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EXPERIMENTAL ARTICLES

Utilization of Dibenzothiophene and Desulfurization of Crude Oil by a New *Klebsiella* sp. Strain BDS24 and the Proposed Metabolic Pathway

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Abstract—A strain capable of efficient biodesulfurization of dibenzothiophene was isolated from oil-contaminated soil samples. The strain, designated BDS24, was a rod-shaped gram-negative bacterium. Molecular identification based on its 16S rDNA gene sequence showed that this strain belonged to the genus *Klebsiella*. Examining its ability to desulfurize dibenzothiophene by gas chromatography revealed that this isolate consumed 0.5 mM of dibenzothiophene within 72 h. Evaluation of growth characteristics by the tetrazolium chloride assay showed that BDS24 isolate reached its maximum growth in the exponential phase after 60 h of growth with DBT. Compared to the reported 4S metabolic pathway, an extended 4S pathway was proposed for the desulfurization of dibenzothiophene, which has not been formerly reported in the literature. A decrease of 88% of the total sulfur content of crude oil sample by a *Klebsiella* strain has not been achieved previously. These results indicate that *Klebsiella* sp. BDS24 is an adequate candidate for deep biodesulfurization of crude oil.

Keywords: biodesulfurization, dibenzothiophene, metabolic pathway, crude oil, *Klebsiella* sp. BDS24

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Sulfur is the third most abundant element in crude oil, and more than 70% of the sulfur in crude oil is in the form of dibenzothiophene (DBT) and its derivatives (Aminsefat et al., 2012; Delegan et al., 2021). Combustion of fossil fuels releases large amounts of sulfur oxides into the atmosphere leading to dangerous environmental hazards such as acid rains (Olmo et al., 2005; Akhtar et al., 2009). Therefore, refineries must decrease the sulfur content of the fuels they produce. The high water content of crude oil makes biodesulfurization (BDS) of crude oil more feasible than that of gasoline and diesel oil. However, few results on biodesulfurization of crude oil have been published (Monticello and Finnerty, 1985; Gupta et al., 2005; Yu et al., 2006). While hydrogen desulfurization (HDS) is a technique ordinarily employed in oil refining companies, requires extreme pressure and temperature conditions. Some refractory heterocyclic sulfur compounds, such as dibenzothiophene, are not eliminated by this method (Park et al., 2011; Omar et al., 2020).

Biodesulfurization (BDS) has been proposed as an economically viable and environmentally safe alternative to desulfurization of difficult-to-decomposable sulfur compounds, which works in mild operating conditions and uses whole-cell biocatalysts instead of expensive enzymes (Canales et al., 2018; Nassar et al., 2020). In various researches, dibenzothiophene has been studied as a model compound for biodesulfurization (Li et al., 2019). *Rhodococcus erythropolis* IGTS8 is the first microorganism known to possess BDS activity and has been extensively studied (Gray et al., 1996, 2003; Parveen et al., 2020). Other desulfurizing bacteria belong to the genera *Corynebacterium*, *Gordonia*, *Nocardia*, *Mycobacterium*, *Paenibacillus*, *Shewanella*, *Sphingomonas*, *Halothiobacillus*, *Bacillus*, etc. (Akhtar et al., 2016a, 2016b, 2018; Nazari et al., 2017; Sadare et al., 2017). The discovery of the aerobic 4S pathway was a turning point for biodesulfurization, as it is a non-destructive pathway in which the calorific value of dibenzothiophene is preserved (Denome et al., 1993; Martínez et al., 2017). The 4S pathway contains four consecutive enzymatic reactions catalyzed by three enzymes encoded by the *dszABC* genes. DBT monooxygenase (DszC) catalyzes the oxidation of DBT to DBT-sulfoxide and then to DBT-sulfone. DBT-sulfone monooxygenase (DszA) catalyzes the oxidation of sulfone to 2-hydroxybiphenyl-2-sulfinate (HBPS), and HBPS sulfinolyase (DszB) catalyzes the conversion of HBPS to 2-hydroxybiphenyl (2-HBP). NADH-FMN oxidoreductase (DszD) also provides the $FMNH_2$ cofactor regeneration required for reac-

tions catalyzed by DszC and DszA (Kodama, 1977; Oldfield et al., 1997; Díaz and García, 2010; Sousa et al., 2020). Nowadays, biodegradation of polycyclic aromatic hydrocarbons (PAHs) are the focus of bioremediation of oil-contaminated soils and seawater, rather than polycyclic aromatic sulfur heterocycles (PASHs). Whereas, complete removal of PASHs without releasing secondary contamination to the environment is mandatory (Li et al., 2019).

