Desulphurization of dibenzothiophene by different bacteria: A case study on model compound

Aniruddha Kumar (aniruddhakumar21@gmail.com)
Babasaheb Bhimrao Ambedkar University https://orcid.org/0000-0003-0553-459X
Asha Lata Singh
Banaras Hindu University
Prakash K Singh
Banaras Hindu University Faculty of Science

Research

Keywords: Dibenzothiophene, Desulphurization, GC-Mass spectrometry, 2-hydroxy biphenyl, Rhodococcus sp.

Posted Date: May 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-29396/v1

Abstract

Bacterial strains are successfully employed to desulfurize dibenzothiophene which may be determined by chromatography, HPLC and GC-Mass spectrometry. Strains of Ralstonia sp ., Pseudoxanthomons sp. and Rhodococcus sp. were used in the present investigation. Only Rhodococcus sp. was able to convert dibenzothiophene (DBT) into 2-hydroxy biphenyl (2-HBP). Gibb's assay blue colour indicates the conversion of DBT into 2-HBP and brown colour indicates complete consumption of DBT by bacteria. After two days of bacterial treatment, there was no further removal of DBT. Conversion of DBT into 2-HBP was monitored through HPLC for six days of entire experiment.

1 Introduction

Dibenzothiophene (DBT) is one of the main contaminants occurring in the organic sulphur compounds in the hydrocarbons of fossil fuels and polycyclic aromatic hydrocarbons (PHAs) (Max Nestler, 1974). It is present as an internal part of the coal matrix, covalently bound in complex form (Marinow et al. 2011). It may be used as a typical model compound for desulphurization studies because of its high boiling point, recalcitrant and resistance to conventional methods of organic sulphur compound (OSC) removal (Mishra et al. 2014; Borzenkova et al. 2013).

DBT is highly toxic and its inhalation leads to lung disorders in human and causes inflammation if it comes in contact with skin (Mishra et al. 2016). During its refining, more amount of sulphur oxides may lead to the acid rain, pollute the environment, biosphere and may cause other health issues (Tailleur et al. 2005 and Ollivier 2005). Heterocyclic compound and its derivatives may persist up to three years after an oil spill and cause severe environmental implications (Gundlach et al. 1983).

Sulphur removal from coal has become a subject of extensive research and it is being given more impetus during last one decade (Singh and Singh 2010; Singh et al. 2012, 2013, 2018; Kumar et al. 2019). The presence of sulphur compounds in coal and oil is a limiting factor for their industrial use because of environmental implications (Demirba 2002). Sulphur dioxide combines with rainwater in the air to form sulphuric acid. Subsequently it falls in form of acid rain which affects agriculture and harms the ecological balance (Ayhan 2004).

Biodesulphurization of organic sulphur compounds is performed in ambient conditions without lowering the calorific value of coal and oil (Soleimani 2007). Strains of several bacteria such as *Pseudomonas*, *Gordonia*, *Paenibacillus* and *Mycobacterium* have been found to be useful for the desulphurization activity. These microorganisms adopt biochemical mechanism, such as Kodama and 4S pathways, to metabolize various aromatic sulphur compounds. Strain converts dibenzothiophene (DBT) into 2-Hydroxybiphenyl (2-HBP). DBT serves as the source of sulphur for biomass and generates 2-Hydroxybiphenyl in the broth and helps in proper growth of the biomass (Kayser et al. 1993; Oldfiel et al. 1997). Several bacteria are reported to desulphurize DBT following 4S pathway like *Rhodococcus erythropolis* IGTS8; (Kilbane 1990). Other DBT desulphurizing microorganisms, mainly mesophilic and

few thermophilic, have also been identified which include *Rhodococcus erythropolis* D-1 (Ohshiro et al. 1999), *Mycobacterium* sp. Strain G3 (Nekodzuka et al. 1997), *Gordonia* sp. CYKS1 (Rhee et al. 1998), *Pseudomonas delafieldii* R-8 (Luo et al. 2003), *Microbacterium* sp. ZD-M2 (Zhang et al. 2005), *Bacillus subtilis* WU-S28 (Kirimura et al. 2001), *Mycobacterium* pheli WU-F1 (Furuya et al. 2001), *Gordonia alkanivorans* RIPI90A (Mohebali et al. 2007), *Pantoea agglomerans* D23W3 (Bhatia and Sharma 2010) and *Sphingomonas subarctica* T7b (Gunam et al. 2013).

In the present paper an attempt has been made to compare the desulphurization activity of the bacterial strains of *Pseudoxanthomonas* sp., *Ralstonia* sp. and *Rhodococcus* sp. These bacteria are able to use various organic sulphur compounds as source of sulphur. In this experiment, Dibenzothiophene has been used as a sulfur compound which is often present in sulphur rich coals.

