum-derived fuels leads to the release of vast amount of sulfur dioxide (SO₂) into the atmosphere, which is a principle source of acid rain and air pollution. Thus, most countries have imposed strict regulations to control these releases mainly by enforcing stringent restrictions on the levels of sulfur in transportation fuels. However, a common problem, petroleum refineries are facing around the world is that crude oil reserves being used as feedstock for refining process are becoming heavier day after day with elevated sulfur contents (Bhatia and Sharma, 2012 and Li and Jiang, 2013).

Hydrodesulfurization (HDS) process has been routinely applied in refineries worldwide. HDS involves the use of chemical catalysts containing metals at high pressures and temperatures to remove sulfur compounds. However, with the different classes of sulfur compounds found in the middle-distillate fraction, Cx-BTHs and Cx-DBTs with alkyl substitutions in positions four and six on the DBT ring are more resistant to HDS treatment than mercaptans and sulfides (Kabe et al., 1992). Compared with HDS process, the biodesulfurization (BDS) process using microorganisms and/or enzymes could be carried out more safely, under mild conditions (Chen et al., 2009).

BDS of petroleum and its fractions offers an attractive alternative to HDS process. For this process to be commercial, microorganisms with high activity and selectivity are required. DBT is widely used as a model sulfur compound for isolation and enrichment of suitable strains (Konishi et al., 1997 and Maghsoudi et al., 2000).
Research on BDS using DBT has resulted in the elucidation of two different biochemical pathways, named Kodama (Kodama et al., 1973) and 4S (Kilbane and Bielaga, 1989). Kodama pathway is considered unsuitable because in this pathway water soluble sulfur compounds are produced which are then unavailable for burning and are therefore forfeited. Through 4S pathway, DBT is first oxidized to DBT sulfoxide (DBTO), then transformed to DBT sulfone (DBTO₂) which in turn to 2-hydroxyphenyl benzene sulfinate (HBPSi) by monooxygenase leading to the cleavage of thiophene ring. Finally HBPSi is reduced to 2-hydroxybiphenyl (2-HBP) by hydrolase enzyme leading to the subsequent release of sulfite or sulfate. In this pathway, sulfur of DBT is selectively removed without destroying the hydrocarbon skeleton so that thermal value of fuels is not decreased.

Various types of bacteria have been recognized to desulfurize DBT via 4S pathway; Rhodococcus erythropolis IGTS8 (Kilbane, 1990). Other DBT desulfurizing microorganisms, mostly mesophilic and a few thermophilic, have been isolated; Rhodococcus erythropolis H-2 (Ohshiro et al., 1996); Mycobacterium sp. G3 (Nekodzuka et al., 1997); Gordonia sp. CYKS1 (Rhee et al., 1998); Pseudomonas delafielddi R-8 (Luo et al., 2003); Microbacterium sp. ZD-M2 (Zhang et al., 2005); Bacillus subtilis WU-S28 (Kirimura et al., 2001) and Mycobacterium pheli WU-F1 (Furuya et al., 2001); Gordonia alkanivorans RIPI90A (Mohebali et al., 2007); Pantoea agglomerans D23W3 (Bhatia and Sharma, 2010); Sphingomonas subarctica T7b (Gunam et al., 2013).

This study aimed to isolate and characterize biodesulfurizing microorganism(s) (BDSM) capable of utilizing DBT in a sulfur specific manner (4S-pathway) relevant to Rhodococcus erythropolis IGTS8.

**MATERIALS AND METHODS**

**Bacterial Strain**

Rhodococcus erythropolis IGTS8 (ATCC 53968) was used as a standard strain for BDS representing positive control.

**Chemicals**

Dibenzothiophene (DBT) (99%), Benzo thiophene (BTH) (97%) and Thiophene (TH) (99%) are products of Merck, Germany. 2-hydroxybipheyl (2-HBP) (98%) and 2,2'-Dihydroxybiphenyl (2,2'-DHBP) (99%) were purchased from Aldrich, United Kingdom. Gibb’s reagent (2,6-dichloroquinone-4-chlorimide) is product of Fluka, Switzerland. Potassium phosphate monobasic (99%), Glycerol (99%), Sodium chloride (99%), Ferric chloride (99%), Ethanol (99%). All other chemicals were of analytical grade, commercially available and used without further purification.

**Media**

Basal Salts Medium (BSM) was prepared according to Piddington et al. (1995) with some modification, as follows; In order to avoid precipitation and turbidity of the medium, the preparation of BSM was performed in 2 parts separately and then these two parts were mixed together to get one liter of BSM (Ansari, 2008).

Part (a) composed of the following: KH$_2$PO$_4$, 2.44 g; Na$_2$HPO$_4$, 5.57 g; NH$_4$Cl, 0.5 g; glycerol, 6.4 mL and 850 mL distilled water. While Part (b) composed of; MgCl$_2$.6H$_2$O, 2.44 g; CaCl$_2$.2H$_2$O, 5.57 g; 2 g FeCl$_3$.6H$_2$O, Yeast extract, 0.1 g and 150 mL distilled water.

