Microtitre Plate Assay for Biofilm Formation, Production and Utilization of Hydroxybiphenyl by *Rhodococcus* sp. Isolated from Gasoline-Contaminated Soil

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Gasoline-contaminated soil from Isfahan, Iran was selected to isolate a bacterium capable of desulfurizing dibenzothiophene (DBT). The isolated strain was named R1 and identified as *Rhodococcus erythropolis* through biochemical tests as well as sequencing of 16S rRNA gene. This strain could efficiently produce 2-hydroxybiphenyl (HBP) from DBT via the 4S metabolic pathway. The highest HBP amount was produced at 2 mM DBT with addition of glucose (10 g l^{-1}), ethanol (3 g l^{-1}), glycerol (2 g l^{-1}) or succinate (10 g l^{-1}) as carbon sources at pH 7. Highest respiration and growth rates were observed by microplate titration on 0.1 mM HBP, and addition of 0.2 mM HBP to glucose (1 g l^{-1}) and DBT (0.3 mM) could inhibite the respiration of the isolate. The isolated strain could grow up to 0.4 mM of HBP when it is used with mineral sulfur as sole sulfur source. To the best of our knowledge this is the first report on a microtiter assay for the production and utilization of HBP by *Rhodococcus.*

Key words: 2-Hydroxybiphenyl, Dibenzothiophene, Microtitre Plate

Introduction

The sulfur-containing compounds present in petroleum are transformed into sulfur oxides by combustion of fuels, causing serious environmental problems such as acid rain (Bustos-Jaimes *et al*., 2003). The thermochemical hydrodesulfurization (HDS) process is presently used in refineries for the removal of sulfur, but some sulfur-containing compounds are recalcitrant to it (Matsubara *et al*., 2001; Tanaka *et al*., 2002). These compounds mainly consist of dibenzothiophene (DBT) and its derivatives and may represent 70% of the sulfur in some crude oil (Monticello, 1998).

Some bacterial species have been isolated which can metabolize DBT via a sulfur-specific degradation pathway, the 4S pathway, and produce 2-hydroxybiphenyl (HBP) as a by-product (Izumi *et al*., 1994; Lee *et al*., 1995; Ohshiro *et al*., 1996; Konishi *et al*., 1997; Kirimura *et al*., 2001). Among these strains, the patented *Rhodococcus erythropolis* strain IGTS8 has been the most extensively studied one (Bustos-Jaimes *et al*., 2003).

HBP is used as hydraulic fluid, plasticized surfactant and flame retardant. Until recently research on biphenyl degradation has focused on Gram-negative bacteria, in particular *Pseudomonas* (Masai *et al*., 1995). Studies on *Pseudomonas* showed less desirable characteristics for use in bioremediation, which shifted the research to *Rhodococcus* (Posada, 2006).

In this study we isolated *Rhodococcus* sp. strain R1 on DBT that biodesulfurized this compound via the 4S pathway and produced HBP. This strain also could grow on HBP. The physiological properties of the isolate were assayed in microtitre plates.

Experimental

Chemicals and reagents

DBT was purchased from Merck-Schuchardt (Hohenbrunn, Germany). HBP was purchased from Fluka (EC No. 2019935). Gibbs reagent or 2,6-dichloroquinone-4-chloroimide was from Merck, Germany. Sterile, 96-well, flat-bottom microtitre plates were used. Other chemicals were of analytical grade and used without further purification and were obtained from Merck, Sigma and Fluka.

Culture media and isolation of bacterial strain

Rhodococcus sp. strain R1 was isolated from gasoline-contaminated soil by a standard culture

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enrichment technique using basal salt medium (BSM) supplemented with 5 g l^{-1} glucose and 5 mM DBT dissolved in ethanol as the sole source of sulfur. BSM contained: 4 g KH_2PO_4, 4 g Na_2HPO_4, 2 g NH_4Cl, 0.2 g $MgCl_2$, 0.001 g $CaCl_2$ and 0.001 g $FeCl_3$ in 1000 ml twice distilled water (Denome *et al*., 1994). The strain R1 was stored in 50% (v/v) glycerol at −80 °C. Each inoculum was grown to the late exponential phase at 30 °C in BSM agar supplemented with glucose (5 g l^{-1}) and DBT (0.3 mM) (Piddington *et al.*, 1995).