2 Materials And Methods

2.1 Chemicals and Reagents

DBT (99% purity), 2-HBP (99% purity), Peptone type-1 (M.B grade), Yeast extracts powder (M.B grade) and glucose, were procured from HiMedia Laboratories Pvt Ltd. (Mumbai, India). Potassium dihydrogen phosphate (KH_2PO_4), Ammonium chloride (NH_4Cl), Magnesium chloride hexahydrate ($MgCl_2.6H_2O$), Disodium Hydrogen Phosphate (Na_2HPO_4), Ethyl Acetate and Methanol (CH_3OH) were purchased from Merck Laboratories Pvt Ltd., Germany. All the chemicals were of analytical grade, commercially available and were used without further purification.

2.2 Culture Media and growth conditions

For the growth of bacteria and treatment of DBT, two different media are prepared and the sulphur free Basal salt medium (BSM) is used for treatment having a composition of 0.2 g glucose, 0.24 g Na_2HPO_4, 0.577 g KH_2PO_4, 0.2 g NH_4Cl, 0.02 g $MgCl_2.6H_2O$ per 100 ml of double distilled water and pH maintained at 7.0 ± 0.2. For proper bacterial growth, 0.5 g peptone, 0.5 g yeast extracts and 1.0 g of glucose per 100 ml of double distilled water were added in the nutrient broth. The entire broth medium was sterilized by autoclave. The stock solution of (100 ppm) DBT and 2-HBP were prepared in methanol.

2.3 Bacterial Isolation, Identification and Maintenance

Three different bacterial strains were isolated from different sources. *Pseudoxanthomonas* sp., *Ralstonia* sp. were isolated from hydrocarbon contaminated soil and coal field sample. The detailed process has been discussed by Singh et al. (2012). *Rhodococcus* sp. was isolated from soil sample collected from Chitrakoot District, U.P, India. A gram of powdered sample was added to 10 ml double distilled water and after thorough stirring it was filtered. One ml filtered water was diluted hundred times with double distilled water and was inoculated on basal salt containing solid agar plates at ambient temperature. Colonies were seen to develop on the solid agar plates within twenty four hours, which were picked up and inoculated in liquid basal salt media. The bacterium was identified as *Ralstonia* sp., *Pseudoxanthomonas*

sp. at Institute of Microbial Technology (IMTECH), Chandigarh, India. The identification of *Rhodococcus* sp. was carried out at the Indian Institute of Vegetable Research (IIVR), Varanasi, U.P., India.

3 Analytical Methods

3.1 Growth estimation of bacteria

Bacterial cell growth was monitored by measuring optical density of the culture at 660 nm, using spectrophotometer (UV/VIS), model HITACHI U-2900.

3.2 HPLC analysis

High-performance liquid chromatography (HPLC) was used for the quantitative assay of DBT (retention time – 7.49 min) and 2-HBP (retention time – 1.43 min) in the dodecane phase. HPLC was performed on HPLC Model Water 600 controller, 717 plus autosampler, Waters 2988 photodiode array detector. The separation was performed with water spherisorb® 5.0 m ODS 24.6 mm x 250 mm column. An isocratic elution was carried out with 75% methanol and 25% water at flow rate of 1.5 ml/min and the detection was realized with Waters 2988 photodiode array detector fixed at 254 nm wavelength. HPLC quantification of DBT and 2-HBP was performed by comparing with standard curve using a series of dilution of these pure compounds.

3.3 UV spectrophotometer and Gibbs assay

Ralstonia sp., *Pseudoxanthomonas* sp. and *Rhodococcus* sp. bacterial species were grown in LB medium at 30 °C. The optical density was maintained at 2.0 at 660_{nm}. The bacteria were harvested by centrifugation and re-suspended in BSM medium for desulphurization.

Gibb's assay was performed for determining the conversion of DBT to phenolic compounds by isolates as per Oldfield et al. (1997). 150 µL of the reaction mixture is transferred into a micro plate and mixed with 30 µL of 1M $NaNCo_3$ (pH 8.0). Twenty µL of Gibbs reagent (1 mg/ml in ethanol) solution is added and the reaction mixture is agitated at room temperature for 45 min so that for full colour is developed. The absorbance of the reaction mixture is determined at 595 nm and converted to parts per million based on 2-HBP generated standard curve.

3.4 GC/MS analysis

JOEL-Q1000GC gas chromatograph/mass spectrometer with capillary column of HP190915-433 type (30.0 m × 250 cm × 0.25 cm) has been used with helium as carrier gas with a flow rate of 1.0 ml/min and split ratio of 20:1, the temperature of 250 °C at injection port, EI source, and ionization voltage of 70 eV and temperature of 230 °C at iron source. The temperature was 50 °C at initial pressure for 2 min and was increased to 300 °C at a heating rate of 10 °C/min and then kept for 10 min.

3.5 DBT degradation study

The degradation activity of DBT through bacterial isolates of *Ralstonia* sp., *Pesudoxanthomonas* sp. and *Rhodococcus* sp. was studied in shake-flasks (100 ml) using DBT in methanol as sole sulphur source in aqueous suspension of cells in Basal Salt medium. The flasks in triplicate were incubated for degradation of DBT.