After mixing these two parts, the pH was adjusted to 7.0. Then sterilized by autoclaving at 121°C and 1.2 bar for 20 min.

Tryptone Glucose Yeast Extract Medium (TGY) is a complex medium used for maintenance and culturing of bacterial strains and was prepared according to Benson (1994).

**Enrichment and Isolation of Dibenzothiophene Biodesulfurizing Microorganisms (DBT-BDSM)**

Coke (C1) with sulfur content of 3.8% was used in this study to isolate BDSM and was collected from El-Nasr Company for Coke and Chemicals, Tebeen, Helwan, Egypt.
100 g of C1 was contaminated artificially by 0.1 g DBT. The mixture was carefully homogenized using a sterile spatula. Then placed in sterile plastic pot, and incubated at 30°C for one month. Sterile distilled water (10 mL) was added regularly (every 7 d) and then mixed well with sterile spatula to keep the humidity constant and to guarantee good aeration, respectively. All the above steps were done under aseptic conditions.

Enrichment (En) was done for detecting and assessing the size of indigenous DBT/BDM in the samples used for isolation. This was done according to Duarte et al. (2001) and Bhatia and Sharma, (2010), but with some modification; 10 g of sample C1 before contamination and after contamination (BC and AC, respectively) were mixed with 100 mL BSM. Then, the flasks were incubated at 30°C for 7 d on a rotary shaker (150 rpm). One mL from each flask was transferred separately to fresh flasks containing 100 mL of En media and the procedure was repeated in a total of three transfers. Serial dilutions (10−1) of each transfer were inoculated on TGY agar plates to enumerate TCFU and onto BSM-DBT agar plates to count BDSM. The Plates were incubated at 30°C, and colonies were enumerated after 48 h on TGY plates and after 7 d on BSM-DBT plates. Separate colonies from BSM-DBT plates were picked and purified on BSM-DBT plates.

The bacteria were maintained by sub-culturing into a liquid medium or plating on a solid medium (BSM-DBT plates) weekly. For long-term storage, 7 mL of culture was transferred to 3 mL of sterile glycerol in a screw cap tube according to Ishii et al. (2005). All tubes were mixed by vortex to ensure that the glycerol was evenly dispersed and kept at -20°C.

Testing the Ability of Isolates for BDS of DBT

DBT-Spray plate Assay

This test was performed as described by Denome et al. (1994) with minor modifications as follows; the selected isolates were transferred separately to TGY agar plates and were incubated at 30°C for 24 h. 500 µL of 1000 ppm DBT solution in ethylether were sprayed individually on BSM/agar plates. Large amounts of cells from these batches were transferred onto BSM-DBT plates. The BSM-DBT plates were incubated at 30°C for 7 d and monitored periodically every day to detect the initial growth.

Clear zones or colored products around the colonies were detected under day light (Krawiec, 1990 and Denome et al., 1994). Fluorescent products around the batches were detected under short-wave (254 nm) UV illumination (Denome et al., 1993). R. erythropolis IGTS8 was used as a positive control for 4S-pathway.

DBT-Bioavailability/Biodegradability Assay

Desulfurization assays were also performed in liquid culture to examine the ability of isolated microorganisms to utilize DBT as a sole-sulfur source. According to Denome et al. (1994), this assay was done as follows; Cells were incubated at 30°C in TGY broth for 24 h in a shaking incubator (150 rpm). Cells were pelleted by centrifugation at 5000 rpm for 15 min and then washed three times with BSM. Washed cells were inoculated into BSM that contained DBT as a sole-sulfur source (dissolved in ethylether and added to BSM in a final concentration of 1000 ppm DBT before sterilization). The inocula were adjusted so that the initial absorbance was (λ600 0.3). The cultures were incubated at 30°C for 7 d, in a shaking incubator (150 rpm). The growth was monitored by measuring optical density at λ600 (JASCO, V570, USA) and non-inoculated BSM was used as a blank. The pH of the cultures was determined using pH-meter (DIGMED DM-22, Brazil). DBT removal was estimated by HPLC and the production of hydroxybiphenyl (a dead end product of 4S-pathway) was evaluated by Gibb’s assay and HPLC analysis.

DBT-Biodegradability assay was done according to Kayser et al. (1993) and Frassinetti et al. (1998), applying the same procedures as mentioned above, but with using BSM free of any C-source other than DBT.

Identification of the Selected DBT-BDSM

Colony morphology was visualized by growth on TGY agar plates. The purity of the most promising isolated bacterium was checked microscopically (Axiolab Carl Zeiss Microscope, USA). The morphology of selected isolate was determined by transmission electron microscope (TEM) (Jeol Jem 2100F, 80 to 200 kV, Japan). Genomic DNA of the selected isolate was extracted using Gene JET Genomic DNA Purification Kit K0729 (Fermentas, USA), according to the manufacturer’s instructions at National Research Center, Cairo, Egypt. A region of
approximately 375 bp from the gene was amplified using the forward primer 5'-AACGTGGAGGAAGTGGGAT-3' and the reverse primer 5'-TACGTTACCTTGGTACGAC-3'.