Identification of isolated strain

Identification of the isolate was based on colony morphology, microscopic observation of the cell cycle, Gram-stain, acid-fast stain, catalase test, oxidase test, oxygen requirement, motility, the ability to grow on different carbon sources, and in the presence of some inhibitors according to standards for microbial identification in "Bergey's Manual of Systematic Bacteriology". Sequence analysis of the 16S rRNA gene was also performed for identification of the strain R1 according to Bustos-Jaimes *et al*. (2003) by MacroGen Company (Seoul, South Korea).

Microtitre plate assay for desulfurization of different carbon sources

The isolate was grown in BSM supplemented with glucose (5 g l^{-1}) and DBT (0.3 mM dissolved in ethanol) for 48 h. The colonies were washed once in BSM and diluted to an optical density of 0.4 at 600 nm with BSM. Then 50 μl of diluted inoculum were used to inoculate individual wells of a 96-well microtitre plate containing 200 μl sterile BSM broth with different carbon sources including: glycerol (2 g l^{-1}), glucose (10 g l^{-1}) and succinate (10 g l^{-1}). 1 μl of the inoculum was streaked on tryptic soy agar (Difco) to check for contamination. The microtitre plates were incubated for 1–4 d at 30 °C. All treatments were done in triplicate with blank wells. After each day of incubation absorbance was measured with a microplate autoreader or ELISA reader (Stat Fax – 2100, Portland, ME, USA) at 630 nm (Johnson *et al*., 2002; Emtiazi *et al*., 2005).

Microplate titration assay for respiration

One of the most frequently used methods to estimate the dehydrogenase activity in soil or bacteria is based on the use of triphenyl tetrazolium chloride (TTC) as an artificial electron acceptor. TTC is water-soluble, has a redox potential of about −0.08 V and functions as an electron acceptor for several dehydrogenases. Many other flavin-containing enzymes catalyzing specialized oxidation-reductions also reduce TTC to triphenyl formazan (TPF). Nearly all microorganisms reduce TTC to TPF, which can be calorimetrically estimated (Alef and Nannipieri, 1995). To determine the dehydrogenase activity, the isolate was grown at 30 °C on DBT with different concentrations in microtitre plates for 3 d. A 50-μl aliquot of an electron acceptor solution, TTC solution containing 0.2 g TTC in 100 ml Tris buffer (100 mM, pH 7), was added to each well. Absorbance was measured with an ELISA reader at 630 nm immediately after the addition of TTC. Then the plates were incubated at room temperature (20–22 °C) for 3 h. Absorbance was measured with an ELISA reader at 490 nm after 3 h. The absorbance at 630 nm was subtracted from the subsequent reading (after 3 h) to obtain the value of respiration (Johnson *et al*., 2002; Emtiazi *et al*., 2005).

Effect of DBT concentration on growth and HBP production

The microtitre plate was inoculated with different concentrations of DBT in BSM containing 10 g l^{-1} glucose (200 μl of each concentration in each well). Then 50 μl of each inoculum, as previously described, were added to each well. The plates were incubated for 5 d at 30 °C. From each concentration 200 μl in wells were used in triplicate as blank wells. The absorbance of each blank well was subtracted from the well by inoculation with the same concentration of DBT to obtain the true absorbance (optical density at 630 nm) that is equal to the growth rate.

Gibbs assay in microtitre plates

The same microtitre plates with different concentrations of DBT were prepared and after 5 d 40 μl of sodium bicarbonate (pH 8) and then 26 μl of Gibbs reagent were added to the wells. After 30 min at room temperature the absorbance at 630 nm was read. The absorbance of plates read before addition of reagent was subtracted from subsequent reading (after 30 min) to obtain the value that is equal to the HBP production (Emtiazi *et al*., 2005).

Growth, respiration, and HBP production on DBT at various pH values

The microtitre plates were inoculated with BSM supplemented with 0.3 mM DBT and 10 g l^{-1} glucose at different pH values. These assays were performed as previously described.

Growth of isolated strain R1 in HBP

BSM medium with various concentrations of HBP and 10 g l^{-1} glucose supplemented with DBT (0.3 mM) or $MgSO_4 \cdot 7H_2O$ as the only sulfur sources was prepared, and the inoculated plates were incubated at 30 °C for 4 d. The growth was measured each day after incubation with an ELISA reader at 630 nm. A respiration assay was also performed as previously described.

