

Biodesulfurization of Dibenzothiophene and Its Derivatives Using Resting and Immobilized Cells of *Sphingomonas subarctica* T7b

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The desulfurization ability of *Sphingomonas subarctica* T7b was evaluated using resting and immobilized cells with dibenzothiophene (DBT), alkyl DBTs, and commercial light gas oil (LGO) as the substrates. The resting cells of *S. subarctica* T7b degraded 239.2 mg of the initial 250 mg of DBT/l (1.36 mM) within 24 h at 27°C, while 127.5 mg of 2-hydroxybiphenyl (2-HBP)/l (0.75 mM) was formed, representing a 55% conversion of the DBT. The DBT desulfurization activity was significantly affected by the aqueous-to-oil phase ratio. In addition, the resting cells of *S. subarctica* T7b were able to desulfurize alkyl DBTs with long alkyl chains, although the desulfurization rate decreased with an increase in the total carbon number of the alkylated DBTs. LGO with a total sulfur content of 280 mg/l was desulfurized to 152 mg/l after 24 h of reaction. Cells immobilized by entrapment with polyvinyl alcohol (PVA) exhibited a high DBT desulfurization activity, including repeated use for more than 8 batch cycles without loss of biodesulfurization activity. The stability of the immobilized cells was better than that of the resting cells at different initial pHs, higher temperatures, and for DBT biodesulfurization in successive degradation cycles. The immobilized cells were also easily separated from the oil and water phases, giving this method great potential for oil biodesulfurization.

Key words: Biodesulfurization, dibenzothiophene, resting cells, immobilized cells, *Sphingomonas subarctica*

Fossil fuels such as light gas oil (LGO) contain various heterocyclic organosulfur compounds, including alkylated forms of dibenzothiophene (DBT) and benzothiophene (BT). Derivatives of these two compounds are the major sulfur compounds in certain types of crude oil [16] that cannot be completely desulfurized using a hydrodesulfurization process with chemical catalysts. However, a biodesulfurization process using microbial biocatalysts capable of desulfurizing hydrodesulfurization-resistant sulfur compounds, such as alkylated DBTs, is suitable for this purpose, and several studies on BT and DBT desulfurizing bacteria and their enzymes involved in DBT desulfurization have been reported [7, 29].

Almost all desulfurizing bacteria are Gram-positive, such as *Rhodococcus* spp. [8, 13, 18, 25, 35], *Paenibacillus* sp. [19], *Gordonia* spp. [2, 31], *Corynebacterium* sp. [30], *Mycobacterium* spp. [14, 28, 36], and *Lysinibacillus* sp. [1] and it has been ascertained that these bacteria have the same desulfurization pathway that produces 2-hydroxybiphenyl from DBT [5, 13, 29]. Notwithstanding, there have been a few reported cases of Gram-negative desulfurizing bacteria, such as *Desulfovibrio* sp. [17], *Pseudomonas* spp. [4, 10, 24], and *Sphingomonas* spp. [9, 15, 23], which can degrade DBT, BT, and their derivatives via a sulfur-specific pathway (4S pathway). Growing cells of *Sphingomonas subarctica* T7b, a Gram-negative bacterium isolated from soil in Toyotomi, Hokkaido, Japan, were shown to desulfurize DBT, alkyl DBTs, and alkyl BTs including an alkyl long chain [9].

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Konishi *et al.* [19] reported that since the biotransformation by growing cells is usually conducted in complex growth media, this can interfere with the product analysis, owing to the presence of a complicated mixture of other metabolites produced by the biocatalysts and medium components. Thus, to avoid these problems, resting or immobilized cells are used for the quantitative analysis of specific desulfurizing activities.

Accordingly, this study evaluated the desulfurization activity of resting and immobilized cells of *S. subarctica* T7b in a two-phase system of a phosphate buffer or saline and *n*-tetradecane containing DBT and its derivatives, and examined the effects of several parameters on the rate of desulfurization. The ability of *S. subarctica* T7b to desulfurize LGO in a resting cell reaction system was also evaluated.

MATERIALS AND METHODS

Chemicals

The DBT was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and the alkylated DBTs were synthesized by Nard Institute Ltd. (Hyogo Japan). The light gas oil was kindly supplied by the Petroleum Energy Center, Shizuoka, Japan. The tetradecane and sodium alginate were purchased from Wako Pure Chemical Co., Osaka, Japan, and the polyvinyl alcohol was supplied by Aldrich (USA). All the other reagents were of available analytical grade.

A concentrated fraction of aromatic compounds (CA) was prepared by fractionation of the commercial light gas oil [9, 32].

