Study of desulfurization rate in *Rhodococcus FMF* native bacterium

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Abstract

The Rhodococcus FMF (R. FMF) native bacterium was isolated from soil contaminated with oil in Tabriz refinery. This bacterium carries three genes sox (dszA, B, C) on its genomic DNA. Preliminary studies have proved that R. FMF strain possess desulfurization activity. In this work soxA and B genes were amplified by PCR, after designing a pair of suitable primers. Analysis of PCR products on agarose gel electrophoresis showed a sharp band in the 2.46 kb region, belonging to soxA and B genes. In addition, dot blotting using the sox4 probe, confirmed the presence of sox operon in the PCR product. The standard Gibbs test was designed for desulfurization activity assay in which production of 2HBP (μM) in four bacterial strains [R. FMF, P. aeruginosa (pESOX4), E.coli DH5α (pESOX3) and E. coli CC118λpir (pESOX4)] was recognized and compared. After 41h of dibenzothiophene (DBT) addition, Pseudomonas aeruginosa (pESOX4) produced 4.8 μM 2HBP and the local R. FMF produced 3.8 μM of DBT. Comparison of 2HBP production in the standard strain of P. aeruginosa (pESOX4) with the isolated R. FMF strain from Tabriz refinery revealed that the later is equally capable of desulfurizing DBT.

Keywords: *Desulfurization, Dibenzothiophene, Rhodococcus*

INTRODUCTION

Crude oil and its distillates contain significant amounts of low-molecular-mass organosulfur compounds such as alkyl and cycloalkyl thiols, alkyl and arylthiophene. This last group includes thiophene itself, benzothiophene, dibenzothiophene, and their alkylated derivatives (Oldfield *et al*. 1997).

Combustion of these compounds result in the release of sulfur oxyacids (SO_2) into the atmosphere, which can cause acid rain (Gallardo *et al*. 1997 and Li *et al*. 1996). Atmospheric SO_2 is a major factor contributing to poor air-quality in the urban environment, producing acid rain and a primary cause of global deforestation; as it lowers the soil pH to levels intolerable for many trees and plants (Maghsoudi *et al*. 2000). Hence, a legislation was sanctioned by Scandinavia, Europe, USA and Japan, which requires progressive annual reduction in the sulfur content of petrochemicals produced by these countries (Oldfield *et al*. 1997).

Currently hydrodesulfurization (HDS) is the method used for desulfurization. In this method, fuel accompanied with hydrogen under high pressure and temperature; and in the presence of a metallic catalyst, reduces organic sulfur and releases H_2S. Although HDS is widely used in the petroleum industries, it has two disadvantages, firstly it is expensive to perform, secondly, some of the compounds containing organic sulfur such as dibenzothiophene (DBT) and their alkylated derivatives are poorly detected with this procedure (Li *et al*. 1996 and Maghsoudi *et al*. 2001).

There are a number of microorganisms which can retain caloric value of crude oil by specific digestion of sulfur-carbon bonds during storage. *Rhodococcus erythropolis* IGTS8 is believed to be the strain of choice for desulfurization studies (Denome *et al*. 1993 and Larose *et al*. 1997). In *Rhodococcus ery-*

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thropolis IGTS8 three desulfurization genes known as *sox (dszA,B,C)* are located on a 120 kb linear plasmid, and regulated by a 4 kb length operon. This gene cluster is responsible for changing DBT to 2-hydroxy biphenyl (2-HBP) and sulfate (Piddington *et al*. 1995).

SoxC codes a 45 KD protein, which is a member of acylCoA dehydrogenase and acts as an initiation factor for oxidation of DBT to DBT sulfone. SoxA and soxB genes code for a 50 KD and a 40 KD protein, respectively. These two proteins are responsible for converting DBT sulfone to 2HBP and sulphate. Conversion of $DBTO_2$ to $HBPsi$ is catalysed by soxA and soxB, which is involved in conversion of HBPsi to hydroxy biphenyl (Denome *et al*. 1994).