3.6 Metabolite extraction

One ml of the aliquots (BSM + DBT + bacteria) was taken from medium to acidify up to pH ≤ 2.0 and same volume of ethyl acetate was added to it and the mixture was vortexed for 5 min. After 5 min of phase separation, ethyl acetate phase was harvested. Extraction was repeated three times and all ethyl acetate phases were centrifuged (5000 rpm, 10 min). The extracted ethyl acetate sample was analyzed by GC/MS JOEL-Q1000GC.

4 Results And Discussion

4.1 Identification of DBT desulphurization bacterium

The bacterial strains were identified by sequencing of 16S rRNA genes, identified at the 16S rRNA level and 99.8% similar to *Ralstonia* sp., *Pseudoxanthomonas* sp. and *Rhodococcus* sp. After purification and sub culturing of microorganism used for desulphurization of DBT, the growth was observed at OD 660_{nm}. After analysis of desulphurization, only *Rhodococcus* sp. is found to be more efficient to desulphurize organic sulphur. This could be due to the capacity of this bacterium to metabolize hydrophobic substrates (Monticello 2000).

4.2 Growth properties of *Rhodococcus sp., Ralstonia sp., Pseudoxanthomonas sp.* in different concentration of Dibenzothiophene

The growth profile of *Ralstonia* sp., *Pseudoxanthomonas* sp. and *Rhodococcus* sp. shows a short lag phase of growth followed by the exponential growth. The Maximum growth was observed in *Rhodococcus* sp. than other bacteria in 10 ppm DBT concentration. However, in the 15 ppm concentration, there was no growth in the bacterial cells (Fig. 1). This corroborates with the studies of Ohshiro et al. (1996) and Chen et al. (2008) who reported that low concentration of DBT enhances the bacterial growth while high concentration inhibits it because of the toxic effect of 2-HBP which is produced as a result of desulfurization of DBT.

4.3 Utilization of DBT as the sole S-source or as C-source

Gibbs's assay was performed by Gibbs's reagent (2, 6-dichloroquinone-4-chloroimine) (kayser et al. 1993) which is used for the determination of phenolic end products after desulphurization of DBT. Only one of the three isolates could produce blue color after one day of treatment in the form of 2-hydroxly biphenyl product while the rest could not. This indicates that only one isolate has the ability to desulphurize DBT

into 2-HBP or phenolic products. The results is 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).

4.4 Desulfurization Profiles of Dibenzothiophene

When growth density (OD 650 nm) was compared among the three isolates, *Rodococcus* sp. was found to be most efficient throughout the incubation period (Fig. 1). The initial result of desulphurization is shown by the growth of *Rhodococcus* sp. in the growth medium in the broth. After treatment with *Rhodococcus* sp. there is a decrease in the concentration of DBT followed by accumulation of phenolic compounds in medium as confirmed by the production of permanent blue color in presence of Gibb's reagents. *Rhodococcus* sp. used DBT as sole S-source, although other isolates were able to desulfurize DBT to full fill their sulphur requirement. Comparison of bond strengths of C-C to C-S bond in thiophenes indicates that heteroatom bonds are weak in these molecules. Thus C-S bond is most susceptible to cleavage (Beskoski et al. 2008). HPLC analysis was performed to detect the intermediate metabolised product of DBT from the free cell culture supernatants of treated sample. The culture extracts revealed the presence of 2-HBP and DBTS. The desulphurized product of DBT i.e. 2-HBP appeared first after the lag phase of 20 hours and then the concentration continued to increase. Further, the chromatographs of treated sample were compared with the standard ones (Fig. 2a&b). The data of desulphurization by *Rhodococcus* sp. strain was further confirmed by the HPLC profile of the desulfurized products. It clearly shows metabolism of DBT which desulfurized to 2-HBP as evidenced by the large peak of 2-HBP at RT-1.435 (Fig. 3). On the other hand, the treatment of DBT with *Pseudoxanthomonas* sp. and *Ralstonia* sp. could not produce 2-HPB and there is complete removal of DBT (Fig. 4a&b). This is confirmed by HPLC profile of metabolised product of DBT which does not show any peak of DBT in the chromatogram.