Bacterial Strains and Cultivation

The *S. subarctica* T7b was grown in a mineral salt sulfur-free (MSSF) medium (pH 7) with CA as the sole sulfur source, as reported in our previous paper [9]. The cultivation of the seed culture was carried out at 27°C for 4 days in a test tube containing 5 ml of the MSSF medium and 10 µl of CA with reciprocal shaking at 273 rpm. To produce a large quantity of cells, the *S. subarctica* T7b was cultivated in 500 ml Sakaguchi flasks containing 100 ml of the MSSF medium with 50 µl of CA as the sole source of sulfur at 27°C for 3–5 days [9].

Preparation of Resting Cells

The cells were harvested after 4 days of incubation, except when evaluating the effect of different harvest times on the desulfurization activity. The cells were collected by centrifugation at 10,000 ×g for 20 min at 4°C. The harvested cells were washed twice with 100 mM potassium phosphate buffer (pH 7) and then resuspended in the same buffer to adjust the cell concentration to an OD₆₆₀ of 30 [25]. The harvested cells were used directly or stored at –80°C.

Entrapment in Calcium Alginate Gel

Four percent of sodium alginate was dissolved in deionized water and sterilized at 121°C for 15 min. After cooling, 6 ml of the sodium alginate solution was mixed with 3 ml of the cell suspension [31.7 mg dry cell weight (DCW)/ml]. The cell immobilization was performed by extruding the mixture through an injector into a gelling solution of 4% (w/v) calcium chloride (CaCl₂) at room temperature

and solidified for 6 h at 4°C [4, 21]. The immobilized beads formed were washed with saline, and then stored at 4°C in a refrigerator until use.

Entrapment in Polyvinyl Alcohol (PVA) and Mixture of PVA with Sodium Alginate

Twelve percent PVA or a mixture of 10% (w/v) PVA with 2% (w/v) sodium alginate was dissolved in deionized water and sterilized at 121°C for 15 min. After cooling, 6 ml of PVA or the mixture of PVA and Na-alginate was mixed with 3 ml of the cell suspension (31.7 mg DCW/ml). An injector was used to drop the mixtures into cold olive oil to form immobilized beads, which were then stored at –25°C for 18 h, followed by gentle thawing at no more than 0.5°C/min to room temperature. The PVA-immobilized beads and PVA-mixture-immobilized beads were subjected to a single freeze-thawing cycle and then washed 4 times with water and 4 times with saline [34]. Finally, the beads were stored at 4°C in a refrigerator until use.

Desulfurization of Oil Using Resting Cells

The reaction was carried out in a test tube containing 17.3 to 28.2 mg of DCW/ml in 1 to 2 ml of a 100 mM potassium phosphate buffer (pH 7). Dead cells were prepared as the control. The reaction was started by adding 0.5 ml of *n*-tetradecane containing 250 mg of DBT/l or its derivatives, except when evaluating the effect of various water/oil ratios on the desulfurization activity (the volume of the added model oil was modified from 0.25 to 1 ml, whereas the volume of the phosphate buffer remained the same). The reactions were performed at 27°C with reciprocal shaking at 273 rpm, and stopped after 24 h by centrifugation to separate the oil phase from the aqueous phase. The oil phases were analyzed by GC to determine the content of DBT and its derivatives.

The biodesulfurization of LGO containing sulfur (280 mg/l) was tested using resting cells of *S. subarctica* T7b. The reaction mixture contained 0.5 ml of LGO and 2 ml of *S. subarctica* T7b cell suspension with an OD₆₆₀ of 25 (28.2 mg DCW/ml). The resting cell reactions were conducted at 27°C for 24 h.

Biodesulfurization Using Immobilized Cells in Model Oil System

The biodesulfurization was carried out using immobilized cells in a 100 ml flask containing 17.5 ml of saline and 2.5 ml of *n*-tetradecane containing 100 mg of DBT/l at 27°C on a rotary shaker at 160 rpm. The immobilized cells were activated for 48 h in a culture medium (MSSF-CA medium) before being used.

The immobilized cells were separated from the medium by transferring the model oil or light gas oil and saline into another tube, while the beads were washed twice with water and twice with saline. The beads were then regenerated based on 20 h of incubation in the MSSF-CA medium at 27°C with shaking at 160 rpm. After being washed 4 times with saline, the beads were used in another cycle to desulfurize fresh oil.

Repeated Biodesulfurization

The immobilized cells were tested in the model oil, as described above, and the biodesulfurization was repeated by replacing the desulfurized oil with fresh oil in combination with reactivation. After completing the biodesulfurization, the reactivation treatment was performed as follows. The immobilized cells were washed twice with water and then twice with saline to remove both the

substrates and the products. Thereafter, the immobilized cells were reactivated in 20 ml of the MSSF-CA medium for 20 h at 27°C with shaking, and then washed again as described above before being used.

