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Biodesulfurization of Dibenzothiophene by a Newly Isolated, *Stenotrophomonas maltophilia* Strain Kho1

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Abstract: A novel desulfurizing bacterium has been isolated from oil-contaminated soils in Khouzestan. The ability for dibenzothiophene desulfurization and its biochemical pathway were investigated. The bacterium was identified as *Stenotrophomonas maltophilia* strain KHO1 (Genbank Accession No, HM367710) by 16S rRNA gene sequencing. HPLC results and Gibb's assay were shown that dibenzothiophene desulfurized via 4S-pathway. Maximum growth (0.391 g dry cells/l) and produced 2-hydroxybiphenyl (58.69 μM) were observed at 128 h of cultivation. Using response surface design procedure the optimization of pH, temperature and rotary shaker round on the desulfurization reaction of isolate KHO1 were performed. The optimum conditions were determined at pH of 7.2, temperature of 29 °C and rotary shaker round of 180 rpm. At these conditions, the dibenzothiophene desulfurization activity was increased and maximum 2-hydroxybiphenyl production was detected 63.15 μM at 96 h. According to results, Isolate KHO1 was capable to desulfurize dibenzothiophene via 4S-pathway and likely it can be useful to reduce organic sulfur contents of crude oil.

Key words: Biodesulfurization · Dibenzothiophene · Optimization · 4S-pathway · Stenotrophomonas.

INTRODUCTION

Sulfur is usually the third most abundant element in crude oil [1], all fossil fuels contain a variety of organic and inorganic sulfur compounds. Sulfur usually accounts for around 0.03-7.89 wt% of crude oil but depending on the sulfur content of any given crude oil supply, the sulfur concentration of the middle-distillate fraction used to make diesel fuels can range widely from <500 to >5000 mg/l [2]. Sulfur dioxide emission through fossil fuel combustion is a major contributor to the generation of acid rain and air pollution [2-5]. Fuel desulfurization represents an opportunity for a cost-effective solution to acid rain and other health hazards caused by sulfurous emissions [2, 6]. Regulation standards against the sulfur content of petroleum products such as gasoline and diesel oil are becoming stricter in view of the need for environmental protection. For instance, the sulfur content in diesel oil will have to be less than 10 or 15 mg/l by 2010 in Europe and the United States [4, 7].

The organic sulfur species in crude oil include both thiophenic (aromatic) and sulfidic (aliphatic) compounds. The relative amounts of these species vary greatly among different oils, e.g. in the maltene fraction of Alberta heavy crude oils the sulfide-to-thiophene ratio, based on weight percent concentration, ranges from 0.22 (Pembina) to 2.1 (Peace River) [8].

To remove sulfur from fossil fuels, refiners now rely on a Hydrodesulfurization (HDS) technique, which converts organic sulfur in the feed to hydrogen sulfide in the presence of a transition metal catalyst and hydrogen. The extent of desulfurization achieved by HDS is determined by the reaction conditions, with higher hydrogen pressures and temperatures giving greater sulfur removal [9-11], this process is costly and energy-intensive and not effective at removing polycyclic sulfur compounds [6].

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Most of the sulfur in crude oil is organically bound, mainly in the form of condensed thiophenes. Up to 70% of the sulfur in petroleum is found as dibenzothiophene (DBT) and substituted DBTs (methylated DBTs and benzo DBTs) that are particularly recalcitrant to conventional HDS [2, 9, 12] especially when they are alkylated at positions 4 and 6 [5, 10].

Biodesulfurization (BDS) processes have been proposed as an alternative technology for removing the recalcitrant organic compounds found after the conventional hydrodesulfurization treatment, mainly polycyclic aromatic hydrocarbons (PAH) as dibenzothiophene [7, 13]. DBT has therefore been widely used as a model compound for biodesulfurization studies [2]. Research on biodesulfurization using dibenzothiophene has resulted in the elucidation of two different biochemical pathways, named Kodama and 4S. 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 from the caloric value of the fuel. Through 4S-pathway, dibenzothiophene is transformed to 2-hydroxybiphenyl and sulfite as end-products. Microbial systems have been reported to selectively take up the sulfur from the DBT molecule by four consecutive enzymatic steps, leaving intact the carbon skeleton [13] and in the pathway the carbon skeleton of DBT is released intact as 2-hydroxybiphenyl (2-HBP); therefore, fuel value is not lost [1, 2]. This process of microbial desulphurization or biodesulphurization is expected to overcome the technical and economic problems associated with HDS as it has the potential benefits of lower capital and operating costs and will produce lesser greenhouse gases [12].