**PCR condition**

The reaction was prepared with 0.5 µL of Dream-Taq 5U/µL (Fermentas, USA), 5 µL of Dream-Taq Buffer 10x, 5 µL of target DNA, 1 µL of dNTP each 20 mM, 1 µL of each appropriate primer 10 pmol/µL and 36.5 µL dH2O were added. The final reaction volume was 50 µL.

After PCR program was completed, for visualizing PCR products, 5 µL of the suspension was electrophoresed on 1% agarose gels in 1X Tris-Acetate EDTA (TAE) buffer, which were then stained with ethidium bromide and examined under UV light. Bands were excised, and DNA was purified from gel slices using QIAquick Gel Extraction Kit, Cat. No. 28704 (Qiagen, USA) at National Research Center, Cairo, Egypt.

The purified PCR products were sequenced with the same primer that has been used in amplification of the target sequence. Sequencing was done by an ABI 3730 XL automatic DNA sequencer (Macrogen Inc., South Korea).

The 16S rDNA sequences (Query sequence) were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.org) using BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and corresponding sequences from database were downloaded. Evolutionary history was inferred using the Neighbor-joining method (Patil et al., 2008). The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree (Dhanve et al., 2009).

**Studying of BDS Pathway of the Most Potent BDSM**

To identify the pathway of DBT utilization by the most efficient DBT-BDSM, the following steps were done; Cells were incubated at 30°C in TGY medium for 24 h in a shaking incubator (150 rpm). Cells were pelleted by centrifugation at 5000 rpm for 15 min and then washed three times with BSM, re-suspended in fresh BSM. Washed cells were inoculated into BSM that contained 1000 ppm DBT as a sole sulfur source. The cultures were incubated at 30°C for 7 d, in a shaking incubator (150 rpm). Culture broth was acidified to pH 2 using 1 M HCl and then extracted with equal volume of ethylacetate, dehydrated over anhydrous Na2SO4, concentrated by evaporation at 60°C and then subjected to GC-MS analysis. *R. erythropolis* IGTS8 was used as a positive control for 4S-pathway.

**Biodesulfurization of Different Thiophenic Compounds by the Most Potent BDSM**

Two groups of eight 250 mL capacity Erlenmeyer flasks each with 100 mL of BSM containing TH, BTH, DBT, DBTO, DBTO2, DMSO, 4-MDBT and 4,6-DMDBT as sole source of sulfur in a final concentration of 1000 ppm were inoculated by the most potent BDSM and incubated at 30°C for 7 d. Preparation of fresh inoculum was done as mentioned before.

Non-inoculated flasks of each sulfur compound were used as negative control and run in parallel with the inoculated flasks through all the experimental procedure. *Rodococcus erythropolis* IGTS8 was used as a positive control for 4S-pathway.

**Analytical Procedures**

The optical densities (O.D.) of cell suspensions were measured using a spectrophotometer (JASCO, V570, USA).

Gibb’s assay was used to screen the conversion of DBT to phenolic compounds by the isolates (Oldfield et al., 1997). For this culture supernatant (5 mL) was taken and incubated with 50 µL of Gibb’s reagent (10 mM ethanol) at 30°C. Positive reactions developed blue to purple color after 30 min of incubation at room temperature and was also monitored at λ610 against a blank containing no DBT.

Quantitative and qualitative estimation of DBT removal was performed using ethyl acetate as the extractant. After extraction, ethyl acetate layer was analyzed using high performance liquid chromatography (HPLC) model waters 600E equipped with a UV detector model waters 2487 (set at 254 nm) and C18 reversed column (4.6X250 mm, 300°A, 5µL). Calibration curves for DBT and 2-HB were done (5 – 1000 ppm).

To study the pathway of DBT utilization by the new isolated BDSM; GC-MS analysis, were performed using a Clarus 500 MS System (USA). Compounds were separated on DB-5 column (5%-phenyl methyl polysiloxane (30 m X 0.25 mm LD X 0.25 µm Film) using helium with flow rate; 2 mL/min. The oven temperature was held isothermally.
at 50°C for 3 min and then programmed to increase to 250°C at a rate of 7°C/min. Injector temperature was set at 200°C.

All the experiments and measurements were done in duplicates and arithmetic averages were taken throughout the data analysis and calculations.

**RESULTS AND DISCUSSION**

**Collection and Preparation of Sample Used for Isolating Biodesulfurizing Microorganisms (BDSM)**

All organisms require sulfur for growth. In bacteria, sulfur makes up to 0.5-1% of the cell dry weight, and is needed primarily as a component of the amino acids cystine and methionine. Sulfur also plays an essential role in a variety of enzyme cofactors including biotin, coenzyme A, coenzyme M, thiamine and lipoic acid, and is critical in many redox processes, both as a building block for iron-sulfur centers and a redox-active component of disulfide bonds. Sulfur for biosynthetic processes is derived from the assimilation of inorganic sulfate by bacteria, yeasts and filamentous fungi (Kertesz, 1999).