Biofilm formation

The bacteria that have good adhesion to the surface are proper for biofilm production. For detection of the microbial adhesion to the surface in the presence of DBT with various carbon sources and at different concentrations of DBT, the microtitre plates were prepared as described in previous sections and incubated at 30 °C for 3 d. After the growth phase, medium was carefully removed by a pipette, the wells of the microtitre plates were then rinsed three times with 200 μl sterile PBS (phosphate buffered saline). After washing, wells were filled with 96% ethanol for 15 min. The microtitre plates were dried in air and 200 μl of 1% crystal violet were added to the wells for 5 min. Then the microtitre plates were washed with distilled water, dried and finally 200 μl of 33% glacial acetic acid were added to the wells and the absorbance was measured with an ELISA reader at 630 nm (Emtiazi *et al*., 2005).

All experiments were done in triplicate with blanks.

Results and Discussion

Isolation and identification of DBT-utilizing bacterium

The DBT-desulfurizing strains were obtained from gasoline-contaminated soil by a standard culture enrichment technique using DBT as the sole source of sulfur. Individual colonies formed zones of clearing when grown on DBT. One isolated strain was identified as *Rhodococcus* sp. by biochemical tests. This isolate was aerobic, Gram-positive, partially acid-fast (mycolate contained cell wall), non-motile, consisted of non-spore forming cocci or short rods, and produced pink colonies. Microscopic observations showed that the isolate presented a morphologic cycle consisting of cocci and short rod cells. The bacteria were bacilli in young culture and cocci in old culture. The biochemical characteristics are shown in Table I. BLAST of the sequences of 16S rRNA gene from R1 strain showed 97% homology with *Rhodococcus erythropolis* in NCBI site; thus the strain R1 was identified as *Rhodococcus erythropolis* strain R1. This strain was selected to continue this study since it presented the highest HBP production rate.

Table I. Biochemical characteristics of *Rhodococcus* sp. strain R1 isolated from diesel oil-contaminated soil and *R. erythropolis*.

	Rhodococcus sp. strain R1	*Rhodococcus erythropolis*
Catalase	+	+
Oxidase	–	–
Anaerobic growth	–	–
Urease	+	+
Growth in sole carbon source:		
Maltose (10 g l^{-1})	+	+
Manitol (10 g l^{-1})	+	+
Rhamnose (10 g l^{-1})	–	–
Sorbitol (10 g l^{-1})	+	+
Sodium lactate (1 g l^{-1})	+	+
Sodium benzoate (1 g l^{-1})	–	–
Sodium citrate (1 g l^{-1})	+	+
Mycolic acid (acid-fast stain)	+	+

+ Positive interaction in test.
– Negative interaction in test.

Effects of carbon source on growth and DBT desulfurization

Ethanol was used to dissolve DBT before its addition to the culture medium; thus the medium inevitably contained a certain amount of ethanol. We found that the isolate did not show any growth on DBT alone and needs another carbon source in a co-metabolism. This result was also observed by other researches on *Rhodococcus erythropolis* (Bustos-Jaimes *et al*., 2003; Izumi *et al.*, 1994). However, the thermophilic bacterium *Panibacillus* should degrade DBT in a C–C bond-targeted fashion because it utilizes DBT as a sole carbon source (Konishi *et al*., 1997).

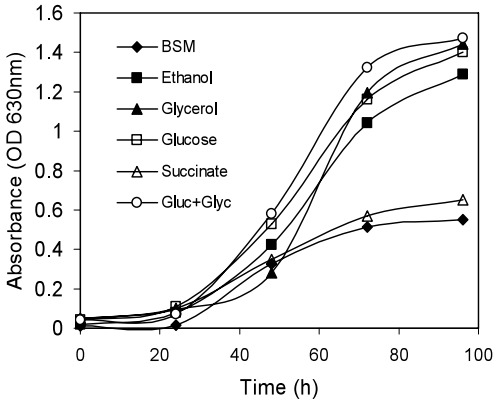

Fig. 1. Growth of strain R1 in the presence of various carbon sources with DBT (0.3 mM) in microtitre plates incubated at 30 °C.

As shown in Fig. 1 additions of ethanol and glucose to BSM-containing DBT as sole sulfur source gave a maximum OD_{630nm} value of 1.5 in the microtiter plate assay. However, addition of ethanol and DBT to BSM gave an OD_{630nm} value of 1.3. The results showed that, for desulfurization of DBT, ethanol or glycerol could be used instead of glucose. Kim *et al.* (2004) showed that *Gordonia* sp. CYKS1 could grow on ethanol as well as glucose and the growth rate on ethanol was observed to be higher than that on glucose. In another study succinate was used as carbon source for desulfurization of DBT as sulfur source by *Rhodococcus erythropolis* Ac-1514D (Zakharyants *et al.*, 2004). These authors also showed that the consecutive passages on the medium containing succinate and DBT as carbon and sulfur sources, respectively, allowed obtaining active cultures with regard to DBT desulfurization.