4.5 GC/MS study of biodesulphurized end product

The desulphurization activities were studies in medium containing DBT. *Rhodococcus* sp. could desulphurize 10 mg/ml of DBT at room temperature after 1 day of treatment (Fig. 5a). GC/MS analysis of the culture extracts in ethanol confirmed that the end product of DBT desulphurization pathway is 2-HBP. The detected metabolite of DBT desulphurization by *Rhodococcus* sp. has a molecular mass ion (m/z) of 170 and its mass spectrum has a similarity to that of 2-HBP (Fig. 5b). This strain grew well in the medium containing 10 mg/ml DBT as a sole sulphur source. Using GC/MS analysis another product in the broth was detected, and it was identified as 2-MBP (2-methoxybiphenyl) with mass ions (m/z) of 184 (Fig. 5c) which is converted from 2-HBP by methylation. As reported earlier, 2-HBP is toxic to bacterial cells and once the concentration of 2-HBP becomes high, the biodesulphurization of DBT is inhibited (Ohshiro et al. 1996 and Honda et al. 1998). The strain was grown in BSM medium with DBT as sole sulphur source to the stationary phase. The culture broth was acidified to pH 2.5 and extracted with ethyl acetate. There was also production of intermediate compound, in order of DBT, DBTS (Dibenzothiophene sulfone), 2-HBP + sulphate (Fig. 5d). This metabolic product is similar to other bacterial product which also has an ability to desulphurize DBT through selective cleavage of its C-S bonds and by conserving its C-C bonds (Izumi et al.1994; Olfield et al. 1997). *Rhodococcus* sp. Contains DszD enzyme, which is classified as an aromatic sulfinic acid hydrolase and may catalyse the following reaction (Oldfield et al.1997).

$HBPS + H_2O \rightarrow 2\text{-}HBP + SO^{2-}{}_3 + 2H^+$

The GC/MS analysis of the extract shows four main peaks (retention time 19.6, 24.8, 29.65, 32.81), which are not observed in the extract prepared from cells grown on inorganic sulphate (Fig. 5e). The strains of *Ralstonia* sp. and *Pseudoxanthomonas* sp. were grown in BSM medium with DBT as the sole sulphur source and the extract did not generate 2-HBP and other products of desulphurization. The GC/MS study of the extract is indicative of complete consumption of the DBT by the bacterial cells (Fig. 6).

5 Conclusions

Based on the present investigation, following conclusions may be drawn:

1. Treatment of DBT with *Rhodococcus* resulted in a decrease in the concentration of DBT followed by accumulation of phenolic compounds in medium. This was confirmed by the production of permanent blue color in presence of Gibb's reagents. *Rhodococcus* sp. used DBT as sole S-source. The desulphurization was further confirmed by the HPLC profile of the desulfurized products. The metabolism of DBT which got desulfurized into 2-HBP was evidenced by large peak of 2-HBP at RT-1.435. GC/MS analysis of the culture extracts in ethanol also supported the view that the end product of DBT desulphurization pathway is 2-HBP. There was also production of intermediate compound and this metabolic product is similar to other bacterial product which also can desulphurize DBT.
2. The treatment of DBT with *Pseudoxanthomonas* and *Ralstonia* sp. strains did not produce 2-HPB and other products of desulphurization but they completely degraded and removed DBT which confirmed by HPLC profile. The GC/MS study of the extract further indicates complete consumption of the DBT by the bacterial cells.

This study may be useful for the desulfurization of sulphur-rich coals from north-east India which contains varied proportions of organic sulphur.

Declarations

Acknowledgements

The authors thankfully acknowledge the University Grants Commission, New Delhi, India, for financial support in the form of DSK Post doctoral Fellowship Research Grant (No.F.4 – 2/2006 (BSR)/ES/18–19/0031) for the study. The authors (ALS and PKS) acknowledge the Departments of Botany and Department of Geology, Banaras Hindu University respectively for the necessary facilities.