In the present study, our objective was to identify a novel desulfurizing bacterium and investigate the ability for desulfurization of dibenzothiophene to produce 2-HBP by using HPLC and Gibb's assay. Finally, the effects of three parameters on the desulfurization reaction were examined and the optimum conditions were determined.

MATERIALS AND METHODS

Materials: Dibenzothiophene were purchased from Merck. 2-hydroxybiphenyl was purchased from Sigma. Acetonitrile was HPLC grade. Other chemicals were of analytical grade and used without further purification [2, 4, 14].

Methods

Media: Minimal Salt Medium (MSM) containing (gram per liter of deionized water) NH_4Cl (2.0), KH_2PO_4 (6.0), Na_2HPO_4 (4.0), $FeCl_3$ (0.001), $MnCl_24H_2O$ (0.004), $MgCl_26H_2O$ (0.75), $CaCl_22H_2O$ (0.001) was used as the medium for cultivation of the strain. The pH was adjusted prior to autoclaving to 7.0. Sodium benzoate (2 g/l) was added to the medium as the sole source of carbon (MSM-S medium). DBT was dissolved in ethanol to a concentration of 1 mM and added to a sterilized MSM as the only sulfur source (MSM-SD medium). All inoculated liquid media were incubated at 30 °C on a rotary shaker operated at 160 rpm. The medium components were selected from chemicals with minimum inorganic sulfur impurity [4, 6, 11].

Microorganism: Several strains which utilized dibenzothiophene as a sole source of sulfur have been isolated from oil-contaminated soil samples by Department of Biology at Shahid Chamran University in Ahvaz, Iran. Among the DBT-utilizing strains, isolate KHO1 was selected for further studies.

Identification of Selected Strain by Using Pcr Amplification and Sequencing: Isolate KHO1 was grown in Luria-Bertoni (LB) culture medium containing 50 μg/ml kanamycine and total DNA was extracted using phenol-chloroform procedure at an early exponential state [15].

PCR was used for amplification of 16S ribosomal RNA gene (16S rRNA gene) by using general eubacterial specific primers FD1 (5□-CCGAATT CGTCGACAACAGAGTTTGATCCTGGCTC AG) and RP1 (5□-CCCGGGATCCAAGCTTACGGTTAC CTTGTTAC GACTT) that are advised to amplify nearly full length of 16S rRNA gene of most eubacteria [16].

The reaction mixture contained 15 pmol of each dNTP, 20 pmol of each primer, 2.5U Taq polymerase (Fermentas) and 15 ng of genomic template DNA. The cycles involve initial denaturation at 94°C for 5 min, followed by 30 amplification cycles (94°C for 1 min, 62°C for 40 s and 72°C for 2 min) and a final extension for 7 min at 72°C. The amplification products were analyzed on a 1% agarose gel electrophoresis. PCR products were purified for DNA sequencing using Gel Band Purification Kit (Bioneer, South Korea) and sequenced using PCR primers. Some bands were also cloned on pDrive Cloning Vector which had a linear form with a U overhang at each end with PCR Product Cloning Kit (Qiagen, Germany).

Vectors were extracted using Rapid Plasmid Miniprep Kit (Bioneer) and sequenced with vector specific universal primers. Sequencing was done by applying automated DNA sequence (Macrogen, South Korea) and the sequence analysis was performed using the BLAST (basic local alignment search tool) in the National Center for Biotechnology Information (NCBI). Sequences manipulation and editing were performed using BioEdit graphic interface [9, 12, 15]. The neighbor-joining (NJ) method was used for constructing phylogenetic trees [17].

Gibb's Assay: Gibb's assay was useful to find bacteria that can selectively remove sulphur from DBT and its derivatives. The test was used to detect and quantify 2-hydroxybiphenyl produced by the strain after incubation with dibenzothiophene. Gibb's reagent reacts with aromatic hydroxyl groups such as 2-hydroxybiphenyl to form a blue-colored complex [2].