Analytical Methods

The cell concentrations were determined from the linear relationship between the optical density at 660 nm (OD_{660}) and the dry cells (drying at 105°C for 36 h). The concentrations of DBT and its derivatives were measured using a GC equipped with a flame ionization detector. The samples were acidified to pH 2 using 6 N HCl, followed by extraction with a 0.5 volume of ethyl acetate. A portion of the ethyl acetate layer was centrifuged, and the supernatant analyzed using a GC (GC-17A, Shimadzu) equipped with a DB-17 column (0.25 mm i.d. \times 30 m length; J&W Scientific, Folsom, CA, USA) and flame ionized detector. The carrier gas was helium, and the injector and detector temperatures were both set at 260°C, as previously described [9]. For the experiments using LGO, the detection of the total sulfur concentrations using a GC-14A (Shimadzu, Japan) equipped with a Zebtron ZB-1 column (60 m \times 0.25 mm \times 0.25 μ m; Phenomenex, USA) and FPD was carried out under the following conditions: the initial temperature of the column oven was 220°C, which was then increased to 280°C at a rate of 3°C/min. The injector and detector temperatures were maintained at 300°C. The content of each alkyl DBT in the LGO was calculated by comparing the GC areas with those of authentic alkyl DBTs. The decrease in the total sulfur content was estimated from the decrease in the sum of each sulfur peak area determined by the GC-FPD, as described by Hernández-Moldonado and Yang [11].

RESULTS AND DISCUSSION

Desulfurization of DBT Using Resting Cells of *S. subarctica* T7b

To investigate the DBT desulfurization by resting cells from the mid-log phase of *S. subarctica* T7b (Fig. 1), the reaction was carried out in a test tube containing 31.7 mg of DCW/ml in 2 ml of a 100 mM potassium phosphate buffer (pH 7) and 0.5 ml of *n*-tetradecane containing 250 mg of DBT/l at 27°C with shaking at 273 rpm. The DBT consumption and 2-hydroxybiphenyl production changed with time, yet the rates of DBT consumption and 2-hydroxybiphenyl formation were not found to be stoichiometrically related (Fig. 1). For example, about 250 mg of DBT/l (1.36 mM) was almost completely degraded within 24 h, while 127.5 mg of 2-hydroxybiphenyl/l (0.75 mM) was formed, representing a 55% conversion of the DBT. No significant decrease of DBT was revealed in the dead-cell controls (data not shown).

As previously reported by Gray *et al.* [7], only 55% of the DBT was converted into 2-HBP, since the degradation is not direct and involves the production of DBT-sulfoxide (DBTO), DBT-sulfone (DBTO₂), and 2-(2'-hydroxyphenyl)-benzene sulfinate (HPBS) as intermediate compounds. Similar results have also been reported by other researchers, where 1.440 μ g/ml of DBT was converted to just 382 μ g/ml of

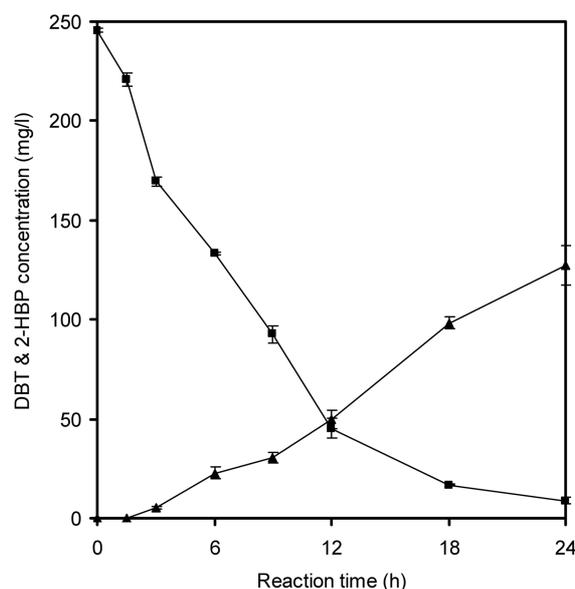


Fig. 1. Time course of DBT degradation and 2-HBP formation by resting cells of *S. subarctica* T7b.

The reaction mixture contained 2 ml of cell suspensions with an OD_{660} of 25 (28.2 g DCW/l) and 0.5 ml *n*-tetradecane containing 250 mg of DBT/l (phase ratio aqueous to oil: 4). Squares, DBT; triangles, 2-HBP.