In the present study, we report a new DBT-desulfurizing bacterial strain, *Klebsiella* sp. BDS24, isolated from soil at the Abadan Oil Refining Company, Iran. Its DBT desulfurization ability, morphological analysis, and growth rate under aerobic conditions were assessed. The metabolites produced via DBT desulfurization by the isolate and the extended metabolic desulfurization pathway were explicated via GC-MS. The decrease of sulfur content in crude oil was also studied.

MATERIALS AND METHODS

Chemicals. DBT (98% purity) and absolute ethanol were purchased from MERCK, Germany. A bacterial Genomic DNA extraction kit was purchased from FAVORGEN, Taiwan. Crude oil samples were procured from the Abadan oil refining company of Iran. All the other chemicals were of the analytical grade and commercially available, and were used without further purification.

Media. The basal salt medium (BSM) was prepared as recommended previously (Izumi et al., 1994; Davoodi-Dehaghani et al., 2010), with some modifications. This culture medium contained the following compounds (g/L distilled water): glycerol, 5; KH_2PO_4, 2; K_2HPO_4, 4; NH_4Cl, 1; $MgCl_2{\cdot}6H_2O$, 0.2; $CaCl_2$, 0.02; and 10 mL of metal solution (pH 7.2). The metal solution contained (g/L distilled water): $FeCl_3{\cdot}6H_2O$, 0.001; $ZnCl_2$, 0.5; $MnCl_2{\cdot}4H_2O$, 0.5; $Na_2MoO_4{\cdot}2H_2O$, 0.1; and $CuCl_2$, 0.05. While containing the necessary growth elements and sources of carbon and nitrogen, this medium does not have a source of sulfur. By adding 0.54 mM of dibenzothiophene as the only source of sulfur, it becomes a selective culture medium for desulfurizing microorganisms. BSM-agar was prepared by adding 1.5% agar.

Enrichment and isolation of DBT-desulfurizing bacteria. From the designated sampling sites in the Abadan oil refining company (Khuzestan province, Iran), 18 samples of oil-contaminated soils were collected. The purpose of enrichment was to obtain microorganisms with high desulfurization capacity. In this method, 2 g of each sample was poured into 100 mL Erlenmeyer flasks containing 20 mL of sterile BSM medium supplemented with dibenzothiophene; then the flasks were incubated on a rotary shaker (150 rpm) at 30°C. After five days, for subcultivation, 2 mL of these primary cultures were added to 20 mL of fresh BSM medium supplemented with DBT and incubated under the same conditions for another 5 days. After five subcultivations, the cultures were prepared in 10-fold dilutions from 10^{-1} to 10^{-5}. The suspensions (100 μL) were spread on BSM agar supplemented with DBT. These plates were incubated at 30°C for three days, and after the growth of microorganisms, each distinctive colony was purified separately onto a BSM-agar medium supplemented with DBT. A total of 25 isolates capable of desulfurization were obtained. Based on its growth which indicated great desulfurization performance, strain BDS24 was chosen for subsequent evaluation.

Identification of the DBT-desulfurizing bacterium. For molecular identification of the selected isolate, template DNA was extracted from fresh bacterial culture using a bacterial DNA extraction kit according to the manufacturer's instructions. The 16S rDNA genes were amplified with the universal primers 27F and 1492R using a Mastercycler ep Gradient S (Eppendorf, Germany). PCR was performed according to the following procedure: denaturation at 94°C for 3 min, 35 cycles of 30 s at 94°C, 30 s at 54°C, and 2 min at 72°C, and a final extension step at 72°C for 5 min.

To confirm PCR by electrophoresis, PCR products and DNA Ladder were investigated on a 0.5% agarose gel in a 1× TAE buffer and stained with Safe stain for visualizing DNA bands using a UVITEC Cambridge Gel Documentation System. The PCR product with a size of about 1500 bp was sent to Microsynth Co. in Switzerland for sequencing. The resulting sequence was submitted into the Blast search of the NCBI database, and species whose 16S rDNA gene had a high degree of similarity with our sequence were introduced. The phylogenetic tree was constructed using MEGA 11 software.