More than 60% of the sulfur in the higher boiling petroleum fractions is present as DBT and substituted DBTs (Kropp and Fedorak, 1998). DBT has accordingly been used as a model compound for most of the performed BDS studies.

Total sulfur content in coke varies from 0.5% to 11%, depending on the geographical location of the coke source (Gogoi and Bezbaruah, 2002). Organic sulfur in coke consist predominantly of dibenzothiophene (DBT) and benzo[b]thiophene (BT) with lower amounts of disulfide joining cyclic structures, sulfide linked to alkyl- and thio-groups attached either to an aromatic ring or to an alkyl chain (Calkins, 1994). That was why; a sample of coke with sulfur content of 3.8% was used in this study to isolate different BDSM.

In the present study; total colony forming unit (TCFU) as well as the culturable microorganisms able to grow on DBT as a sole source of sulfur (BDSM) were enumerated on TGY plates and BSM-DBT plates respectively, cultivation was carried out directly after collection and after three weeks of enrichment on En broth medium containing 1000 ppm DBT as a sole-sulfur source.

### Enumeration of Total Colony Forming Units and BDSM

The total viable count on TGY plates (CFU/mL) and the count of BDSM (CFU/mL) on BSM-DBT plates directly after collection showed good microbial population in the collected sample. According to Madigan et al. (1998); contaminants are often potential energy sources for microorganisms, and according to Ilyina et al. (2003); microorganisms could survive in contaminated habitat because they are metabolically capable of utilizing its resources and can occupy a suitable niche.

In general, artificial contamination with 0.1% (w/w) DBT recorded an increase in both BDSM (CFU/mL) and TCFU (CFU/mL) either before or after En. This might be due to the adaptation of the indigenous microbial populations in the

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBT</td>
<td>Golden yellow</td>
</tr>
<tr>
<td>DBTO</td>
<td>V.V. weak purple</td>
</tr>
<tr>
<td>DBTO2</td>
<td>Weak purple</td>
</tr>
<tr>
<td>2,2'-BHP</td>
<td>Strong Purple</td>
</tr>
<tr>
<td>2-HBP</td>
<td>Bright white blue</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>V.V. bright green-blue</td>
</tr>
</tbody>
</table>

Table 1. Fluorescence of the standard desulfurization products of DBT under UV-illumination
samples used for isolation to DBT as a model compound for organic sulfur compounds found in crude oil and its distillates. Similar observation was reported by Abed et al. (2002).

Carman et al. (1995); Abed et al. (2002) and Margesin et al. (2003) reported that, the chronic exposure to relatively high levels of DBT in contaminated environments, have resulted in the higher concentrations of DBT utilizing microorganisms that would be well adapted to DBT.

Generally, either before or after contamination (BC and AC, respectively); the

### Table 2. Results of DBT-Spray Plate Assay

<table>
<thead>
<tr>
<th>Isolate</th>
<th>I.G.</th>
<th>Fluorescence under 254nm UV-illumination</th>
<th>Clear Zone</th>
<th>Colored Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGTS8</td>
<td>2 days</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>1 day</td>
<td>Blue</td>
<td>+ve</td>
<td>Orange-red</td>
</tr>
<tr>
<td>C2</td>
<td>2 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>2 days</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>2 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>2 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C6</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C7</td>
<td>1 day</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>3 days</td>
<td>Blue</td>
<td>+ve</td>
<td>Orange-red</td>
</tr>
<tr>
<td>C9</td>
<td>2 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>1 day</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C11</td>
<td>3 days</td>
<td>Strong Purple</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C12</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C13</td>
<td>3 days</td>
<td>Bright Blue</td>
<td>+ve</td>
<td>Orange-red</td>
</tr>
<tr>
<td>C14</td>
<td>3 days</td>
<td>Blue</td>
<td>+ve</td>
<td>Orange-red</td>
</tr>
<tr>
<td>C15</td>
<td>1 day</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C16</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C17</td>
<td>3 days</td>
<td>Bright Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C19</td>
<td>1 day</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C20</td>
<td>1 day</td>
<td>Blue</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C21</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C22</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C23</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C24</td>
<td>3 days</td>
<td>Green-Purple</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C25</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C26</td>
<td>2 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C27</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>C28</td>
<td>3 days</td>
<td>-</td>
<td>+ve</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. GC-MS mass spectral data of DBT-BDS metabolites by *R. erythropolis* IGTS8 and *Brevibacillus invocatus* C19

