RESEARCH ARTICLE

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Biological removal of organic sulphur by bacterial strains isolated in Thailand

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ABSTRACT: Three bacterial strains isolated in Thailand, *Bacillus circulans* MS2, *Rhodococcus gordoniae* R3, and *Rhizobium* sp. MS4, were investigated for their potential to remove organosulphur compounds. These strains were shown to be very effective at removing dibenzothiophene, which was not metabolized via a sulphur-specific pathway, as revealed by Gibb's assay. The substrate specificity of each strain was investigated by studying the growth and the ability to remove the growing and resting cells on various substrates. R3 had a broader range of substrate specificity than MS2 and MS4, making it a good strain for bioremediation of oil-contaminated environments.

KEYWORDS: dibenzothiophene, organosulphur compounds, bioremediation

INTRODUCTION

Sulphur is the third most abundant element in a typical crude oil. In addition to elemental sulphur and hydrogen sulphide, over 200 organic sulphur compounds, classified as thiols, sulphides, and thiophenes, have been identified from crude oils. However, the organosulphur compounds that predominate in the higher boiling point fractions are primarily in the form of condensed thiophenes[1].

Dibenzothiophene (DBT) and its derivatives are present in most crude oils and are found to be among the most persistent compounds in the oil-contaminated environment[2]. DBT is therefore widely accepted as a model sulphur heterocyclic compound in biodegradation and biodesulphurization studies[3–7]. Three pathways for DBT metabolism have been reported. The first is the ring-destructive pathway or the Kodama pathway. Through this pathway, one benzene ring of DBT is oxidized and cleaved to yield 3-hydroxy-2-formylbenzothiophene as the major product[8]. The second is the complete pathway, in which DBT is mineralized to carbon dioxide, sulphite, and water[9]. The third is the sulphur-specific pathway or 4S pathway, in which only sulphur is removed from DBT, releasing 2-hydroxybiphenyl as the end product so that decrease in carbon content does not occur[3, 4, 10–13]. This pathway is ideal for biodesulphurization, but is unsuitable for bioremediation of oil-contaminated environments because sulphate ions and other species of inorganic sulphur present in the environment inhibit the expression of the DBT-degrading enzymes[13–15].

To date, several studies have been performed with DBT and alkyl-substituted DBTs using ring-destructive DBT-degrading bacteria. Saftić et al[16] reported that three *Pseudomonas* species metabolize all four isomers of methyl-DBT to form the respective methyl-substituted products through degradation of the unsubstituted benzene ring. The resting cells of *Sphingomonas paucimobilis* strain TZS-7 were shown to be able to degrade DBT and 4,6-dimethyl-DBT via a ring-destructive pathway[6]. DBT degradation of *Arthrobacter* sp. strain P1-1 was also investigated and three catabolic pathways of DBT degradation were proposed[17]. However, it is likely that the bacteria would be exposed to more than one organic compound in the natural environment[18]. Thus, it is imperative to identify microorganisms which are able to use a wider range of substrates.

In this study, bacterial strains which could utilize organosulphur compounds were isolated from oil sludge and soil samples collected from various oil-contaminated sites in Thailand. Strains with good ability to remove DBT were selected and identified. The ability of resting and growing cells of the selected strains in removing several substrates was also investigated.

MATERIALS AND METHODS

Chemicals

Benzothiophene (BT), DBT, 3,4-benzoDBT, 4,6-dimethylDBT (4,6-DMDBT), methyl phenyl sulphide, phenyl sulphide, and phenyl disulphide (>97% purity) were purchased from Sigma-Aldrich. Chromatography grade ethyl acetate was purchased from Merck. All other chemicals were of analytical grade, commercially available, and used without further purification.

Source of bacteria

The sulphur-removing bacteria in this study were isolated from oil sludge and soil samples from oil-contaminated areas in Bangkok and Ratchaburi, Thailand. *Rhodococcus erythropolis* IGTS8 (ATCC 53968, formerly *Rhodococcus rhodochrous*) purchased from the American Type Culture Collection was used as a control strain in Gibb's assay.