Investigation of bacterial growth and desulfurization of DBT by BDS24 strain. To investigate the decrease of DBT and identify the metabolites produced during the desulfurization process by gas chromatography-mass spectrometry (GC-MS), a bacterial suspension with $OD_{600} = 2.0$ was prepared in the Ringer solution, and 200 μL of this suspension was inoculated to each Erlenmeyer flask with 20 mL of the medium. The flasks were placed on a rotary shaker (150 rpm) at 30°C. The cultures were removed from the shaker after 24, 44, 64, and 72 h, respectively.

Due to the nature of growth and biofilm formation by the isolate in the medium with DBT, the growth rate could not be measured by spectrophotometry. Owing to the presence of grains, the resulting culture was not homogeneous causing errors in the test results. Therefore, growth was measured by the triphenyl tetrazolium chloride (TTC) colorimetric assay (Hayashi et al., 2003; McCluskey et al., 2005; Sabaeifard et al., 2014). After optimizing the TTC assay conditions for this isolate using the Taguchi method (data

not shown), two BSM media with DBT were prepared in 100-mL volumes. These media were inoculated from a 48-h culture of strain BDS24. The flasks's were placed on a rotary shaker (150 rpm) at 30°C, and samples were collected every 6 h. For the TTC test, the samples (2.5 mL) were transferred to sterile test tubes-wrapped in aluminum foil to prevent light access. The TTC stock solution (0.1%) was added (250 μL), and the tubes were placed on a rotary shaker for 5 h. After this time, the liquid was discarded, and the resulting red formazan precipitate was dissolved in 2.5 mL of dimethyl sulfoxide. The absorbance (OD_{480}) of the samples was then measured spectrophotometrically (T60U, PG Instruments LTD., Leicestershire, United Kingdom).

GC-MS-based analysis. To determine the degree of DBT decrease and identify the compounds resulting from its desulfurization, a 7890B GC/5977A MS instrument (Agilent Company, United States) was used, equipped with the split/splitless injection system and electron impact ionization model; it had NIST and WILEY mass libraries. To analyze the desired compounds, an HP5-MS capillary column with a length of 60 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm was used. Injection site temperature, interface temperature, and ionization site temperature were 270, 299, and 230°C, respectively. The temperature program of the column started with the initial temperature of 50°C. It was kept at this temperature for 7 min, then the temperature of the column reached 299°C with a slope of 13°C/min, and for 10 min remained at this temperature. The split ratio was set to 1 to 10, and the injection volume was 1 μL.

Broth cultures were acidified to pH 2–3, and the extraction process was performed for 45 min on a magnetic stirrer at 450 rpm. The extracting solvent was the hexane/dichloromethane mixture (0.3/0.7 vol/vol).

Investigation of sulfur consumption from crude oil by strain BDS24. Nutrient broth medium was prepared in a volume of 100 mL and inoculated with a single colony of strain BDS24. The flask was incubated on a rotary shaker (150 rpm) at 30°C until the OD_{600} nm reached to 0.8. Then, cells were harvested by centrifugation at 2870 *g* for 15 min and washed with 100 mM phosphate buffer (pH 7.0).

Two BSM media supplemented with 2% crude oil were prepared in volumes of 100 mL. One of these flasks was inoculated with the bacterial suspension, and the other flask was considered as a control. Both flasks were incubated for 72 h in a rotary shaker (150 rpm) at 30°C.

Crude oil was extracted from the aqueous phase using normal hexane. Then the total sulfur of the crude oil present in the control sample and the sample treated with BDS24 isolate was measured according to the ASTM D4294 Standard Test Method for Sulfur in Petroleum and Petroleum Products by Energy Dispersive X-ray Fluorescence Spectrometry.

RESULTS AND DISCUSSION

Isolation and characterization of DBT-desulfurizing bacteria. Oil-contaminated soil samples were collected from different parts of the Abadan refinery. After 30 days of enrichment, a total of 28 isolates with the ability to grow in a desulfurization medium were purified. After five transfers of the BRS13 sample, strain BDS24 was isolated. In the course of consecutive transfers, the dominance of some colonies and elimination of other colonies was observed. The BDS24 isolate was selected for subsequent analyzes based on its growth rate and high desulfurization activity when grown on DBT. The BDS24 colonies on BSM medium with DBT were colorless, opaque, spherical, medium in size, with a smooth margin, a mucoid surface and a sticky form (Fig 1b). After Gram staining, the cells of this isolate appeared as relatively short gram-negative rods. The colonies of this isolate on EMB agar medium were pink, spherical, large, and have a mucoid form (Fig. 1c). The BDS24 cells were rod-shaped and approximately 1.5 μm in length (Fig. 1d).