The Gibb's assay was carried out as follows: the pH of 5 ml of microbial culture was adjusted to 8.0 using 10% (w/v) sodium carbonate (Na_2CO_3). Then, 100 µl of Gibb's reagent (10 mg of 2,6-dichloroquinone-4-chloroimide dissolved in 1 ml of ethanol) was added to each sample and the reaction mixture was incubated at 30 °C for 30 min. To separate the bacterial cells, the reaction mixture was centrifuged (4000 rpm, 4°C, 20 min) and the absorbance of the supernatant was measured at 610 nm using spectrometry (Biochrom, Model Biowave ^{22}, UK). The standard curve was created using 2-hydroxybiphenyl versus Gibb's assay results; the linear correlation between them was occurred at 2-hydroxybiphenyl concentrations ranged between 0 and 12 mg/l [1, 4, 14]. The assay was verified by using *Rhodococcus erythropolis* IGTS8 (ATCC 53968) with DBT as the substrate in a positive control experiment [1].

High-performance Liquid Chromatography (HPLC): Detection and quantification of 2-HBP was carried out by HPLC on a Waters instrument (model Pump 600E), using a NOVA PAK C18 (300 mm × 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). Acetonitrile/water (70:30 v/v) was the mobile phase with a flow rate of 1 ml/min. The column was maintained at 40°C [2, 18].

For HPLC analysis, liquid samples were acidified to pH 2.0 with 1N HCl and extracted three times with equal volumes of ethyl acetate [4, 9, 18].

Dbt-desulfurization by Growing Cells: For desulfurization experiments by growing cells, cultivations were carried out in 100 ml erlenmeyer flasks containing 50 ml of MSM-SD medium at 30°C and under rotary shaking at 160 rpm. The time course of dibenzothiophene utilization was obtained by sampling at defined time intervals and analyzing the various parameters including optical density (OD_{660}), pH and Gibb's assay. HPLC analysis was performed for quanitification of 2-HBP [2, 14].

Cell Concentration: Cell concentration was determined by converting the optical density value obtained in a Biochrom spectrophotometer at 660 nm (OD_{660}) in grams dry cell weight per liter (g dry cells/l) [4, 14, 18]. The sterilized MSM-SD medium sample was used as the blank for measurement of biomass production (OD_{660}).

Energy Dispersive Spectrometry (EDS): To detect any mineral sulfur contamination, 5 ml of sulfur free MSM-S medium was dried at 60°C and room pressure. The dried salts were analyzed using Scanning Electron Microscope (SEM), Equipped with EDS analyzer.

Optimization of Conditions for Desulfurization Activity of Isolate Kho1: To determine the best conditions for DBT-desulfurization activity of isolate KHO1 on MSM-S containing 1 mM dibenzothiophene as the sole source of sulfur, response surface design procedure was carried out using three factors including pH, temperature and rotary shaker round. Effects of these parameters on desulfurization activity at three levels each, as presented in Table 1, were studied [4, 19].

All the reaction mixtures were used duplicate and incubated on a rotary shaker (160 rpm) at 30 °C for 144 h. After 120 h of incubation, 1 ml of the reaction mixtures were centrifuged (4000 rpm, 20 min) and the supernatants were analyzed for DBT-biodesulfurization by Gibb's assay. After analysis of data, optimal conditions were determined [4].

Table 1: Design variables and their levels for optimization of biodsulfurization conditions.

Variable	Level 1	Level 2	Level 3
pH	6.5	7	7.5
Temperature (°C)	28	30	32
Rotary shaker round (rpm)	140	160	180

RESULTS AND DISCUSSION

Dentification of Microorganism: Among isolated strains, one strain, designated KHO1, showed a desirable potent DBT-utilizing ability. Identification of strain KHO1 was on the basis of its morphological and physiological properties and analysis of 16S rRNA gene sequence. Strain KHO1 was an aerobic and Gram-negative bacterium. The isolate was motile, catalase-positive, oxidase-negative. Colonies of the strain on nutrient agar were round, smooth, slightly mucoid and yellow.

Agarose gel electrophoresis of PCR product indicated a sharp band in 1500 bp (Figure 1). For analysis of sequencing result, the sequence of KHO1 was first compared with others in a nonredundant sequence database at the National Center for Biotechnology Information by using the BLASTN program. The BLAST results of the 16S rRNA gene sequence indicate the strain is closely related to *Stenotrophomonas* species. Figure 2 illustrates the phylogenetic tree reconstructing by the NJ method based on 16S rRNA gene sequences. In this tree, isolate KHO1 and *Stenotrophomonas maltophilia* WS12 (Genbank Accession No, HM137729) constituted a branch

Fig. 1: Agarose gel electrophoresis of 16S rRNA-PCR product indicated two sharp bands in 1500 bp for *Rhodococcus erythropolis* IGTS8 and isolate KHO1.