<table>
<thead>
<tr>
<th>ID</th>
<th>Chemical name</th>
<th>RT(min)</th>
<th>MS Fragmentation pattern (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dibenzothiophene</td>
<td>32.71</td>
<td>184,152,139,113</td>
</tr>
<tr>
<td>2</td>
<td>Dibenzothiophene sulfoxide</td>
<td>38.73</td>
<td>200,184,171,152,139,118</td>
</tr>
<tr>
<td>4</td>
<td>2-HBP-2- sulfinic acid</td>
<td>30.96</td>
<td>234,216,126,111,97</td>
</tr>
<tr>
<td>5</td>
<td>Sultine</td>
<td>35.75</td>
<td>232,168,152,139,113</td>
</tr>
<tr>
<td>6</td>
<td>2-HBP</td>
<td>27.24</td>
<td>186,139,129,78</td>
</tr>
</tbody>
</table>
microbial count increase with 1st En cycle then decreased thereafter up to the 3rd En cycle as shown in Fig. (1).

Chen et al. (2008) reported that; repeated exposure to a compound usually increases the adaptive capabilities of microorganisms. In addition, the longer En period would enhance the proliferation of bacteria capable of utilization of a certain compound.

The obvious increase in the count of BDSM and TCFU after En for one week in En medium, as illustrated in Fig. (1), might be due to the adaptation of the indigenous microbial populations in the C1 sample used for isolation to organic sulfur compound (DBT) and/or due to the production of the sulfate starvation-induced proteins (SSI proteins), as according to the concept of sulfur starvation and the theory of enrichment culture; microorganisms capable of desulfurizing DBT (BDSM) can be selected by depriving the microbial populations of all sources of sulfur except DBT where only the organism(s) with the
necessary DBT utilizing abilities would grow significantly under these conditions and these organisms would outgrow the very large number of other organisms found at the start of the experiment (Krawiec, 1990; Soto et al., 1998 and Abbad-Andaloussi et al., 2003). Under sulfate-limited conditions a set of extra proteins are synthesized by several species of bacteria, yeasts and fungi. In this study, also the presence of yeast extract in the En medium could accelerate the adaptation of the bacterial strains with high rate and capacity of DBT utilization. Konishi et al. (1997); Kishimoto et al. (2000) and Nassar, (2009), reported similar observation during isolating different BDSMs using different substrates for En.

The viable counts of BDSM either before or after En were less than TCFU indicating that; not all the indigenous microorganisms have the enzymatic system capable to degrade DBT and only microorganisms that have the required enzymatic system to metabolize DBT would grow on the BSM/DBT plates.

**Purification and Selection of the Tolerant DBT-BDSM**

From the local environmental sample (C1), 28 different aerobic culturable microorganisms, able to grow on BSM-DBT plates using DBT as sole source of sulfur were isolated. About 32.14% (10.71% BC and 21.42% AC) of the isolates were obtained directly after sample collection (before En) and more than 67.86% (10.71% BC and 57.14% AC) obtained throughout the successive En cycles in En medium. This indicates an increase of the microbial diversity after artificial contamination either before or after En.

This mirrored the succession in the adaptation of the isolated BDSM for the continuous exposure to DBT which is in agreement with the results obtained by Duarte et al. (2001); El-Gendy, (2004) and Xiaojuan et al. (2008).

**Selection of the Most Potent DBT-BDSM**

**DBT-Spray Plate Assay**

Spray plate assay for rapid screening of organisms able to degrade water-insoluble hydrocarbons, was originally described by Kiyohara et al. (1982). This assay had been modified by Denome et al. (1994), in order to allow a rapid screening of organisms with the presumptive ability to transform DBT either through Kodama or 4S pathway.

Separate different colonies were incubated on BSM agar plates sprayed with 1000 ppm DBT solution in ethylether (BSM-DBT plates). The test substrate precipitated out as a white film immediately when sprayed onto the plate surface and fluoresced golden yellow under short wave (254 nm) UV-illumination, as mentioned in Table 1. After the 7th day of incubation at 30°C, BSM-DBT plates were viewed in a day light and under (254 nm) UV-illumination for the presumptive detection of the obtained products.

Colonies of *R. erythropolis* IGTS8
surrounded with clear zones appeared on BSM-DBT plates after 2 d incubation.

Four isolates (C1, C7, C10 and C15) appeared on BSM-DBT plates with clear zones around their colonies after only 1 d of incubation period, which might indicate a shorter lag phase than that of *R. erythropolis* IGTS8. Shorter lag phase, may indicate that these isolates would be well adapted to DBT.

Five isolates (C2, C3, C4, C9 and C26) appeared on BSM-DBT plates with clear zones surrounding their colonies after 2 d of incubation period, which might indicate that they could have nearly the same lag phase as *R. erythropolis* IGTS8.

The rest of the isolates appeared on BSM-DBT plates with clear zones surrounding their colonies after 3 d of incubation period, which might indicate that they require longer lag period than that of *R. erythropolis* IGTS8.