Based on the results of the 16S rDNA gene sequencing of the isolate BDS24 (1417 bp), this strain belonged to the *Klebsiella* sp. The sequence was deposited in GenBank under accession number OL823066 (https://www.ncbi.nlm.nih.gov/nucleotide/OL823066.1). The phylogenic tree of strain BDS24 and closely related bacteria constructed by the neighbor-joining method using the 16S rDNA gene sequences is shown on Fig. 2. The BDS24 strain was closely related to *Klebsiella variicola* CCFM8387 (KJ803944.1) (Long et al., 2022), with a 99% sequence identity. In the study of Bhatia and Sharma (2012), a thermophilic strain of *Klebsiella* sp. 13T was isolated by enrichment method from contaminated soil samples collected from an oil refinery. This *Klebsiella* sp. strain was isolated from an environmental sample and we expect it to be different from clinical isolates in terms of pathogenicity, so that it can be used industrially. Of course, this claim requires additional identification and investigation of pathogenic genes in this strain.

Evaluation of growth and efficiency of the BDS24 isolate in desulfurization of DBT. Growth and cell viability analysis of strain BDS24 was assessed by TTC assay, during which sampling of cultures continued for 120 h. The results are shown as the growth curve graph on Fig. 3. In the first stage of growth, there was a lag phase. The highest growth rate of the isolate observed at 60 h when OD_{480} was 0.538, and then its growth decreased. It is worth mentioning that in 108 to 120 h, there was an increase in OD, comparable to the values for 18–24 h.

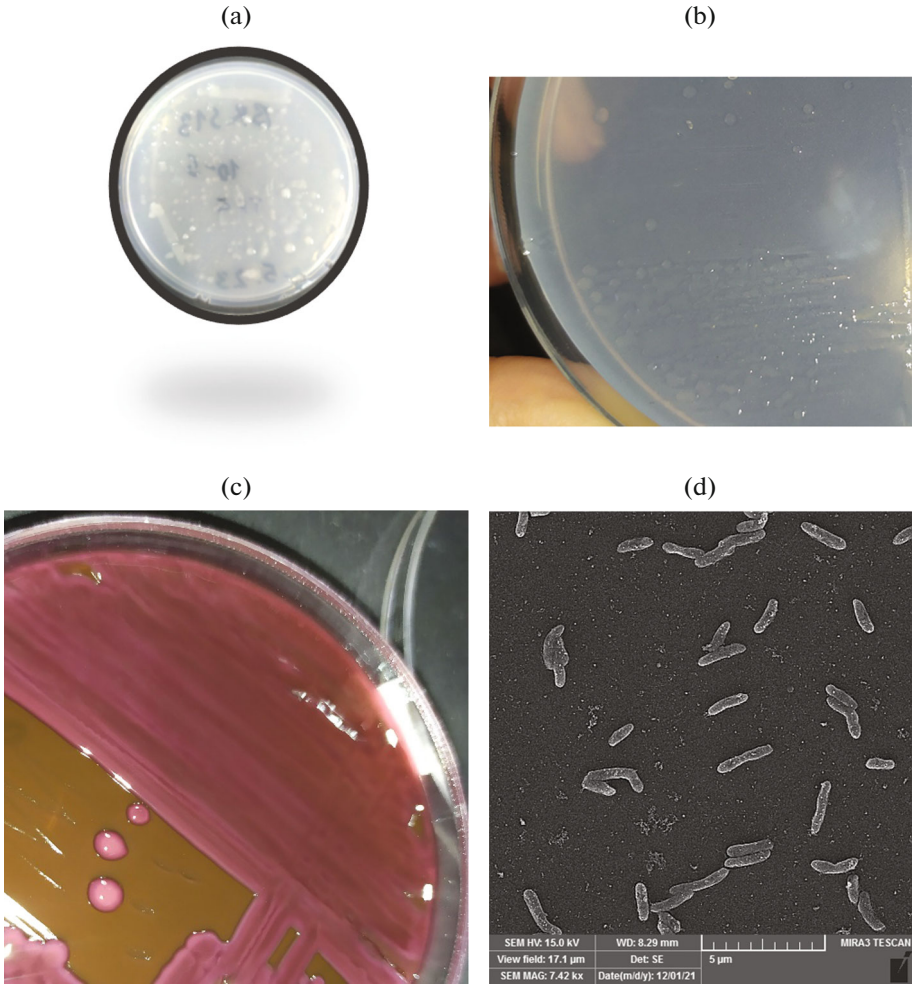

Fig. 1. Photographs of the colonies obtained on different media and of the cells: fifth transfer of BRS13 sample from 10^{-5} dilution (a); the BDS24 isolate colonies on BSM agar with DBT (b); colonies of BDS24 on EMB agar (c); and scanning electron microscope micrograph of the BDS24 cells (d).