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Figures

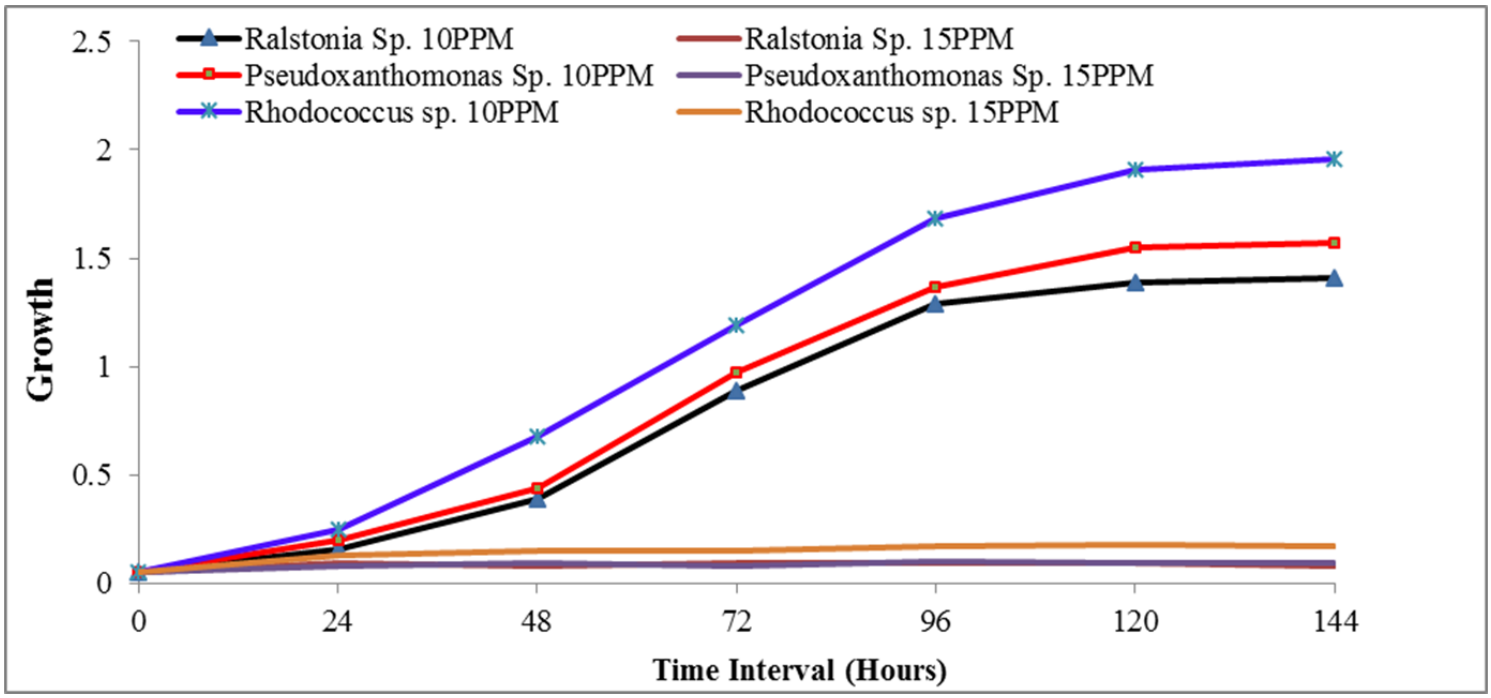

Figure 1

Comparative studies of growth profile by different bacteria in presence of Dibenzothiophene.

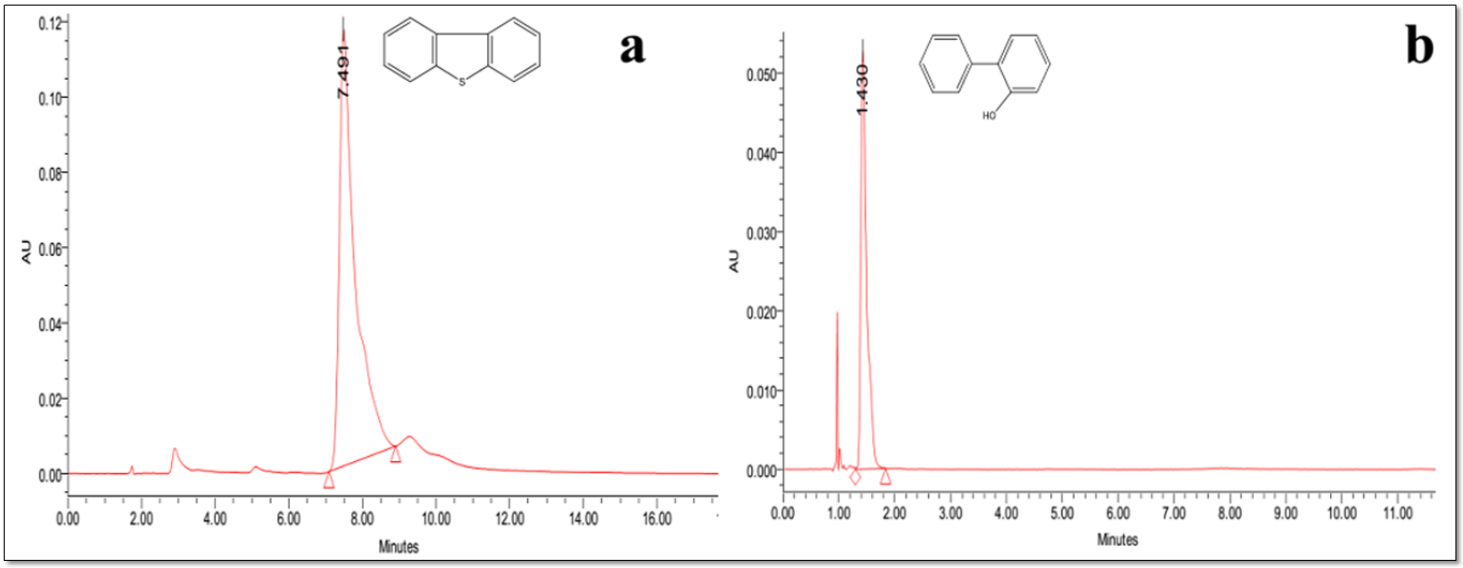

Figure 2

(a) HPLC chromatogram of DBT and (b) chromatogram of 2- HBP.