The results of the Gibbs assay for HBP production showed that carbon sources do not have any significant effect on the production of HBP and equal amounts of HBP were produced from these carbon sources in the isolated bacterium (data not shown). Gilbert *et al.* (1998) also found that mannitol, pyruvate, citrate and glycerol could be substituted by fructose in *Rhodococcus* medium for desulfurization of DBT by *Gordonia* sp. strain 213E. However, Kim *et al.* (2004) showed that about 0.07 mM of DBT was desulfurized in ethanol as carbon source, and when ethanol and glucose were used together, the DBT concentration started to decrease more rapidly.

Effect of DBT concentration on growth and HBP production

DBT is intrinsically a xenobiotic compound, and there have been several reports addressing that a high concentration of DBT shows inhibition of cell growth and desulfurization activity (Yoshikawa *et al.*, 2002; Kim *et al.*, 2004). The effect of initial DBT on the growth rate is shown in Fig. 2. As shown 0.3 mM DBT gave maximum growth (OD_{630nm} 1.7), while addition of 4 mM DBT decreased the OD_{630nm} value to 0.7 and 10 mM DBT inhibited the growth rate. Although the strain R1 has its maximum growth on 0.3 mM DBT, the highest production of HBP was on 2 mM DBT, which was obtained by the Gibbs assay. Yoshikawa *et al.* (2002) showed high desulfurizing activity of *Rhodococcus erythropolis* when DBT was added at 0.1 mM, but above 1 mM DBT the bacterial cell growth was inhibited. The results obtained by Kim *et al.* (2004) showed, that when 4 mM DBT was added, the rate of HBP production was only 4.5 μmol l^{-1} h^{-1}. Such an inhibitory effect of DBT on biodesulfurization has been reported elsewhere (Setti *et al.*, 1999; Ohshiro *et al.*, 1995). The results of this study showed that the strain R1 has a high tolerance to DBT and grows in high concentrations of DBT (up to 4 mM); thus this strain could be used efficiently for desulfurization of DBT.

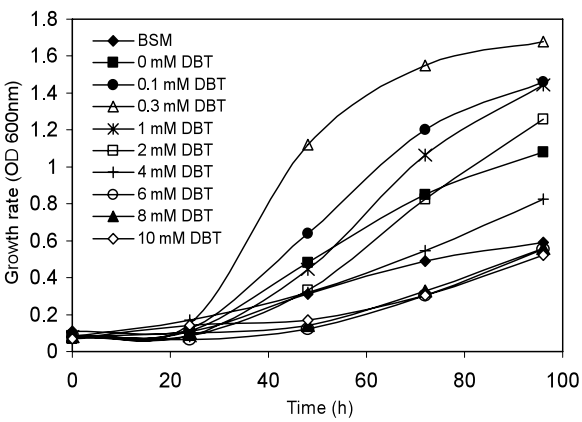

Fig. 2. Growth of strain R1 in the presence of different concentrations of DBT in BSM supplemented with 10 g l^{-1} glucose in microtitre plates incubated at 30 °C.

Effect of pH value

To determine the optimal pH value for cell growth and desulfurization activity of the isolate, the effect of the initial pH value was studied. A

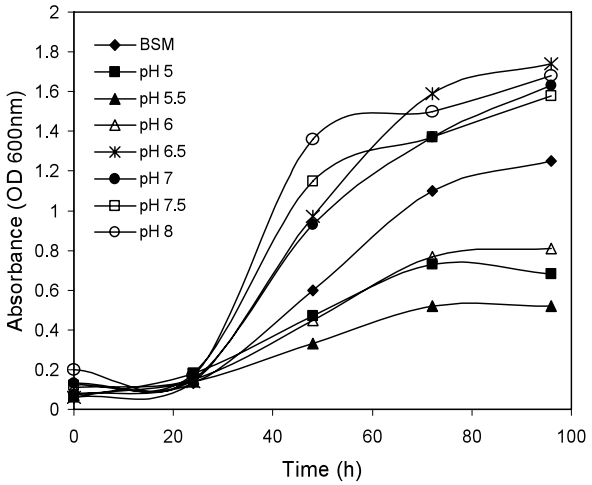

Fig 3. Effect of pH value on growth of strain R1 in the presence of medium containing DBT (0.3 mM) with 10 g l^{-1} glucose in microtitre plates incubated at 30 °C.