Cultures of strain BDS24 were analyzed by GC-MS at 24, 44, 64, and 72 h. DBT in absolute ethanol solution with a final concentration of 0.5 mM was added to BSM culture media. Figures 4a and 4b show the chromatograms of the control sample and of the sample inoculated with the isolate after 72 h of incubation. The decrease of DBT content and the shortening of its peak can be seen compared to the control sample. DBT was recorded at retention times of 18–19 min. The DBT concentrations at different time intervals, determined based on the decrease of its sub-peak area in the chromatograms, are shown on Fig. 5. The percentage of DBT desulfurization 24, 44, 64, and 72 h after inoculation was calculated based on the area under its peak in the samples treated with the BDS24 isolate and the control sample, which were 37, 55, 62, and 97.5%, respectively.

New proposed extended 4S pathway for DBT desulfurization. To determine the possible metabolic pathway for DBT desulfurization by the isolate BDS24, intermediates were extracted from different time intervals and analyzed by GC-MS.

Phthalic acid was found in this experiment at the retention time of 18.004 min. This metabolite was also detected during the desulfurization of DBT by *Pseudomonas* sp. LKY-5 (Li et al., 2019). 1,3-Dimethyl benzene was recorded at the retention time of 6.974 min, which had the highest abundance among metabolites. Phenol, 2-methyl-5-(1-methyl ethyl) was also detected at the retention time of 14.117 min.

Based on the reported metabolites and metabolic pathways of DBT, the metabolic pathway for desulfurizing of DBT by *Klebsiella* sp. BDS24 is proposed as shown in Fig. 6. In studies such as by El-Gendy et al., (2014) and Elmi et al. (2015), extended 4S pathways have been proposed, in which 2-hydroxy biphenyl, the end product of the 4S pathway, is broken down into other metabolites. During the metabolic pathway proposed in the present study, sulfur is released in the form of SO_2 from dibenzothiophene. This is an advan-