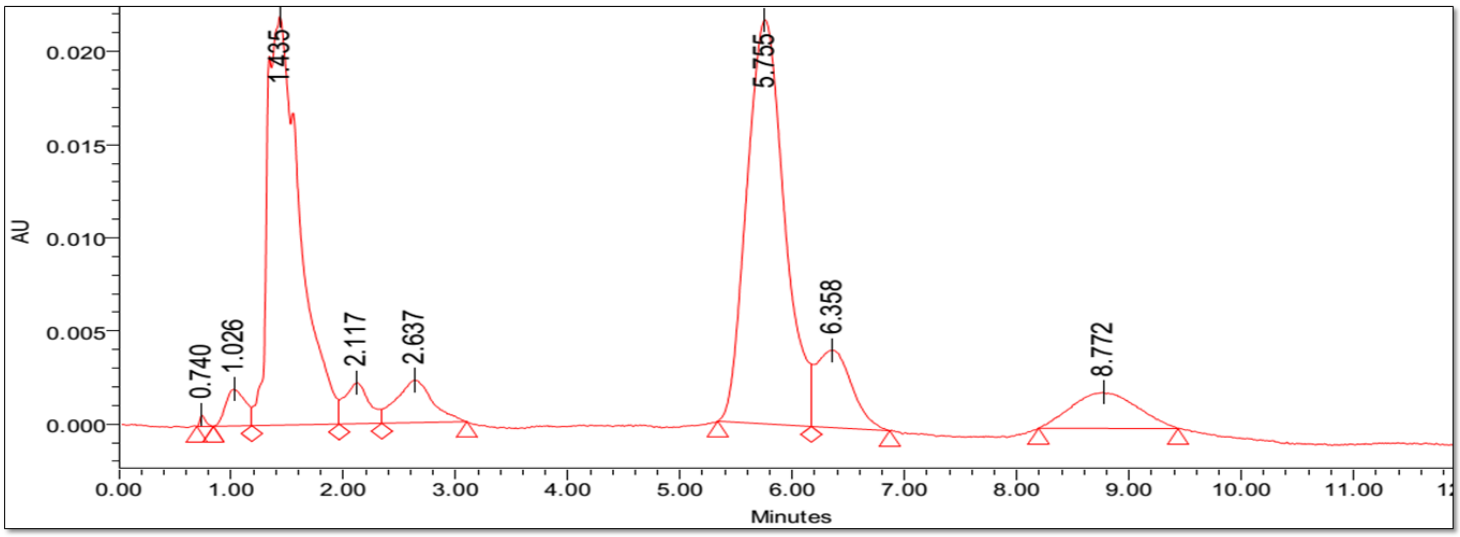

Figure 3

HPLC chromatogram showing desulfurization of DBT after 1 day of treatment with Rhodococcus sp.