Generally, for all the 28 isolates and IGTS8, as shown in Table (2); the substrate sprayed on the BSM-DBT plates completely disappeared, beginning as a clear zone around the colonies after their appearance on the BSM-DBT plates and spreading out across the entire plate throughout the incubation period up to the 7th day. The clear zones around the colonies and the disappearance of DBT indicated that the isolated microorganisms were capable of metabolizing DBT but with different rates.

The obtained results in this study can be confirmed by the results of other researchers; Fought and Westlake (1990); Denome *et al.* (1993); Rhee *et al.* (1998); Castorena *et al.* (2002) and Arulazhagan *et al.* (2010).

Only one isolate (C24) showed clear zones without colored products in day light but showed green-purple fluorescent product around their colonies which spread out across the entire plate when viewed under (254 nm) UV-illumination.

Four isolates; C1, C8, C13 and C14 showed a blue UV-Fluorescence product under (245nm) UV-illumination and orange-red product when viewed in day light. Similar observations had been reported by Krawiec, (1990) and Denome *et al.* (1993). These results might indicate that these isolates utilize DBT through Kodama pathway (Fought and Westlake, 1990 and Nassar, 2009).

According to Krawiec, (1988); colonies of organisms which transform DBT by the sulfoxide/sulfone/sulfonate/sulfate pathway (4S-pathway) are surrounded by fluorescent rings which are clearly blue when illuminated with ultraviolet light. According to the fluorescence of

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**Fig. 5.** Gram stain and transmission electron micrograph of isolated bacteria

**Fig. 6.** DNA agarose gel electrophoresis of PCR amplified 16S rDNA
the tested standard compounds listed in Table (1) the bright blue fluorescence observed around the colonies of C3, C10, C19 and C20 which spread out across the entire plate when viewed under (254nm) UV illumination might indicate the production of 2-HBP. Similar observations had been reported by Krawiec, (1990); Li et al. (1996) and El-Gendy, (2004).

The very weak purple fluorescent product and the clear zone observed closely around the colonies of C11 which did not spread out across the entire plate might indicate the ability of isolates to oxidize DBT to DBTO and/or DBTO₂ without further metabolism to 2-HBP or 2,2′-BHBP. A similar observation was reported by Denome et al. (1994).

The rest of the isolates produced neither color nor fluorescence but only clear zones around the colonies were observed. Similar results were obtained by Krawiec, (1988) and El-Gendy, (2004).

Briefly, four different distinct results were illustrated in Table. (2), 14.28% of the isolates (C1, C8, C13 and C14) produced both colour and fluorescence which might indicate Kodama pathway. 25% (C3, C10, C11, C15, C17, C19 and C20) produced only blue or purple fluorescence which might indicate 4S-pathway. While only one isolate (C24) produced green/purple fluorescence which might indicate production of benzoic acid. All other isolates produced neither color nor fluorescence but only cleared zones.

In conclusion, DBT-spray plate assay demonstrated the presence of fluorescence but it did not establish whether the fluorescence arose from 2-HBP and/or 2,2′-BHBP or other metabolites. Further tests were carried out in order to confirm these results.

**DBT-Bioavailability Assay**

This assay was done according to Lu et al. (1999) to examine the ability of the isolated microorganisms to utilize DBT as a sole sulfur source for the growth in a liquid culture.

Growth of the 28 tested microorganisms revealed their abilities to utilize DBT as a sole sulfur source to a varying extent. But these results did not exclude that DBT might also serve as a carbon source (Soleimani et al., 2007).

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**Fig. 7.** Nucleotide sequence of the 350 bp fragment containing the *Brevibacillus invocatus* C19 16S rDNA structural genes

**Fig. 8.** Phylogenetic tree reconstructing by neighbor joining method of 16S rDNA gene from C19 and closely related bacteria
The pH of the different cultures showed slight decrease from the initial pH 7 as it ranged for all the isolates between pH 6.06-pH 6.98, except in case of C3 (pH4.86) and C13 (pH5.07). This decrease in pH value could be explained by the production of intermediates or dead end products which might cause the decline in the pH of the media.

*R. erythropolis* IGTS8 gave positive blue results with Gibb’s assay which might indicate the production of 2-HBP and/or 2,2'-BHBP. These results were in agreement with those obtained by Gilbert *et al.* (1998); Wang and Krawiec, (1994); Ansari, (2008); Bhatia and Sharma, (2010) and Rath *et al.* (2012).

Nine isolates showed positive blue results with Gibb’s assay (C3, C7, C10, C11, C18, C19, C20, C25 and C26), while all the rest of the isolates gave negative results.

HPLC analysis using system managed to separate DBT, intermediates (DBTO and DBTO2) and the end products of the 4S-pathway (2-HBP and/or 2,2'-BHBP), is illustrated in HPLC chromatogram Fig. (2).

The ability of the 28 isolates and *R. erythropolis* IGTS8 to utilize DBT as a sole source of sulfur was additionally monitored by HPLC analysis after 7 d of incubation as shown in histogram (Fig. 3).