Isolation and culture conditions

To culture the bacteria, 10 g of each sample were dispensed into 500 ml Erlenmeyer flasks containing 100 ml of MS medium, a sulphur-free medium which is a modified form of S medium [13] in which the carbon source (10 g/l of ethanol) is replaced by 5 g/l of glucose and the yeast extract is omitted. The medium with bacteria was agitated on an orbital shaker at room temperature (28 ± 2 °C) for 1 h. Then, 1 ml of the aliquot was inoculated into a set of test tubes each containing 5 ml of MS medium with 1 mM of one of the following sulphur sources: BT, DBT, 3,4-benzoDBT, 4,6-DMDBT, methyl phenyl sulphide, phenyl sulphide, and phenyl disulphide. The bacteria were then incubated on the shaker at 150 rpm at 30 °C. Successive subcultivations were carried out every 4 days. Single colony isolation from the enrichment cultures was performed by plating appropriate diluted culture samples onto LB medium [19]. Each isolate was tested for ability to remove DBT, and those with a high activity were selected for characterization.

Identification of selected strains

The selected bacterial strains were characterized by microscopy, Gram staining, and 16s rDNA sequencing. The 16s rDNA sequencing of the bacteria was performed at the Mahidol University - Osaka University Collaborative Research Center for Bioscience and Biotechnology. The DNA sequences (500–1500 nucleotides) were then compared to GenBank sequences using the NCBI BLAST program. A phylogenetic tree was constructed using PAUP 4.0 b1 [20].

Growth of the selected bacteria on various substrates as a sulphur source

The abilities of the selected strains to metabolize BT, DBT, 3,4-benzoDBT, 4,6-DMDBT, phenyl sulphide, methyl phenyl sulphide, and phenyl disulphide, were investigated. The bacterial colony was inoculated into a set of tubes, each containing MS medium with 1 mM of substrate or Na_2SO_4 (positive control). Another set of tubes containing the medium without a sulphur source was used as a negative control. All test tubes were incubated on the shaker at 150 rpm at 30 °C. After 3 days of incubation, 0.1 ml aliquots of bacteria were transferred into a new set of tubes and then incubated for another 7 days. The growth of the selected bacteria as compared to the strain IGTS8 was monitored by measuring the absorbance at 600 nm.

Gibb's assay

To find bacteria that can selectively remove sulphur from DBT and its derivatives, Gibb's assay [21] was used for testing the presence of 2-hydroxybiphenyl, the end product of DBT desulphurization via the sulphur-specific pathway. The Gibb's assay in this study was carried out as follows: the pH of the sample was adjusted to 8.0 using 10% (w/v) Na_2CO_3. Then, 50 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 (10000 rpm, 4 °C, 20 min) and the absorbance of the supernatant was measured at 610 nm. The assay was verified by using *R. erythropolis* strain IGTS8 with DBT as the substrate in a positive control experiment.

Removal of various substrates by growing cells

The bacterial cells (6–10 mg dry wt) were placed in 500 ml Erlenmeyer flasks each containing 50 ml of MS medium with 1 mM of one of the substrates. The flasks were then incubated on the shaker at 150 rpm at 30 °C. Samples were drawn from the flasks after 0, 5, and 72 h of incubation. The concentrations of the substrates were analysed by gas chromatography-mass spectrometry (GC-MS). The MS medium containing each substrate without inoculation was used as a control.

Removal of various substrates by resting cells

The bacterial cells (20–32 mg dry wt) were placed in a set of test tubes each containing phosphate buffer with 0.5 mM of each substrate. The test tubes were then incubated on the shaker at 150 rpm at 30 °C. After 5 h

of incubation, the reactions were stopped by acidification (pH 2.0). The quantities of the substrates were analysed by GC-MS. The reaction mixtures without the bacteria and with the sterilized cell suspension were used as controls.

Analytical methods

The reaction mixtures were extracted twice with an equal volume of ethyl acetate containing 1 mM carbazole as an internal standard. A small amount of anhydrous Na_2SO_4 was added to the ethyl acetate extracts to remove residual water. All samples were evaporated under a stream of nitrogen gas. The quantities of organosulphur compounds and their metabolites were determined using an Agilent gas chromatograph (model 6890N) coupled to an Agilent mass selective detector (model 5973). The chromatograph system was equipped with a DB-5MS column (0.25 mm $\times$ 30 m $\times$ 0.25 µm). The injector and detector temperatures were 220 and 230 °C, respectively. The oven temperature was started at 80 °C, then programmed to increase to 190 °C at a rate of 10 °C/min, followed by a second ramp at 5 °C/min to 215 °C, and a third ramp at 15 °C/min to 230 °C and then held for 18 min.

The growth of the bacteria was determined by monitoring the absorbance at 600 nm using a UV spectrophotometer (Aquarius, Cecil model CE 7200).