2HBP when using *M. phlei* [14]. Matsui *et al.* [26] also reported that only 65% of the metabolized DBT was converted to 2-HBP when using *M. phlei* GTIS10. One explanation could be a time lag between the DBT uptake by the bacterial cells and the release of the produced 2-HBP [19].

Desulfurization of Alkylated Forms of DBTs

The desulfurization of alkylated DBTs was investigated using a two-layer system of water and oil phases with resting cells. The reactivity of the desulfurization rate

Table 1. Substrate specificity of desulfurization for resting cells of *S. subarctica* T7b.

Sulfur compounds	Degradation	
	(%)	mg/(kg DCW·h)
DBT	81.5	293.5
4,6-Dimethyl DBT	48.2	231.4
4,6-Dipropyl DBT	22.6	108.1
4,6-Dibutyl DBT	10.8	51.1
4,6-Dipentyl DBT	8.8	43.9
4-Hexyl DBT	30.0	144.0
2,3-Dihexyl DBT	15.0	71.9
3-Propyl-4,8-dimethyl DBT	16.9	79.4

Phase ratio: 4 (aqueous to oil).

The initial DBT concentration in *n*-tetradecane was 250 mg/l and initial cell concentration was 21.7 g dry cell weight (DCW)/l. Incubation was at 27°C, for 24 h with shaking at 273 rpm.

Table 2. Effects of sulfur sources in growth media on the desulfurization activity of resting cells of *S. subarctica* T7b^a.

Sulfur source in medium	DBT degradation ^b	2-HBP production	4-Hexyl DBT degradation ^b
	(mg/kg DCW·h)	(mg/kg DCW·h)	(mg/kg DCW·h)
CA	303.6	267.2	56.3
DBT	276.9	228.5	47.2
4-Hexyl DBT	236.6	141.7	49.4
Na ₂ SO ₄	8.9	3.4	14.7
MgSO ₄	47.4	18.9	20.7
Methionine	163.1	77.5	28.2
Cysteine	74.4	44.8	8.2

^aCultivation in 100 ml of MSSF medium containing 100 mg/l of sulfur source at 27°C with shaking at 273 rpm.

^bInitial DBT or 4-hexyl DBT concentration in *n*-tetradecane: 250 mg/l; cell concentration: 18.9 g DCW/l; phase ratio of water-to-oil: 4; incubation at 27°C for 24 h with shaking.

decreased when increasing the total carbon number of the alkylated DBTs (Table 1). As such, *S. subarctica* T7b was able to degrade the alkyl DBTs with long chains, such as 4,6-dipropyl DBT, 4,6-dibutyl DBT, 4,6-dipentyl DBT, 4-hexyl DBT, and 2,3-dihexyl DBT, indicating that *S. subarctica* T7b had a broad substrate specificity for high molecular weight alkyl DBTs in the resting cell system [9].

Watanabe *et al.* [36] reported that the desulfurization of C_x-DBTs by resting cells depends on the carbon number substituted at positions 4 and 6, and that the rate-limiting step in the desulfurization reaction of highly alkylated C_x-DBTs is the transfer process from the oil phase into the cell.

Effects of Sulfur Sources on Desulfurization Activity

To determine the effect of the sulfur source on the desulfurization activity of *S. subarctica* T7b, the strain was grown in MSSF media containing various sulfur sources. Whereas *S. subarctica* T7b was able to grow in the MSSF media with different sulfur sources, the highest growth occurred in the MSSF medium containing CA as the sole sulfur source (data not shown).

After preparing the resting cells, their desulfurization of DBT and 4-hexyl DBT was measured (Table 2). The resting cells grown in the MSSF-CA medium showed the highest desulfurization of DBT and 4-hexyl DBT as substrates at 303.6 mg of DBT/kg DCW·h and 56.3 mg of

4-hexyl DBT/kg DCW·h, respectively. Moreover, the cells grown in the MSSF medium with methionine (organic sulfur compound) or MgSO₄ (inorganic sulfur compound) as the sole sulfur source also showed a high desulfurization activity at 163.1 mg of DBT/kg DCW·h and 47.4 mg of DBT/kg DCW·h, respectively. In contrast, a lower desulfurization activity was recorded for the cells grown in the MSSF medium with Na₂SO₄ as the sole sulfur source. Tanaka *et al.* [35] also reported that the amount of 2-HBP produced by the desulfurization of DBT by *R. erythropolis* KA2-5-1 decreased when increasing the concentration of Na₂SO₄, although the growth yield was not affected.