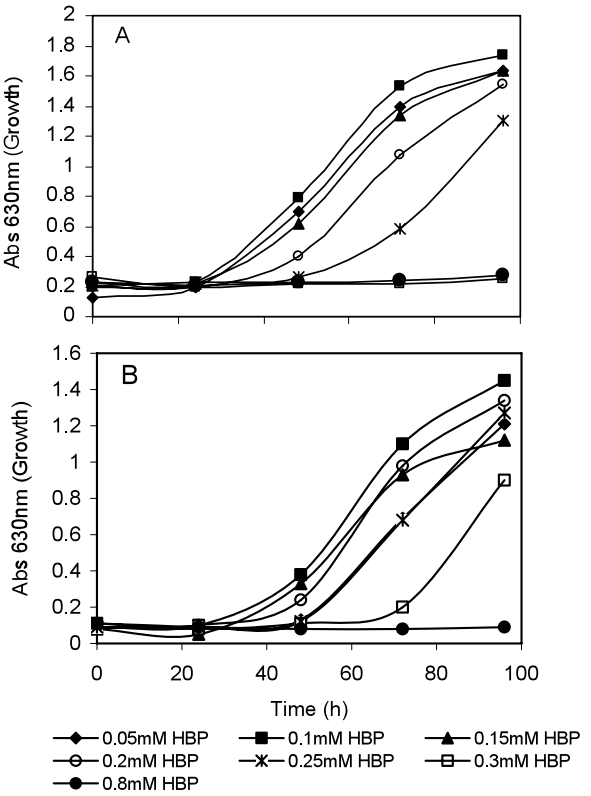

Fig. 4. Growth of strain R1 in the presence of different concentrations of HBP in medium containing (A) 0.3 mM DBT or (B) sulfate as sole sulfur source in microtitre plates incubated at 30 °C.

good cell growth and thus HBP production from DBT was observed at pH 7–8. As it is shown in Fig. 3, pH 6 reduced the growth by 73%. The microtitre plate assay showed that maximum respiration is at pH 8 and raising the pH value from 7 to 8 increased respiration from 1.2 to 1.6 (data not shown). The same results are shown by Kim *et al.* (2004). The maximum HBP production by *Rhodococcus* sp. strain R1 was observed at pH 7 (data not shown) when BSM was supplemented with 0.3 mM DBT and 10 g l^{-1} glucose.

Growth on HBP

It is surprising that the isolate grew on HBP in the presence of other carbon sources such as glucose but could not utilize HBP as the sole source of carbon. Growth of the isolate in different concentrations of HBP is shown in Fig. 4 with DBT (A) or sulfate (B) as sole sulfur sources. When DBT was used as sole sulfur source, 0.2 mM HBP inhibited the growth. However, when sulfate was used as sole sulfur source, the maximum growth was on 0.1–0.25 mM HBP, and 0.8 mM HBP inhibited completely the growth of the isolate. The microtitre plate assay showed that 0.2 mM of HBP in media containing DBT and glucose (10 g l^{-1}) inhibite the respiratory activity (data not shown). The results indicated that 0.3 mM of HBP are toxic to the *Rhodococcus erythropolis* strain R1, when it is grown on DBT as sole sulfur source. However, without DBT, HBP is less toxic and the isolate could tolerate higher concentrations of HBP. Degradation of biphenyl ethylbenzene and phenylacetate by *Rhodococcus* RHA1 was shown by Goncalves *et al.* (2006). In this paper, for the first time, the biodesulfurization of DBT was assayed by microtitre plates, and utilization as well as production of HBP by a Gram-positive strain identified as *Rhodococcus erythropolis* strain R1 was reported.

Biofilm formation

The strain R1 had a strongly hydrophobic surface and differentially adhered to the glass walls of the flask and microtiter plates. Microplate assay titration showed that the adherence to microplate wells is maximal when succinate or glucose are used as sole carbon source (OD_{630nm} 0.93 or 0.85), and with ethanol as sole carbon source the lowest

biofilm production was observed (OD_{630nm} 0.56). Maximum biofilm formation in DBT with different concentrations was seen at 1–2 mM DBT (data not shown). The adherence is important for desulfurization of DBT in a reactor since occasionally the microbial film peels off from the surface or the carrier. Oda and Ohta (2002) used a UV mutant strain to solve this problem.

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