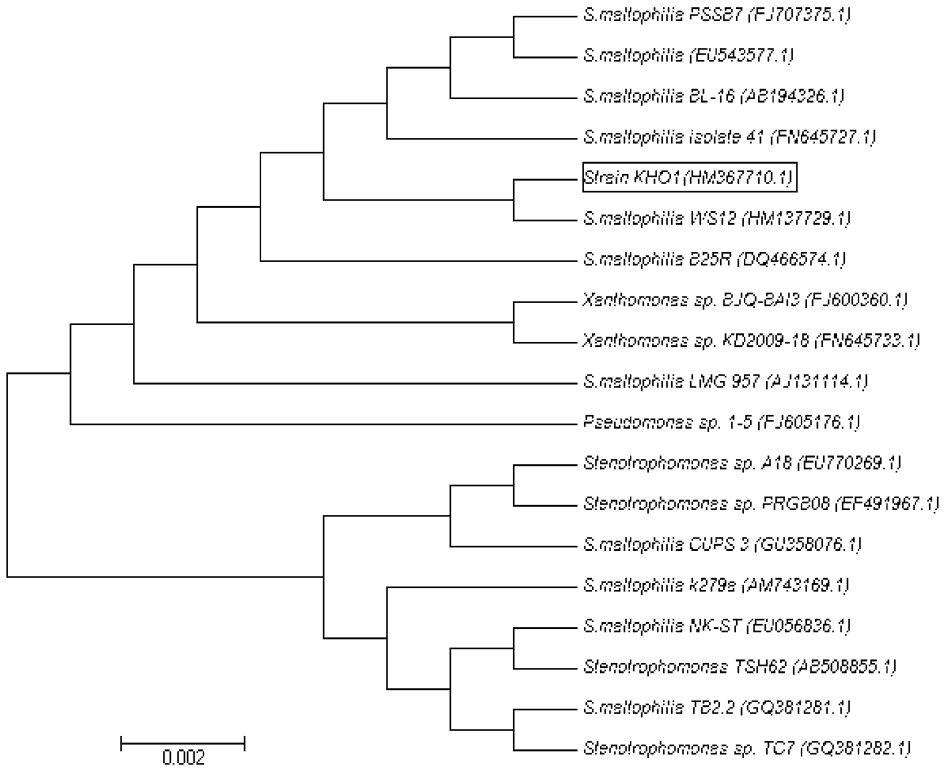

Fig. 2: Phylogenetic tree reconstructing by neighbor joining method of 16S rRNA gene from isolate KHO1 and closely related bacteria. Sequences were obtained from the Genbank sequence database. Accession numbers were shown in parentheses

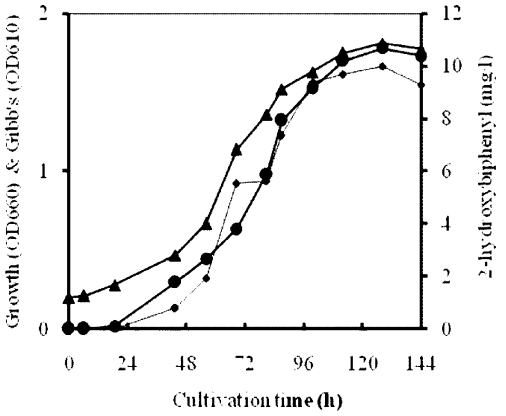

Fig. 3: The typical growth profile and the time course of 2-HBP production by *Stenotrophomonas maltophilia* strain KHO1 in MSM-SD medium. Symbols: (▲), growth (OD_{660}); (●), optical densities at 610 nm of 2-HBP (Gibb's assay); (■), concentration of 2-HPB (HPLC).

of phylogenetic. Many *Stenotrophomonas maltophilia* strains were found to have a high identity with the KHO1 sequence. The homology levels for the 16S rRNA genes of strain KHO1 and its closely related strains were all 99%. Based on combination of these analyses, KHO1 was identified as one strain of *Stenotrophomonas maltophilia*. Considering its isolation from oilfield in Khouzestan province of Iran, we have named it *Stenotrophomonas maltophilia* strain KHO1. The isolate was recorded in the National Center for Biotechnology Information as *Stenotrophomonas maltophilia* strain KHO1 (Genbank Accession No, HM367710).

DBT-desulfurization by Isolate Kho1: The metabolite that accumulated in the medium was identified and quantified using HPLC as 2-HBP. HPLC results and observation of blue-color in Gibb's assay showed that considerable amount of dibenzothiophene desulfurized via 4S-pathway and 2-hydroxybiphenyl formed.