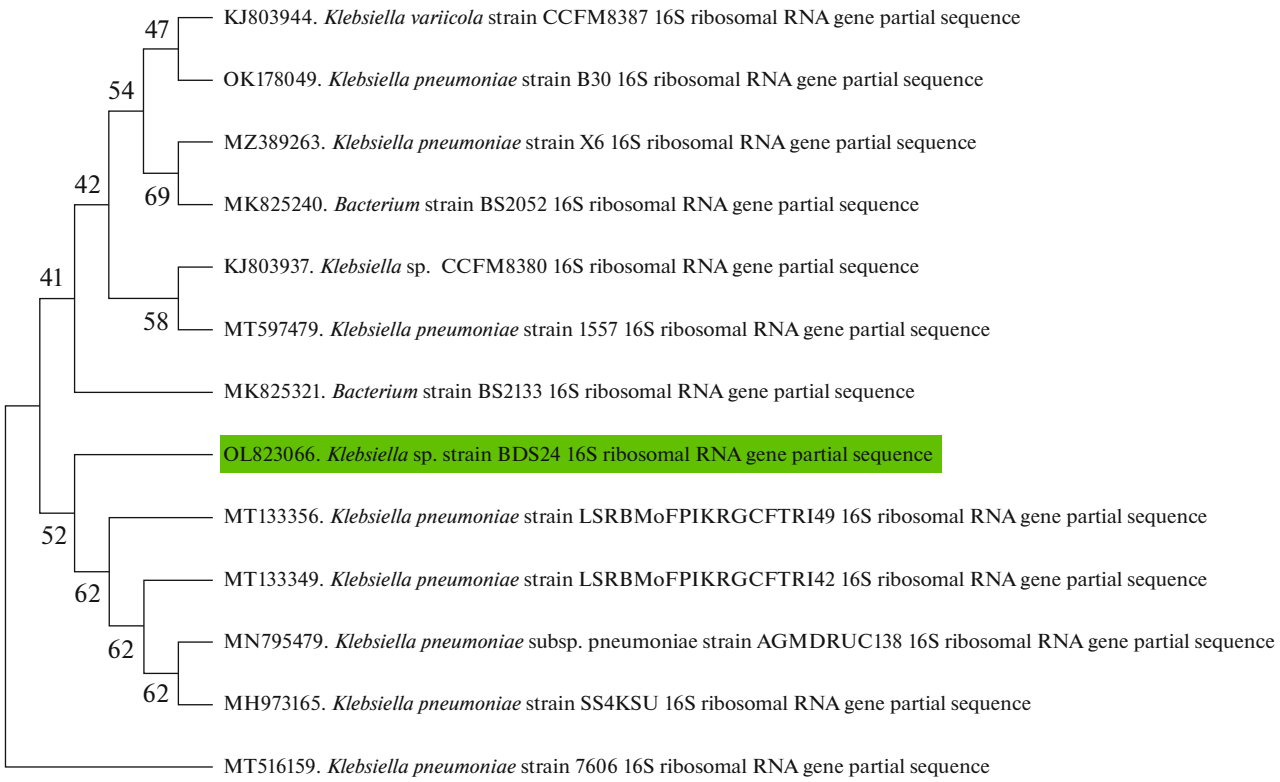

Fig. 2. The phylogenetic tree showing the taxonomic position of *Klebsiella* sp. BDS24. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985).

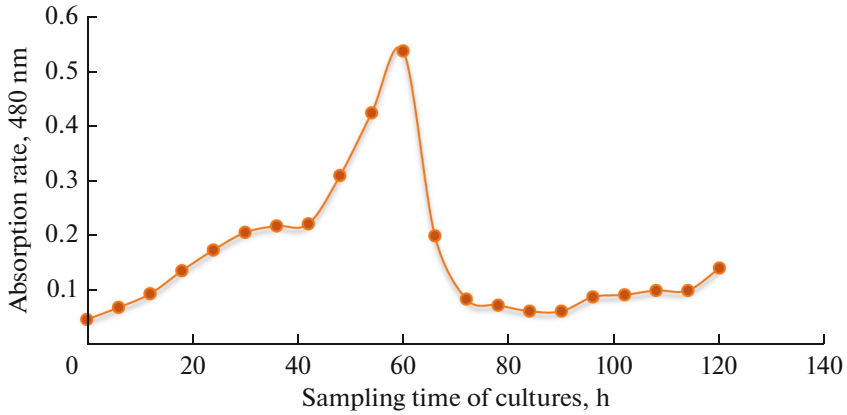

Fig. 3. Growth curve of strain BDS24 obtained using the TTC assay.

tage of biodesulfurization, as in hydrogen desulfurization sulfur is released from sulfur compounds in the form of H_2S, which has higher toxicity. One of the advantages of this pathway for desulfurization is that it is not destructive; that is, the integrity of the carbon structure of DBT is not attacked by enzymes, and the end products such as toluene (RT 6.662 min), ethylbenzene (RT 8.125 min), and xylene (RT 7.077 min) are produced, which have a calorific value in crude oil.

Evaluation of crude oil sulfur content decrease by strain BDS24. Oil refineries commonly separate crude oil into several fractions and then desulfurize them separately. Refineries can make significant cost savings if most of the sulfur is removed from crude oil before it is fractionated (Yu et al., 2006). Therefore, the ability of strain BDS24 to remove sulfur compounds from crude oil was determined. After biphasic batch desulfurization of crude oil, the total sulfur con-