Microbiological methods used for desulfurization are associated with certain advantages over the conventional chemical methods provided that a suitable microbial strain is available. In this study we have attempted to isolate a wild type *R. FMF* strain from soil contaminated with crude oil, which can potentially produce 2HBP from DBT.

MATERIALS AND METHODS

Bacterial strains: *Rhodococcus FMF* native bacterium was isolated from soil contaminated with oil, Tabriz refinery, Iran. The *R. FMF* used for study was donated by Iranian Research Organization for Science and Technology (IROST) to this center. It was capable of using dibenzothiophene as a source of sulfure. Positive controls used were *E. coli DH5α (pESOX3)* and *E. coli CC118λpir (pESOX4)* (genetically engineered strains of *Rhodococcus erythropolis IGTS8* containing sox (dsz) operon) and *Pseudomonas aeruginosa (pESOX4)* were gifted from University of Madrid, Spain.

Bacterial culture and separation of genomic DNA: *Rhodococcus FMF* native bacteria was grown in LB culture in presence of kanamycine antibiotic for 3-4 days in a shaker incubator 220 rpm at 30°C and its bacterial precipitate was used for separation of genomic DNA. DNA was purified by proteinase K and phenol/chloroform treatment (Sambrook, 1989).

PCR method: PCR was used for amplification of 2 genes in the sox operon (dszA,B) in *R. FMF*. Proper primers were designed as follows:

5' GAATTCCGCGATGACTCAACAACGAC 3'	forward
5' AAGCTTCTATCGGTGGCGATTGAGGC 3'	reverse

Amplification was done by Corbett Research set using the following program.

Denaturation	94 °C	1 min
Annealing	65 °C	1 min
Extension	72 °C	3 min
For 30 cycles		

Simple and standard Gibbs test: The Gibbs assay and standard Gibbs tests were used for qualitative and quantitative measurement of 2HBP produced, using DBT as a sulfur source in desulfurized bacteria.

For standard Gibbs test, the 5 strains of bacteria were cultured in BSM media containing suitable antibiotics and collected by centrifugation, the obtained precipitate were diluted with HEPES buffer so that an OD of 1 at 610 nm, was obtained for 40 ml of each bacterium. 25 µl stock of 40 mM DBT in acetone was added to each culture, to reach a final concentrate of 25 µm and incubated on a shaker at 220 rpm at 30°C. The first sampling was done after 5 hours and the next after 12 hours. 1 ml of each sample was removed and centrifuged at 8000 g for 3 min and the supernatant transferred to a new vial. 10 µl of 10 mM Gibbs reagent (2,6 dichloroquinon 4-chloroamid) in acetone was added to the samples and stored at 4°C to the end of experiment time.

In order to prepare a standard curve 0, 1, 3, 5, 7, 15, 20 and 30 µM of 2-HBP was diluted with the HEPES buffer and then 10 µl of Gibbs reagent was added to them and incubated at 30°C overnight. Absorbance of each standard was read at 610 nm and the absorption curve obtained (Oldfield *et al*. 1997).

RESULTS

Existence of 4S biochemical pathway genes in *Rhodococcus FMF* was confirmed by PCR. Primarily, using the database from Gene bank and DNA star software, suitable primers were designed to amplify a 2460 bp fragment from *soxA, B*. The dsz operon in *R. FMF* is located on the chromosome and