A *chs* mutant strain of *R. erythropolis* KA2-5-1 also expressed high levels of *Dsz* enzymes when methionine was used as the sole source of sulfur [35]. Therefore, based on these results, an effective strategy would be to use methionine or MgSO₄ as the sulfur source in the first stage to obtain a high cell density, and then to use CA as the sulfur source in the second stage to induce the desulfurization activity of the cells.

Effect of Cell Age on Desulfurization Activity of Resting Cells

To investigate the effect of cell age on the desulfurization activity, the cells were harvested at different ages: early log phase (3 days cultivation), mid-log phase (4 days cultivation), and late log phase (5 days cultivation). The initial DBT

Table 3. Effect of different cell ages on the desulfurization activity of *S. subarctica* T7b.

Cell age (h)	pH	Growth ^a		DBT degradation ^b	
		OD ₆₆₀	DCW (mg/ml)	(%)	(mg/kg DCW·h)
72	6.5	2.8	17.3	72.0	337.0
96	6.3	3.9	17.7	84.3	401.4
120	6.2	4.8	18.4	79.4	382.9

^aCultivation in 100 ml of MSSF medium containing 50 µl of CA (Sakaguchi flask) at 27°C with shaking at 273 rpm.

^bInitial DBT concentration in *n*-tetradecane: 250 mg/l; cell concentration: 18.9 mg/kg DCW/l; phase ratio of water-to-oil: 4; incubation at 27°C for 24 h with shaking at 273 rpm.

concentration in the model oil was 250 mg/l or 1.36 mM (1 mM of DBT is equivalent to 0.184 g DBT/l). The resting cells harvested in the middle of the log phase showed the highest desulfurization rate at 401.4 mg of DBT/kg DCW·h (Table 3), whereas only 337.0 mg of DBT/kg DCW·h and 382.9 mg of DBT/kg DCW·h were desulfurized by the cells from the early and late log phases, respectively. Kayser *et al.* [14] also reported that the best desulfurization activity of *Mycobacterium phlei* GTIS10 was obtained from mid-to-late log phase cultures.

Effect of Cell Density on Desulfurization in Two-Phase System

To study the effect of the cell concentration on the desulfurization rate, a reaction solution containing a 0.1 M

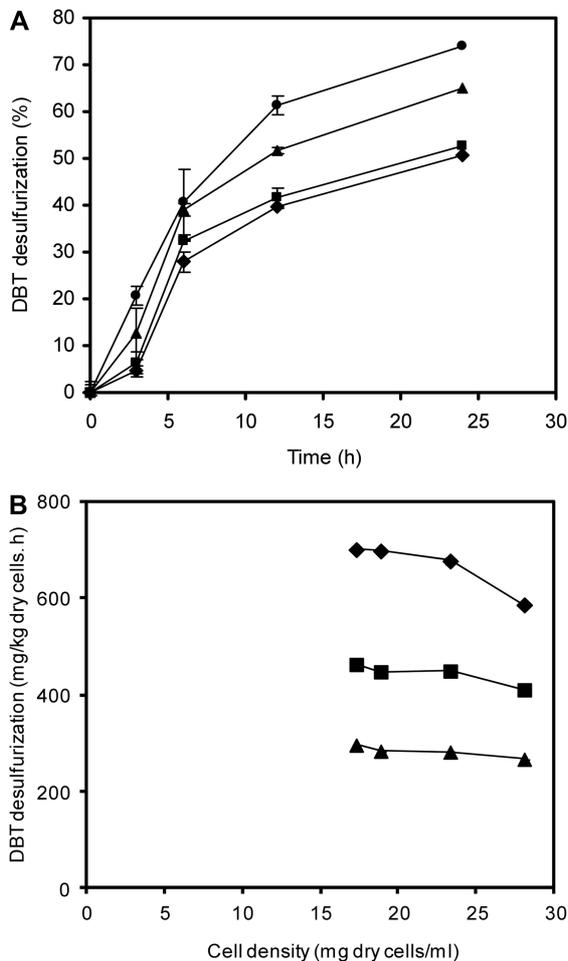


Fig. 2. Time course of DBT desulfurization with different cell amounts.

(A) Diamond, 17.3 mg of DCW/ml; square, 18.9 mg of DCW/ml; triangle, 23.3 mg of DCW/ml; circle, 28.2 mg of DCW/ml. (B) Time course of specific desulfurization rate of DBT. Diamond, 6 h; square, 12 h; triangle, 24 h of incubation. Initial DBT concentration in oil phase was 250 mg/ml; phase ratio water-to-oil: 4.

phosphate buffer (pH 7) and *n*-tetradecane containing 250 mg of DBT/l was prepared, and the cell concentrations were varied from 17.3 to 28.2 mg of DCW/ml in the buffer. While the total amount of desulfurized DBT increased with an increasing cell mass, the specific DBT degradation rate decreased at a high cell concentration and after a prolonged reaction time (Fig. 2). It is likely that the higher cell concentrations limited the mass transfer of the substrate and cells, as the penetration of the substrates, such as the DBT, into the cells was more restricted at a high cell concentration. Kaufman *et al.* [12] speculated that the desulfurization process may be limited by the rate of cell surface regeneration, as the biocatalyst interacts with the organic-aqueous interface to acquire the DBT substrate.