All data shown are the mean values of data collected from triplicate experiments.

RESULTS AND DISCUSSION

Selection of DBT degrading bacteria

In this study, 25 bacterial strains which could grow in the media containing organosulphur compounds as a sole sulphur source were isolated. Among them, 3 bacterial strains (MS2, MS4, and R3) showed a high ability to degrade DBT in preliminary tests, so they were chosen for further experimentation.

Based on 16s rDNA sequence homology and the affiliation shown in Fig. 1, MS2, MS4, and R3 were identified as *Bacillus circulans*, *Rhizobium* sp., and *Rhodococcus gordoniae*, respectively.

Growth of the selected bacteria on various substrates as a sulphur source

All of the organosulphur compounds listed in Table 1 were examined for their ability to serve as a sole sulphur source for growth of the selected strains. The data revealed that benzothiophene (BT), phenyl sulphide, and phenyl disulphide were unable to support growth of any bacteria. Similar results were also obtained from the study of Kayser et al[21]. It was found that R3 could utilize more substrates than MS2 and MS4.

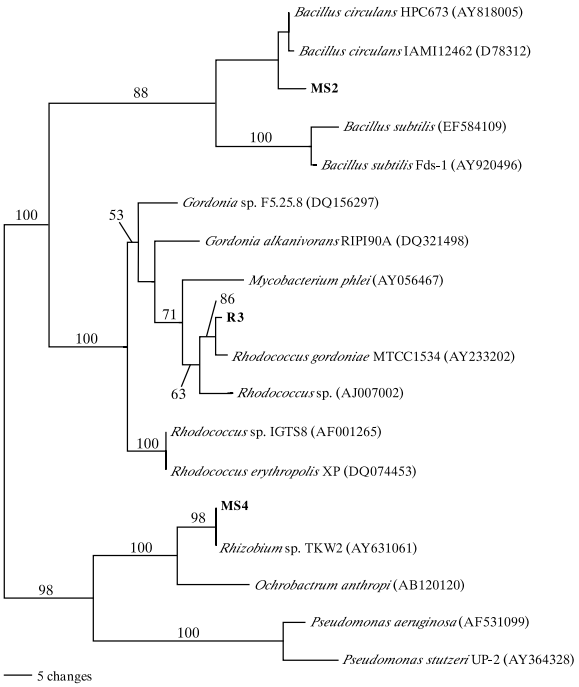

Fig. 1 Phylogenetic tree based on partial sequences of 16s rDNA of the selected strains (MS2, MS4, and R3) and reference strains from GenBank. Numbers at the nodes represent bootstrap values based on 1000 samplings. Only bootstrap values >50% are shown. The scale bar indicates 5 changes per nucleotide.

Table 1 Growth of the bacteria on various substrates as a sulphur source.

Substrate	MS2	MS4	R3
BT	-	-	-
DBT	+	-	+
3,4-benzoDBT	+	+	+
4,6-DMDBT	+	+	+
Methyl phenyl sulphide	-	-	+
Phenyl sulphide	-	-	-
Phenyl disulphide	-	-	-

+ indicates growth of bacteria observed after 7 days of incubation.
- indicates no growth of bacteria observed after 7 days of incubation.

Gibb's assay

To check whether the selected bacteria were able to metabolize organosulphur compounds via the sulphur-

specific pathway, Gibb's assay was used. The results indicate that the product of DBT desulphurization via the sulphur-specific pathway (2-hydroxybiphenyl) was absent from the cultures of the selected strains. Moreover, the phylogenetic tree shown in Fig. 1 revealed that none of the selected strains clustered together with the sulphur-selective desulphurizing bacteria (*Bacillus subtilis* Fds-1, *R. erythropolis* XP, *Rhodococcus* sp. IGTS8, *Gordonia* sp. F5.25.8, *G. alkanivorans* RIPI90A, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, and *P. stutzeri* UP-2). It can be concluded that none of the selected bacteria could desulphurize DBT via the sulphur-specific pathway. MS2 belongs to the genus *Bacillus* (Fig. 1) which was previously considered to be an oil-degrading bacteria[22,23]. *Rhodococcus gordoniae*, which is capable of degrading phenol[24] and trichloroethylene[25], was the closest relative to the strain R3. In addition, *Rhizobium* sp. TKW2, which was clustered with the strain MS4, has been reported to be able to degrade a metal lubricant[26]. These relations suggest that all the selected strains may convert DBT via a ring-destructive pathway.