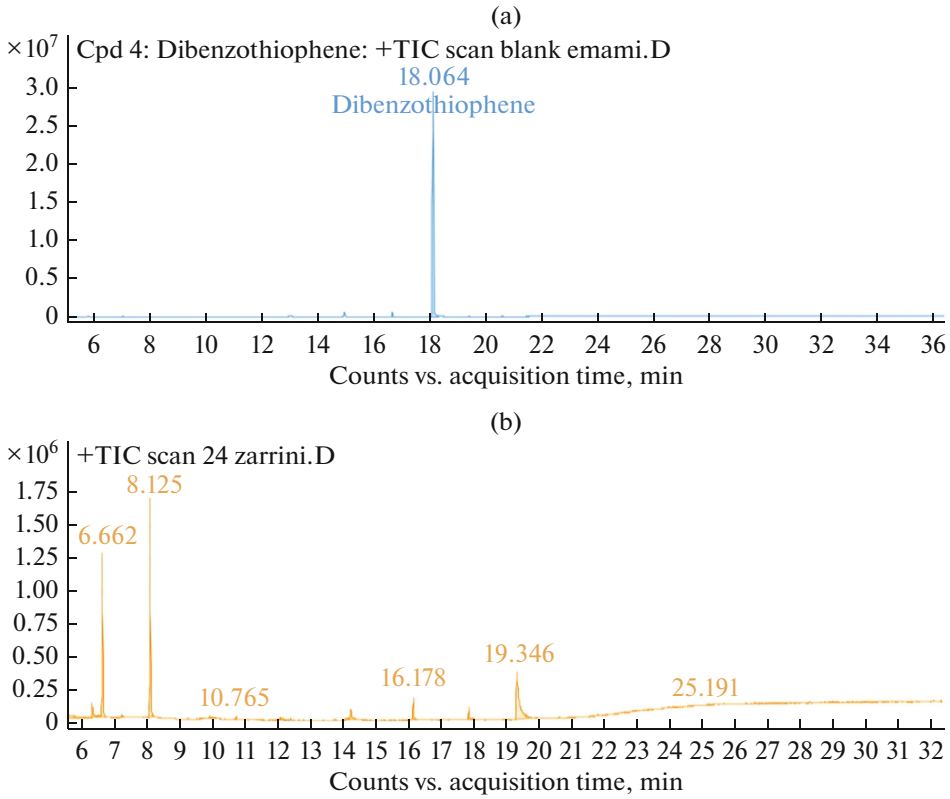

Fig. 4. Chromatograms of the control sample without microorganisms (a) and of the sample treated with the BDS24 isolate for 72 h (b).

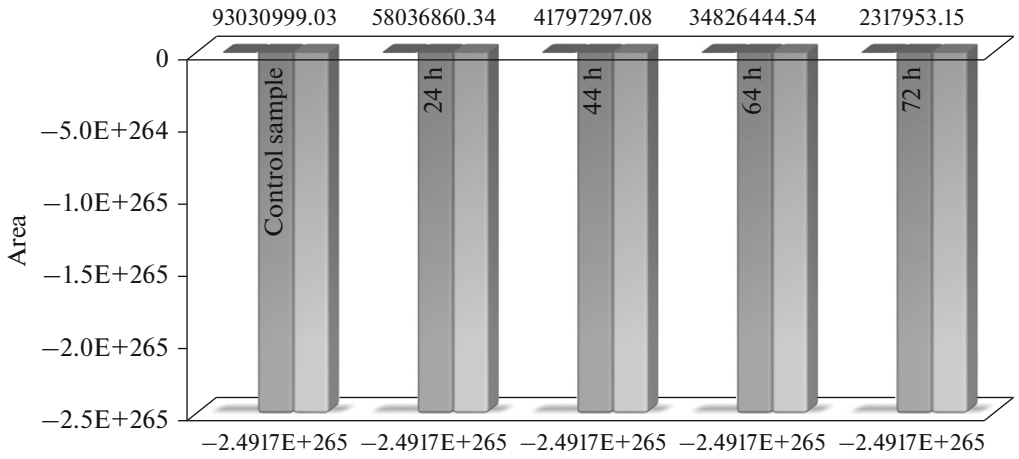

Fig. 5. The rate of DBT consumption in the samples treated strain the BDS24 at different time intervals compared to the control sample.

tent decreased from 0.265 to 0.030 mass % within the 72-h period. The percentage of crude oil sulfur content decrease compared to the control sample was calculated using the following formula:

$$R = S_i - S_f / S_i \times 100.$$

In this formula, R is desulfurization efficiency, S_i is total sulfur of crude oil present in the control sample, and S_f is total sulfur of the crude oil present in the desulfurized sample. According to this calculation, the BDS24 isolate can removed 88% of the sulfur content of crude oil present in the sample treated with this isolate for 72 h.