Effect of Water-to-Oil Ratio on Desulfurization

The effect of the water-to-oil ratio on the desulfurization rate is a factor in determining the reactor productivity and reactor volume (Fig. 3). To investigate the effect of the phase ratio, the desulfurization reaction solution contained 1 ml of the cells suspended in a 0.1 M potassium phosphate buffer (28.2 mg of DCW/ml) and *n*-tetradecane containing about 250 mg of DBT/l in a volume of 0.25, 0.5, and 1 ml corresponding to a water-to-oil ratio of 4: 2: 1, respectively. The desulfurization rates were found to increase when increasing the water-to-oil ratio. As such, 250 mg of DBT/l was completely desulfurized within 24 h when the water-to-oil ratio (w/o) was 4; however, the DBT degraded slowly when the phase ratio (w/o) was 2 and 1.

When using the same initial concentration of DBT in the oil, if the volume of the oil phase containing the DBT was

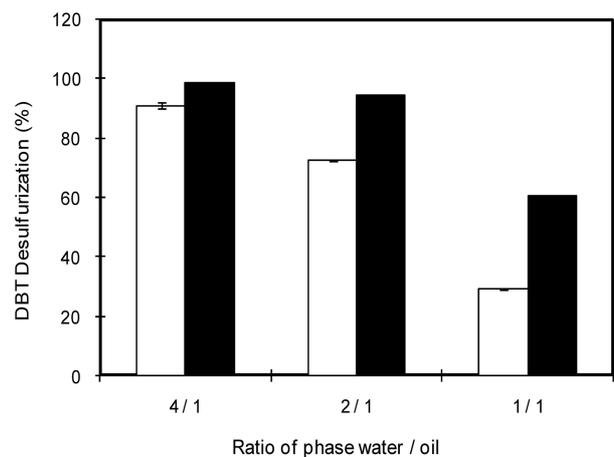


Fig. 3. Effect of phase ratios of water-to-oil on desulfurization of DBT by resting cells of *S. subarctica* T7b.

The reaction mixture contained 1 ml of a cell suspension with an OD₆₆₀ of 25 (28.2 g of DCW/l) as the aqueous phase and 0.25–1 ml of *n*-tetradecane containing 250 mg/l of DBT as the oil phase. White columns, 12 h of incubation; black columns, 24 h of incubation.

increased, the total sulfur content also increased. This indicates that the possibility of the substrate coming into contact with the cells decreased, thereby decreasing the specific desulfurization activity. Maghsoudi *et al.* [25] also reported that when the phase ratio (w/o) was decreased, the substrate became less available to the cells through a reduction of the interfacial area per unit volume of the organic phase.

Biodesulfurization of Light Gas Oil

A sample of LGO with a sulfur content of 280 mg/l was used for the biodesulfurization with *S. subarctica* T7b. An organic phase ratio (w/o) of 4:1 and cell concentration of 28.2 mg of DCW/ml were selected for a higher rate of reaction and conversion. As a result, the *S. subarctica* T7b desulfurized the DBT, its derivatives, and the LGO, where the total sulfur concentration in the LGO was reduced from 280 to 152 mg/l after 24 h of reaction at 27°C, corresponding to 45.7% desulfurization.

Paenibacillus sp. strains A11-1 and A11-2 have been shown to grow in the presence of oil, and the sulfur content decreased from 800 to 720 mg/l [19]. Resting cells of *Rhodococcus* sp. strain P32C1 (80 mg of DCW/ml) have also been reported to degrade the sulfur content in diesel oil from 303 to 156 mg/l (48.5%) within 24 h [25]. In the present study, the desulfurization ability of *S. subarctica* T7b in the two-phase system was comparable with these strains and showed a higher activity.