The results confirmed that 9 isolates (C3, C7, C10, C11, C18, C19, C20, C25 and C26) had the ability to utilize DBT as a sole-sulfur source to a varying extent. The difference in the conversion of DBT by different isolates might be due to different enzyme systems utilized by each strain to desulfurize DBT and/or might be due to other factors other than kinetics, physical and chemical parameters required by each isolate to utilize DBT. Several factors could also be responsible for this behavior, such as substrate mass transfer through the cell wall, the pathway through which they could utilize DBT and/or inhibition of activity by the intermediates and products produced from DBT metabolism (Monticello, 2000; Caro *et al.*, 2007 and Irani *et al.*, 2011).

Losses of DBT due to abiotic processes were calculated, which represented 3.47%. Any observed loss exceeding this value in the inoculated flasks can be attributed to BDS processes.

HPLC analysis constituted a more exacting tool for the identification of the intermediates and the final product as indicated from the results showed in Fig. (2). The presence of DBT intermediates (DBTO and DBTO2) and the final product (2-HBP and/or 2,2'-BHBP) of the 4S-pathway had been confirmed with this analytical technique after comparing the retention time of the obtained separated compounds from the

![GC chromatogram](image)
different cultures extract to that of the available standard compounds of the 4S-pathway (DBT, DBTO, DBTO₂, 2-HBP and 2,2'-BHBP). A further advantage of the HPLC analysis; the amount of DBT was also quantified to determine the BDS abilities of the isolated BDSM comparing them with the ability of the standard bacterium; *R. erythropolis* IGTS8 to desulfurize DBT. *R. erythropolis* IGTS8 produced DBTO, DBTO₂ and 2-HBP and not 2,2'-BHBP. Similar results were obtained by Gallagher et al. (1993); Monticello, (1998); Kobayashi et al. (2001); Castorena et al. (2002); Raheb et al. (2009) and Amin, (2011).

All the isolates (C3, C7, C10, C11, C18, C25 and C26) which showed lower BDS abilities than that of *R. erythropolis* IGTS8 were excluded from further BDS studies (Fig. 3).

In conclusion, only one isolate; C19 showed higher abilities to desulfurize DBT relative to *R. erythropolis* IGTS8. They removed up to 66.85% and 50% of the initial 1000 ppm DBT and produced 31.98 ppm and 31.34 ppm 2-HBP, respectively (Fig. 3). C19 was chosen for further BDS studies.

HPLC chromatograms (Fig. 4) illustrate the identified peaks of DBT and its metabolites for IGTS8 and C19 cultures.

**Testing the Ability of Selected BDSM to Utilize DBT as a Sole Carbon and Sulfur Source**

DBT-spray plate assay and DBT-bioavailability assay lacking glycerol were performed to determine if the selected BDSM could utilize DBT as a sole carbon and sulfur source.

*R. erythropolis* IGTS8 was used as a negative control since IGTS8 is reported to be.
unable to use any of the organosulfur compounds as carbon sources (Kayser et al., 1993) i.e.; DBT is not degraded but only transformed into 2-HBP and sulfate (Olson et al., 1993; Oldfield et al., 1997; Ohshiro and Izumi, 1999 and Castorena et al., 2002).

C19 and R. erythropolis IGTS8 showed no growth on DBT-spray plate assay or DBT-bioavailability assay in absence of glycerol, and gave negative results with Gibb’s assay. Moreover, the pH of their media did not undergo any significant change from the initial pH7.

In conclusion, these results confirmed the ability of the isolate C19 to utilize DBT as a sole sulfur source without altering its hydrocarbon skeleton.

Identification of the Most Efficient BDSM Morphological and Physiological Characteristics

Identification of the most promising biodesulfurizing isolate (C19) was first done on the basis of their morphological and physiological properties.

Identification of the Most Efficient BDSM Morphological and Physiological Characteristics

The isolate C19 on the TGY plate is white round colony, edge entire, raised and opaque. The strain is Gram-positive, long bacilli. The cell dimensions of C19 are (1.9 x 0.4 µm) as determined using TEM (Fig. 5).

16S rDNA Sequencing and Phylogenetic Analysis of C19:

Agarose gel electrophoresis of PCR product indicated a sharp band in 350bp area as shown in Fig. (6).

For analysis of sequencing result, the sequence of C19 (Fig. 7) was first compared with others in a non-redundant sequence database at NCBI server (http://www.ncbi.nlm.nih.org) by using the BLAST program. The BLAST results of the 16S rDNA gene sequence indicate the strain is closely related to Brevibacillus invocatus LMG 18962 by 99.05% as indicated in Fig. (8).

The Gene Bank accession number for the isolated strain Brevibacillus invocatus C19 16S rDNA gene sequence is KC999852.
Jiang et al. (2002) reported the ability of *Bacillus brevis* R6 and *Bacillus sphaericus* R16 to metabolize DBT to DBTO₂ and 2-HBP.