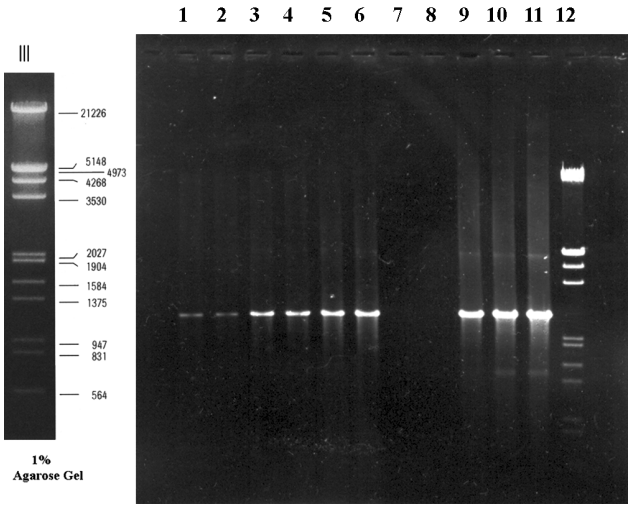

Figure 1. **Amplification of genomic fragments *sox* (*dsz A, B)* by PCR***:*** Lanes 1-4, A, B gene fragments produced from *R.FMF;* 5-6, A, B gene fragments produced from *CC118 pESOX4* as positive control; 7-8, *DH5α PUC18* as negative control; 9-11, A, B gene fragment produced from *R. FMF* after purification and concentration; 12, molecular weight markers.

this was shown as no plasmid was extracted from the sample. Chromosomal DNA was used as template in the PCR mix after extraction and purification. The best results were obtained at 65°C annealing and 3 min extension. Under such conditions, a sharp band in the 2.46 kb region was seen. In this test *CC118 pESOX4* was used as positive control and *DH5α PUC18* was used as negative control (Fig. 1).

Final verification for existence of *sox (dsz A, B)* genes was obtained by dot blot test and labeling of obtained fragment of *SOX4* plasmid by Dig DNA labeling and detection kit which was used as a probe (Fig. 2).

Gibbs test was used for monitoring enzyme activity for desulfurization, this showed that the bacteria used DBT as sulfur source and could change it to 2HBP via 4S pathway as shown in figure 3.

The rate of desulfurization by native strain *R. FMF* was high as compared to other positive controls (Fig. 4). Measurement of produced 2HBP showed logarithmic increase until 41h to reach approximately 3.8 µM whereas, the rates of DBT uptake by *P.aeruginosa (pESOX4), E.coli DH5α (pESOX3)* and *E.coli CC118λpir (pESOX4)* was 4.8, 4.2 and 4.3 µM.

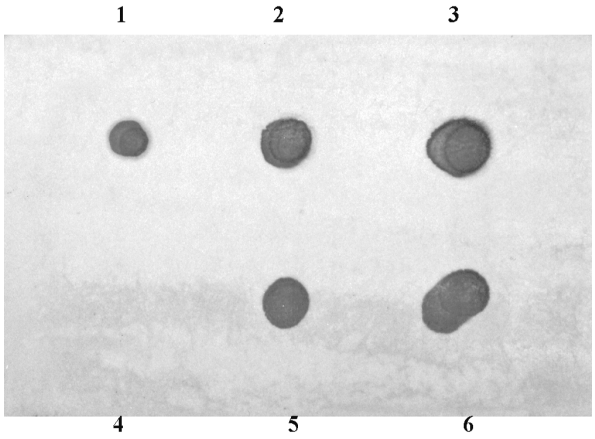

Figure 2. Dot blotting test confirmation of produced genomic fragments from PCR. Spot No. 1 gDNA produced from *R.FMF.* Spot No. 2 and 5, AB gene fragments from *R. FMF* amplified by PCR. Spot No. 3 and 6, AB gene fragments from *pESOX4* plasmid amplified by PCR as a positive control. Spot No. 4, *PUC18* plasmid as negative control.

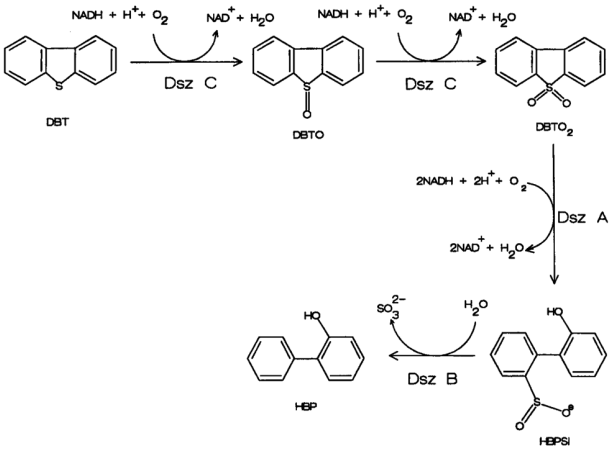

Figure 3. Role of major enzymes in changing DBT to 2HBP in 4S pathway.