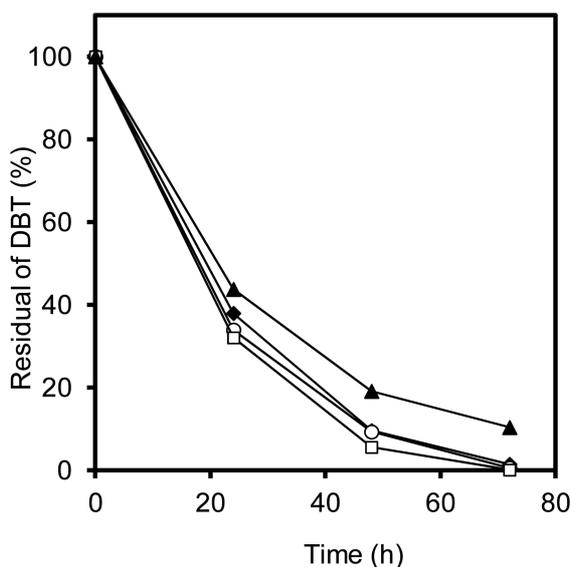


Fig. 4. Degradation of DBT by immobilized cells in model oil. Cells with an OD of 33 (31.7 g of DCW/l) were immobilized by each material and used in 2.5 ml of *n*-tetradecane containing 100 mg/l (0.54 mM) of DBT and 17.5 ml of a saline solution in a 100 ml flask at 27°C for 24 h with shaking at 160 rpm. Open square, 4% Na-alginate; open circle, 10% PVA + 2% Na-alginate; filled diamond, 12% PVA; filled triangle, resting cells.

Biodesulfurization Using Immobilized Cells of *S. subarctica* T7b

Immobilized cells were prepared using 4 kinds of support material: polyvinyl alcohol (PVA), poly (ethylene-co-vinyl) alcohol, sodium alginate, and sodium silicate. Only PVA and sodium alginate showed a good performance and were easy to prepare (data not shown).

A comparison was made of the biodesulfurization activities of the cells immobilized in PVA, Na-alginate, a mixture of PVA and Na-alginate, and resting cells, and the results are shown in Fig. 4. The cells immobilized in 4% (w/v) sodium alginate, 12% (w/v) PVA, and a mixture of 10% (w/v) PVA and 2% (w/v) sodium alginate exhibited a better activity in the model oil and showed a slightly higher activity than the resting cells.

In addition, comparative biotransformations using resting cells or free cells provided similar results, demonstrating that the immobilization procedure did not affect the enzyme activity. When the cells were immobilized with sodium alginate, 2.5 ml of *n*-tetradecane containing 100 mg of DBT/l was completely degraded after 72 h of operation. The cells entrapped in PVA or a mixture of PVA and Na-alginate also exhibited a similar biodesulfurization activity, indicating that all the support materials were appropriate for cell immobilization and that cell immobilization is an essential technique for the degradation of sulfur in oil.

Turbidity of Immobilized Cells

To examine the turbidity of the immobilized cells, the turbidity of the aqueous phase was measured at 660 nm after the first and the second biodegradation cycles (Fig. 5). After the first biodegradation cycle, only minimal cell leakage from the immobilized cells was observed, whereas

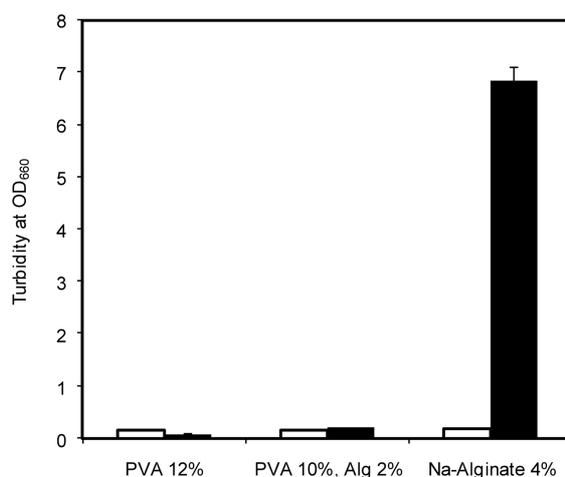


Fig. 5. Turbidity of immobilized cells from immobilized preparations in model oil.

The turbidity of the aqueous phase was measured at 660 nm. The reaction conditions were the same as those described in Fig. 4. White columns, first degradation cycle; black columns, second degradation cycle.

after the second cycle, the turbidity of the aqueous phase of the batch containing the Ca-alginate gel was high. Thus, the turbidity of the aqueous phase in this system indicated that the immobilization material had broken into tiny particles and cell leakage occurred.

Giuliano *et al.* [6] previously reported that experiments with sodium-alginate-immobilized cells resulted in complete disruption of the beads, especially when using a sodium-acetate buffer containing the substrate. These materials are also known to have a weak mechanical strength [27]. Furthermore, Fernandes *et al.* [4] reported that calcium alginate is easily damaged by phosphoric acid salt and tends to erode or dissolve when used in reaction systems. In contrast, the PVA-immobilized biocatalyst showed good stability and degradation activities. Therefore, PVA and a mixture of PVA and Na-alginate were selected as appropriate materials for immobilization and used in the subsequent experiments.