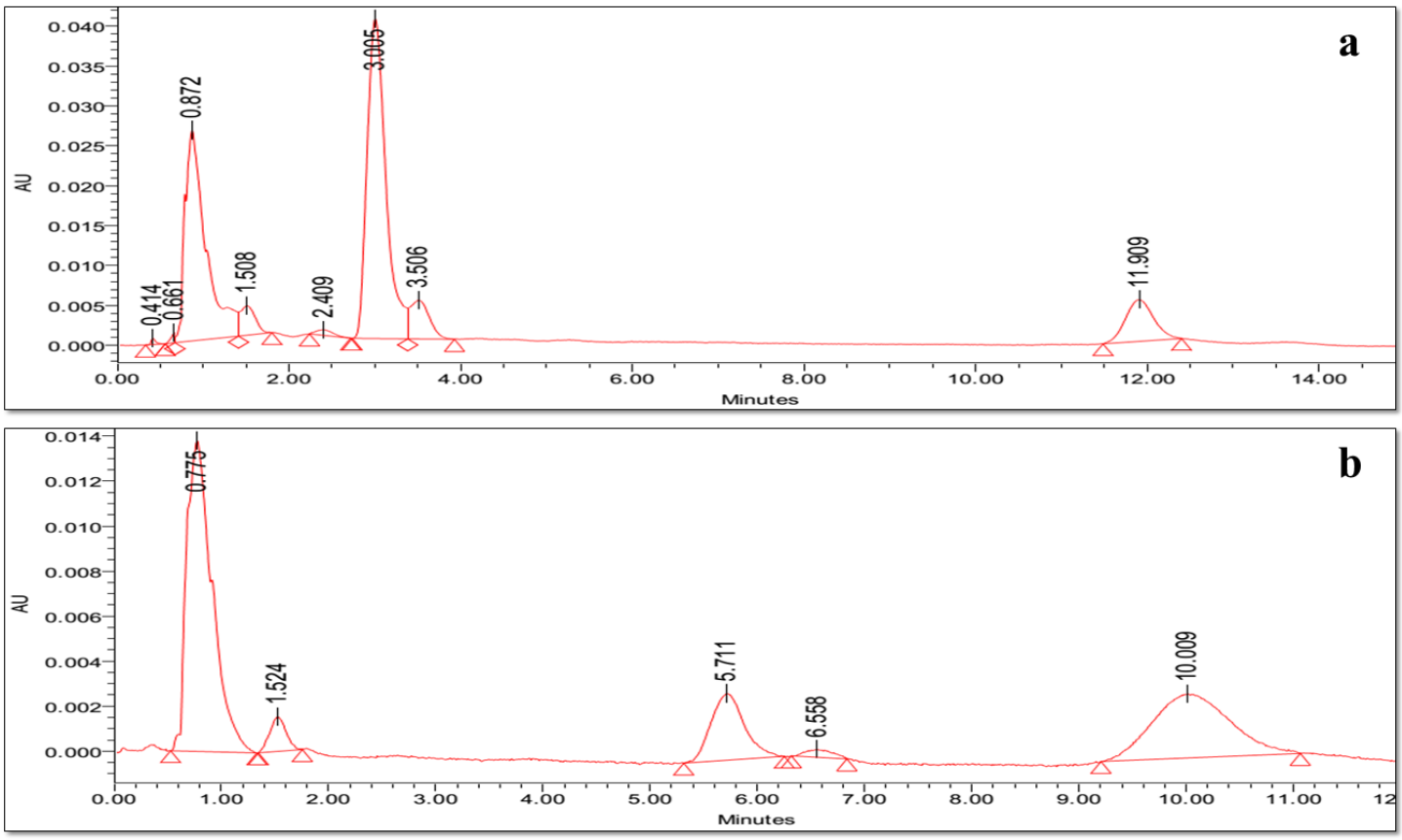

Figure 4

Utilization of DBT as a Source of Carbon by Ralstonia sp. (a) and Pseudoxanthomonas sp. (b).