DISCUSSION

Rhodococcus FMF native bacterium is a strain, which was isolated from soil contaminated with oil, Tabriz refinery, and can desulfurize DBT as a model molecule. Biochemical studies were done for approving major enzyme activity of these bacteria.

In the beginning, simple Gibbs test on *R. FMF*

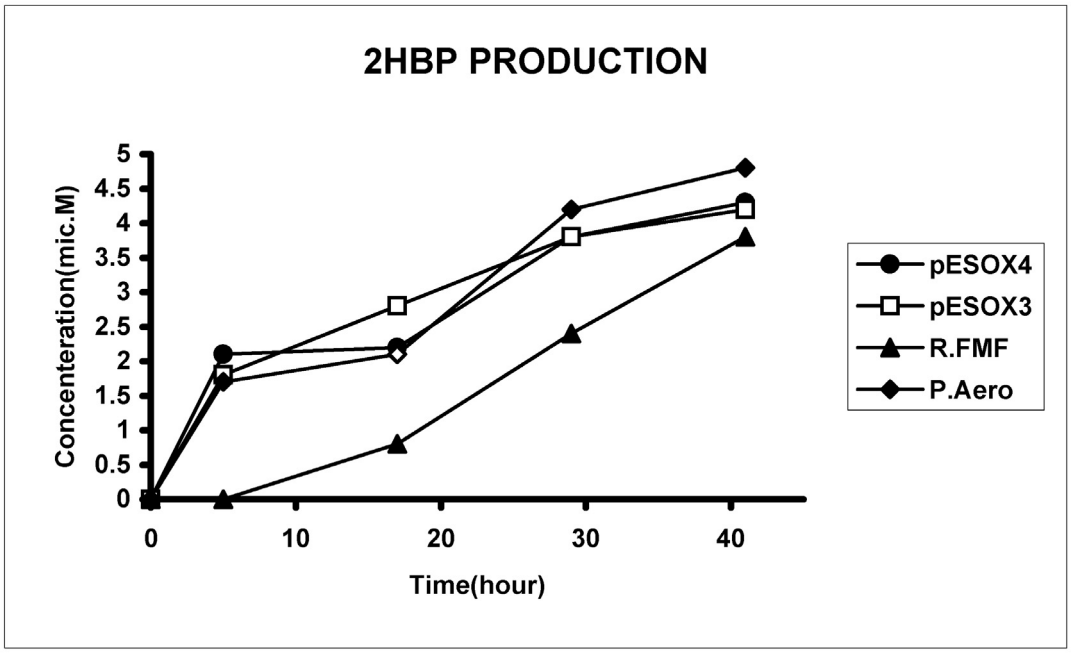

Figure 4. 2HBP production. Activity curve of R. FMF, P. aeroginosa (pESOX4), E.coli DH5α (pESOX3) and E.coli CC118λpir (pESOX4) rate of 2HBP consumption.

strain approved that the bacteria uses DBT as a sulfur source and can convert it to 2HBP via the 4S pathway. Standard Gibbs test was performed by Oldfield and co-workers method in order to determine the produced 2HBP.

In this test, genetically-engineered strains of *Rhodococcus erythropolis IGTS8* [*E.coli DH5α(pESOX3)* and *E.coli CC118αpir (pESOX4)* strains] were used as control strains.

PESOX3 and pESOX4 plasmids were produced by cloning dsz operon into pVLT31 and pBSL118 vectors, respectively, which were under tac promoter control. Produced amount of 2HBP by *R. FMF* has an ascending rate and a maximum consumption of 3.8 µM until hour 41h; where as maximum consumption of DBT by *P. aeruginosa (pESOX4)* is equal to 4.8 µM 2HBP. This result expresses the activity of the enzymatic system responsible for conversion of DBT to 2HBP. Our results indicated that native strain *Rhodococcus FMF* had 2HBP production activity comparable to positive controls.

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