Removal of various sulphur substrates by growing and resting cells of the selected strains

Phenyl sulphide could not be degraded by any bacterial strains (Figs. 2 and 3). This has also been found to be the case with other bacteria[21,27]. Growing bacterial cells were unable to metabolize BT and phenyl disulphide (Fig. 2). However, a decrease in the concentration of BT and phenyl disulphide was observed in the reactions containing resting cells of all strains (Fig. 3). In previous reports, BT was found to inhibit the growth of several bacteria[28–30] and could not be metabolized by several bacteria[3,27,31]. In the reaction with no inoculation (control), the BT concentration was also found to be reduced. Therefore, the decrease in BT concentration in the reactions may be the consequence of a physical loss of BT (such as volatilization). Such loss was also reported in a study by Fedorak and Grbić-Galić[30].

Phenyl disulphide is not volatile and therefore the decrease in its concentration could be due to bacterial activity. The conversion of organosulphur compounds into oxidized forms by some bacteria has been reported[8,32–34].

The extent of substrate removal by R3 in the reaction with methyl phenyl sulphide was high. Although a slight loss of the substrate in the control was observed, the results from the growth studies and sulphur removal experiments indicate that R3 could metabolize and utilize methyl phenyl sulphide as a sulphur source.

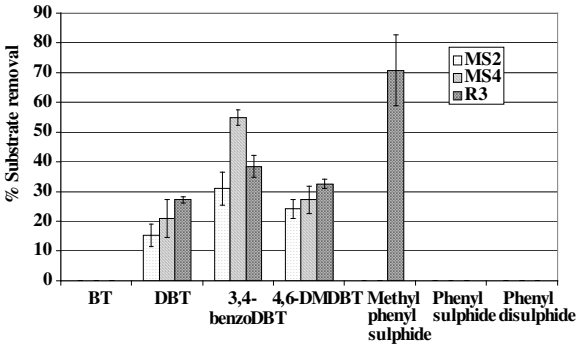

Fig. 2 Percentages of substrate removal by growing cells of the selected strains after 72 h of incubation. Bars represent the standard deviations of means.

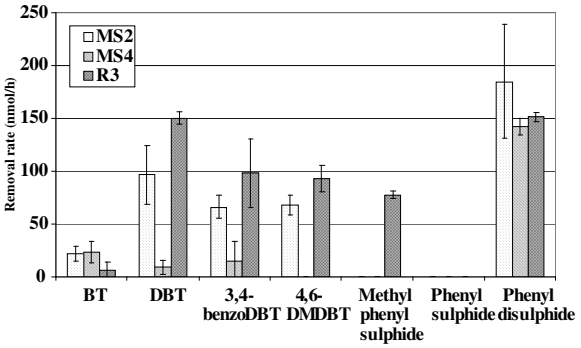

Fig. 3 Rate of substrate removal by resting cells of the selected strains during 5 h of incubation.

For DBT, 3,4-benzoDBT, and 4,6-DMDBT, the results obtained from the reactions with growing cells and resting cells show that R3 had the highest removal ability when compared with those of MS2 and MS4 in almost all of these three compounds except in 3,4-benzoDBT with growing cells where MS4 did better. The removal extent of R3 on these three substrates are in descending order as follows: 3,4-benzoDBT, 4,6-DMDBT, and DBT. Lower removal extent of MS4 was obtained with the resting cells than with the growing cells. Similar results were obtained from the reports on *Gordonia* sp. ZD-7, which was attributed to more desulphurization enzymes and co-enzymes produced during the growth of the bacteria[35].

Based on the data from this experiment, R3 was the best strain for sulphur removal and may have potential in sulphur removal from mixtures of petroleum wastes containing a wide range of organosulphur substrates. However, the rate of organosulphur removal has to be increased and its metabolic pathway needs to be investigated further. Although GC-MS is the most

frequent analytical tool for identifying transformation products, this method has limitations when it comes to analysing very polar, less volatile and thermally unstable compounds[36]. In several studies[31,34,37,38], besides GC-MS, other analyses (e.g., nuclear magnetic resonance spectroscopy) were also used for identifying the metabolites from organosulphur compounds and their derivatives. Use of alternative techniques are needed to identify potential metabolites.

At present, the R3 strain is held in the preserve at the Department of Biology laboratory for possible commercialization with future collaborators.

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