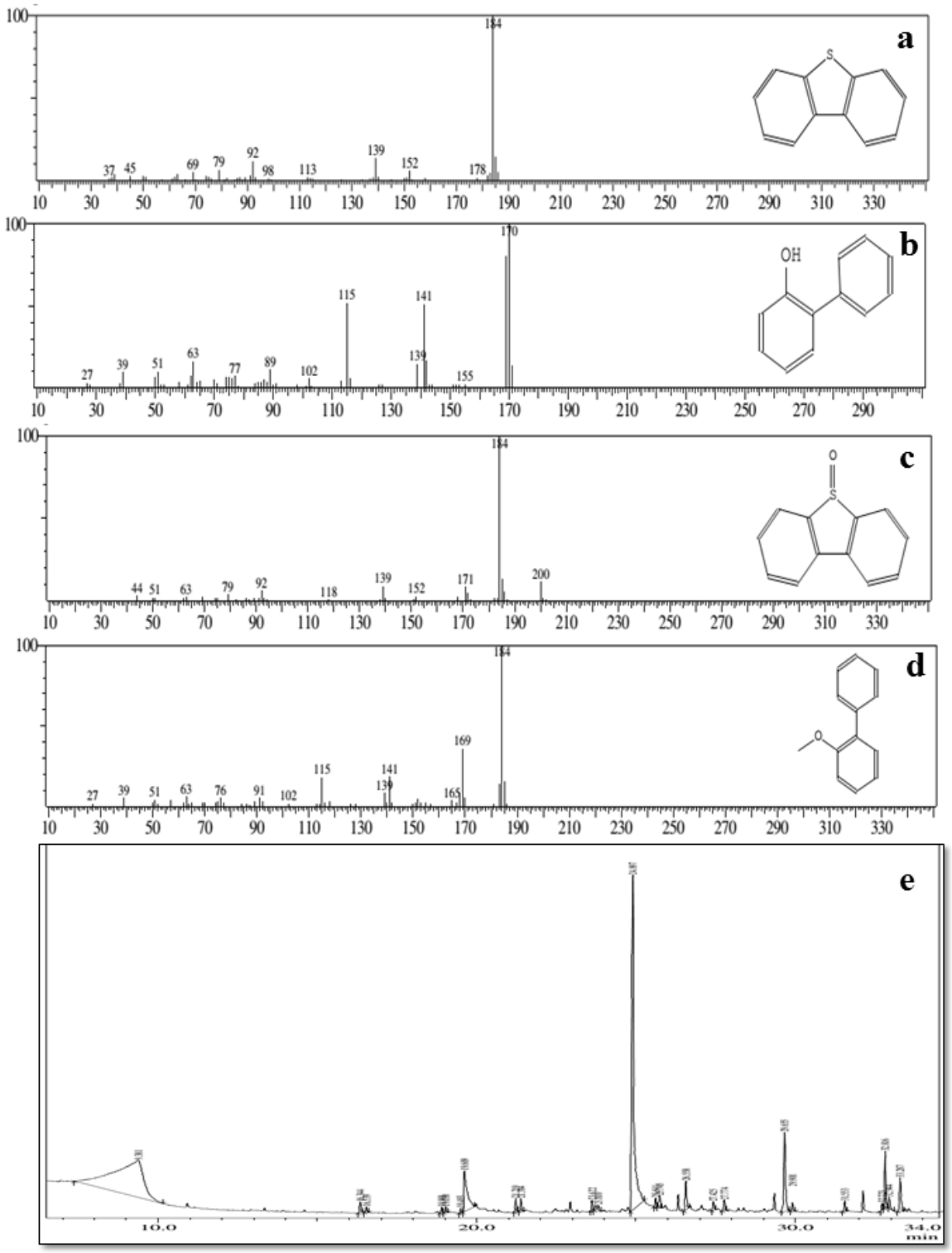

Figure 5

Mass spectrogram of the metabolite formed in the degradation of 10ppm of dibenzothiophene DBT by Rhodococcus sp. a) Mass spectrum of DBT(m/z-184); b) Mass spectrum of 2-HBP (m/z-170);c) c) Mass spectrum of Dibenzothiophene monoxygenase (m/z-184); d) Mass spectrum of 2-methoxy-3,3-dimethyl-biphenyl (m/z-184); e) Total GC Chromatography of the culture extract of Rhodococcus sp.

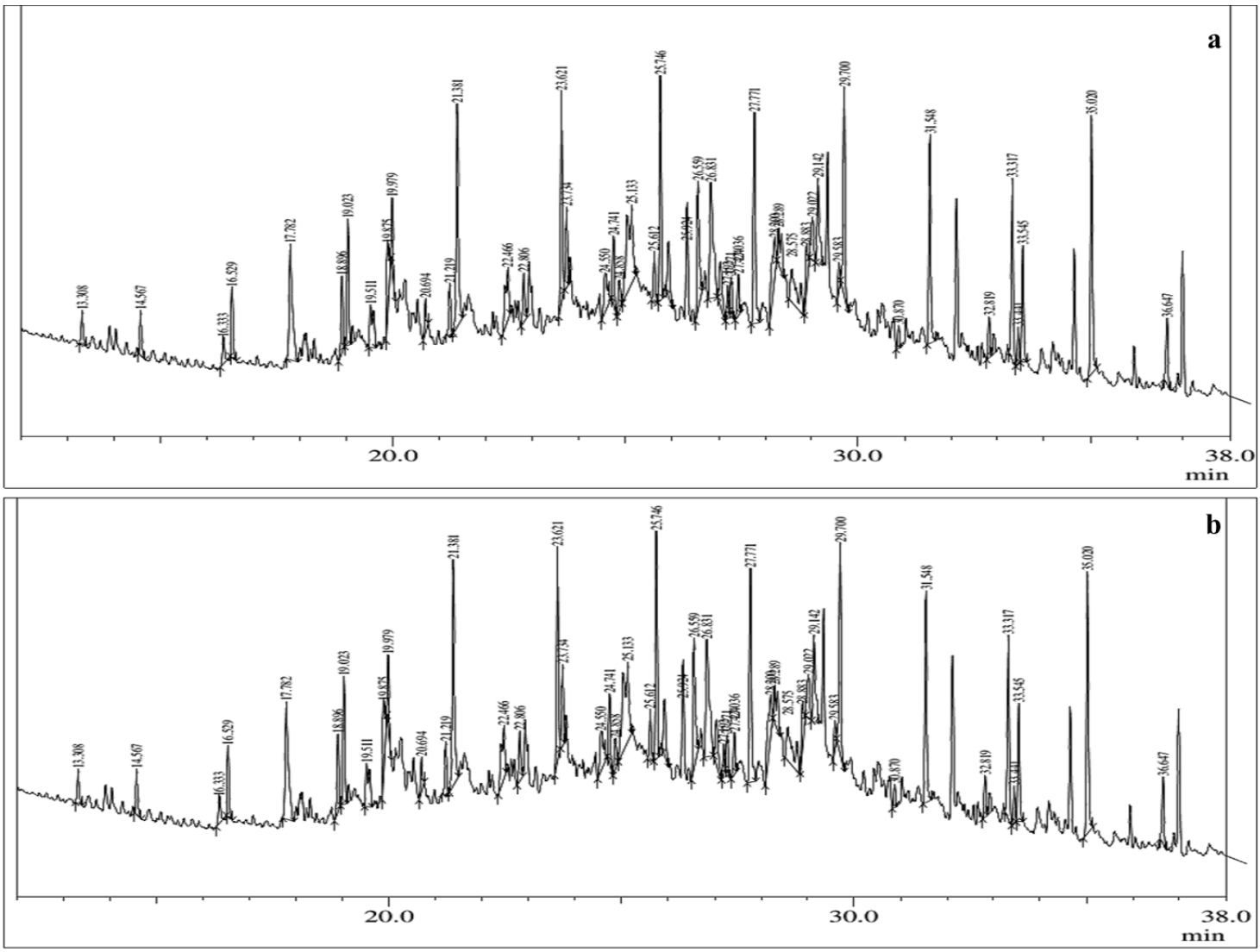

Figure 6

Total GC chromatography of the culture extract of Ralstonia sp. and Pseudoxanthomonas sp.