Concomitant with growth, the concentration of 2-hydroxybiphenyl increased. The yield of 2-hydroxybiphenyl was maximum (9.99 mg/l equals 58.69 μM) at the time of the transition from the late exponential phase to stationary phase. The strain showed maximum growth 0.391 g dry cells/l (OD_{660}=1.82) at 128 h of cultivation (Figure 3). The pH decreased from 7.0 to 6.25.

EDS analysis of 5 ml of dried sulfur free MSM-S showed no sulfur moiety by a certainty of 0.01%.

By now, several aerobic DBT-desulfurizing bacteria such as *Rhodococcus* sp. [9], *Nocardia* sp. strain CYKS2 [20], *Rhodococcus erythropolis* D-1 [18], *Gordona* Strain CYKS1 [6], *Microbacterium* strain ZD-M2 [12], *Gordonia alkanivorans* RIPI90A [14] and *Mycobacterium goodie* X7B [3] have been reported to date that able to desulfurize DBT via a sulfur-specific pathway. In particular, *Rhodococcus erythropolis* IGTS8 which was the first strain found able to use DBT through 4S-pathway, has been widely used for BDS and the genes related to the 4S-pathway have been studied [21]. However, according to our knowledge, no studies have ever been reported using *Stenotrophomonas* sp. cells for dibenzothiophene biodesulfurization via 4S-pathway. Therefore, strain KHO1 is perhaps the first *Stenotrophomonas* strain reported to be capable of dibenzothiophene desulfurization, without carbon skeleton cleavage. Evidence of sulfur-specific desulfurization was obtained by the Gibb's assay and high-performance liquid chromatography (HPLC) analysis of samples taken from the MSM-SD. The decision was based on the presence of 4S-pathway in the strain and also the rate and extent of desulfurization. In addition, there have been other reports on the isolation of DBT-desulfurizing bacteria. Species of *Brevibacterium* and *Pseudomonas* utilize dibenzothiophene as the sole source of carbon, sulfur and energy; these bacteria were cleavaged C-C bond of dibenzothiophene. Therefore, they were unsuitable for biodesulphurization because resulting in loss of caloric value of the fuel [1, 6]. *Stenotrophomonas maltophilia* strain KHO1, was desulfurized dibenzothiophene to 2-hydroxybiphenyl. Considering that 2-hydroxybiphenyl has valuable combustible compounds, likely strain KHO1 could be a much more promising biocatalyst for the treatment of fuel oils other than strains that use dibenzothiophene as a carbon and energy source and thus cause a fuel value loss.

Optimization and Effects of Three Parameters on Desulfurization Activity of Isolate KHO1: The amount of 2-HBP production was measured in the samples as a result of each trial conditions, which repeated two times. After performing the experiments, the analysis of data was accomplished and optimum desulfurization conditions for the isolate were determined. Using MINITAB 15 software, the optimum conditions were determined at pH of 7.2, temperature of 29°C and shaking round of 180 rpm. Other levels of these conditions did not significantly influence the desulfurization.

On the basis of the determined optimum desulfurization conditions, the biodesulfurization pattern of DBT was studied and the yield of 2-HBP production was detected. The results were shown, biodesulfurization rate was increased at the optimum conditions; therefore,

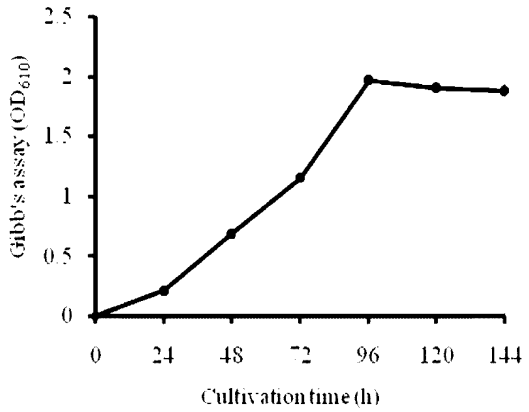

Fig. 4: Biodesulfurization activity of isolate KHO1 at determined optimum conditions. Symbol: (●), optical densities at 610 nm of 2-HBP (Gibb's assay).

these conditions were significantly effective on DBT-desulfurization activity of isolate KHO1 and the high production of 2-HBP was 63.15 μM (OD_{610}= 1.964) at 96 h of cultivation (Figure 4).

The results suggest that *Stenotrophomonas maltophilia* strain KHO1 had a good potential to be applied to the DBT-desulfurization and bio-refining processes.

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