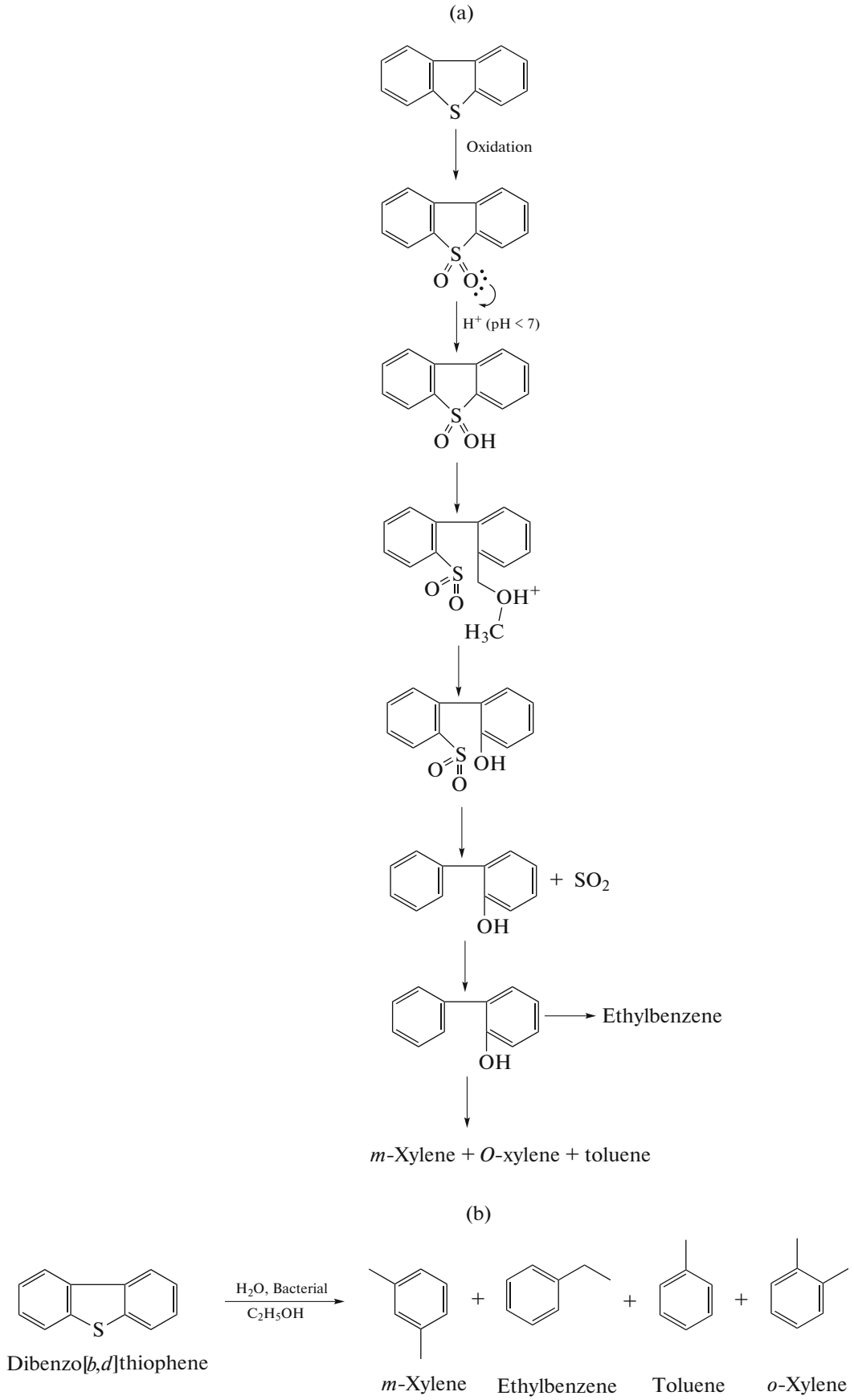

Fig. 6. Proposed metabolic pathway for desulfurization of DBT by strain BDS24 (a) and the end products of proposed extended 4S pathway (b).

In the present study, a gram-negative bacterium, *Klebsiella* sp. BDS24, able to desulfurize DBT, was isolated. This bacterium is the first strain of *Klebsiella* with very high desulfurization efficiency, i.e., removal of 97.5% of DBT in 72 h, which was obtained for the first time in this study. An extended 4S pathway for desulfurization of DBT by this strain has been proposed, which has not ever been mentioned in the literature. The isolate was also able to decrease the sulfur content of crude oil by 88% without affecting its calorific value, which was one of its advantages. These results indicate that *Klebsiella* sp. BDS24 is an adequate candidate for biodesulfurization of crude oil.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest.

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