A biosurfactant-producing bacterium was isolated from petrochemical contaminated site, identified as *Brevibacillus* sp. PDM-3 has the ability to grow on the expense of phenanthrene, anthracene and DBT. For DBT desulfurization after the optimization of different growth parameters, bacteria showed 93% degradation in six days (Reddy et al., 2010).

**Studying of BDS Pathway of *Brevibacillus invocatus* C19 and *Rhodococcus erythropolis* IGTS8**

Fig. (9) shows that 6 major peaks other than DBT (1) were detected in the GC chromatogram at different retention times as listed in Table (3).

DBT is first oxidized through “4S”-pathway to DBT-sulfoxide (2), DBT-sulfone (3) then to 2’-HBP-2-sulfinic acid (4) and sultine (5) which led to the production of 2-HBP (6). These results confirmed those of the DBT-spray plate assay and also explained the blue coloration developed in Gibb’s assay and confirmed results of HPLC analysis in DBT bio-availability assay.

The presence of sultine may have been formed by the cyclization of 2’-HBP-2-sulfinic acid under the acidic conditions used in the work up and preparation of samples for analysis (Oldfield et al., 1997; Gilbert et al., 1998; Acero et al., 2003 and Mohebali and Ball, 2008).

In case of *Brevibacillus invocatus* C19, the accumulation of 2’-HBP-2-sulfinic acid is higher than 2-HBP. This may suggest that the desulfination step which is catalyzed by DszB (Piddington et al., 1995 and Zhongxuan et al., 2002) is the rate limiting step. Other studies by McFarland et al., (1998) and Nakayama et al., (2002) supported this conclusion.

Data obtained from GC/MS analysis of ethyl acetate extract of DBT culture with *Brevibacillus invocatus* C19 and *Rhodococcus erythropolis* IGTS8 (Fig. 9) and its mass spectra (Fig. 10) suggested the same desulfurization pathway as illustrated in Fig. (11).

In conclusion, *R. erythropolis* IGTS8 and *Brevibacillus invocatus* C19 have the ability to desulfurize DBT while conserving its hydrocarbon skeleton, and producing 2-HBP as dead end product through the 4S-pathway.

**Biosulfurization of Different Thiophenic Compounds by *Brevibacillus invocatus* C19**

This experiment was done after optimization (data not shown), to investigate the ability of C19 to grow on different thiophenic compounds as the sole-sulfur source as compared to *R. erythropolis* IGTS8.

The BDS efficiency of C19 decreased in the following order; DMSO > DBTO₂ > BTH > DBTO > TH > 4,6-DMDBT > 4-MDBT, recording; 99.79%, 98.14%, 96.94%, 96.89%, 96.55%, 93.25%, 90.05% and 69.22%, respectively. While the BDS efficiency of IGTS8 decreased in the following order; TH > DBTO₂ > BTH > DBTO > DBT > DMSO > 4-MDBT > 4,6-DMDBT, recording; 91.65%, 85.06%, 80.88%, 78.10%, 74.68%, 74.56%, 70.96% and 69.64%, respectively as shown in Fig. (12).

Several bacteria have been isolated for the desulfurization of DBT, BT, and their alkylated derivatives (Xu et al., 2009).

Kirkwood et al. (2007) reported that methylation decreases aqueous solubility and increases toxicity. Lower aqueous concentrations due to reduced aqueous solubility, and lower reaction rates due to mass transfer limitations and possibly steric hindrance for the more hydrophobic 4-MDBT and 4,6-DMDBT, would limit the efficiency of detoxification and could therefore be responsible for the retardation in growth and BDS of these compounds.

Fedorak and Westlake, (1983 and 1984) showed that the susceptibility of DBTs in prodhoe Bay crude oil to biodegradation decreased with increasing alkyl substitution.

The results of IGTS8 obtained in this study are consistent with the results reported by Kropp et al. (1997) and Zhang et al. (2013); C₂-DBTs are more susceptible to BDS than C₃-DBTs. Thus, in environments contaminated with crude oil, BT and methyl-DBT will be depleted before the isomers of dimethyl-DBTs. But C19 showed more efficiency to desulfurize 4,6-DMDBT than 4-MDBT, which adds to its advantages.

In conclusion, the relatively broad range of specificity for organic sulfur compounds showed by strain *Brevibacillus invocatus* C19 compared to *R. erythropolis* IGTS8 suggests its potentiality to be used in the BDS of petroleum...
and its fractions.

CONCLUSIONS

The suggested enrichment technique has proven to be effective for isolation of biodesulfurizing microorganisms (BDSMs) with capabilities to remove sulfur from dibenzothiophene (DBT) without altering its hydrocarbon skeleton.

The higher BDS abilities of the isolated Gram +ve, long bacilli, Brevibacillus invocatus C19 relevant to the standard BDS strain R. erythropolis IGTS8, would suggest its potentiality to be used in the BDS of different sulfur compounds in petroleum and its fractions.

Further work is undertaken now in EPRI Biotechnology Lab., to enhance the BDS efficiency of C19 throughout immobilization and applying nano-technology.

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