Repeated Batch Biodesulfurization

The stability and durability of the cells immobilized in PVA and a mixture of PVA and Na-alginate for the biodesulfurization of the model oil were investigated in the case of a repeated batch operation. For DBT degradation, the cells immobilized in PVA were used for biodesulfurization of the model oil in repeated batch cycles of 24 h. As shown in Fig. 6, the desulfurization activity of the immobilized cells was about 60% and remained stable until the 8th batch cycle. As expected, the biotransformation capacities declined during the repeated DBT degradation cycles, yet the initial activity was recovered after passage through a fresh complete medium (MSSF-CA). The desulfurization activity

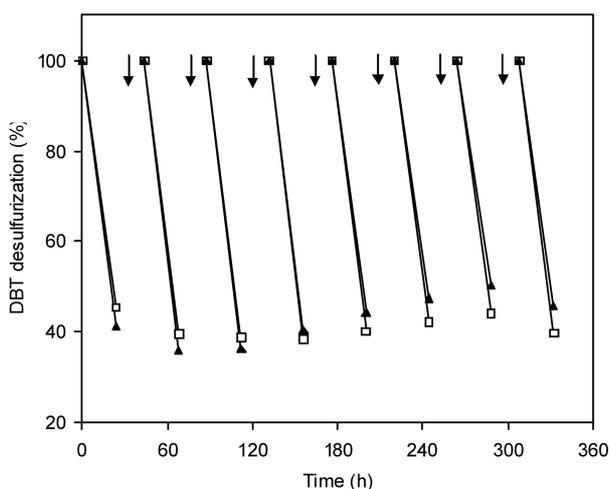


Fig. 6. Repeated biodesulfurization with PVA and mixture of PVA and Na-alginate-immobilized cells.

Each reaction was carried out as in Fig. 4. The reaction was performed as described in the text. Filled triangle, mixture of 10% PVA and 2% SA; open square, 12% PVA; arrows, reactivation for 20 h.

of the cells immobilized in a mixture of PVA and Na-alginate was initially slightly higher than that of the cells immobilized in PVA until the third batch cycle. Thereafter, the desulfurization activity of the cells immobilized in a mixture of PVA and Na-alginate decreased slightly, whereas the desulfurization activity of the cells immobilized in PVA remained more stable, thereby suggesting that the PVA remained stable for a longer period than the mixture of PVA and Na-alginate. Giuliano *et al.* [6] previously reported that entrapment in PVA helped to stabilize a biocatalyst, resulting in a better operational stability when performing consecutive degradation cycles. Therefore, 12% (w/v) PVA was selected as the appropriate support material for immobilization and used in the subsequent experiments.

When the biotransformation capacity declined, the beads were regenerated based on 20 h of incubation in a complete growth medium (MSSF-CA medium). Following this regeneration, the medium turbidity reflected the growth of microorganisms on the surface of the immobilization material. Thus, the immobilized cells were washed extensively with a saline solution (four times) before starting a new series of biodegradation cycles. In addition, the cell content in the immobilized beads constantly changed owing to continuous cell growth and death during the cultivation and reaction [10].

Effect of Initial pH on Biodesulfurization Rate

The resting cells of *S. subarctica* T7b exhibited a significant catalytic activity toward DBT at a pH value

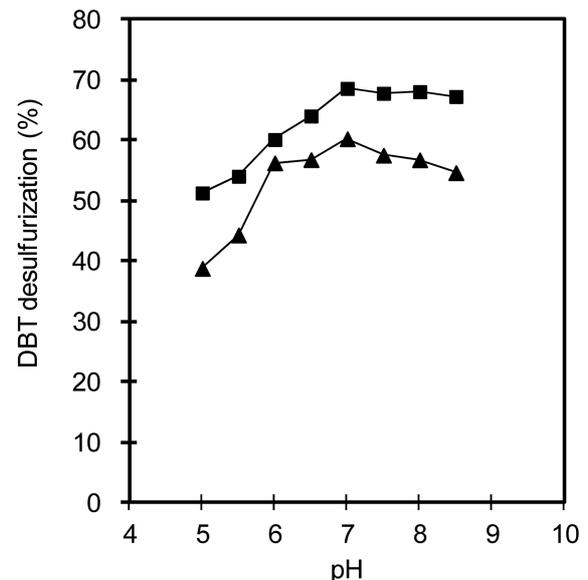


Fig. 7. Effect of initial pH on desulfurization activity of resting and immobilized cell systems.

The reaction was performed at 27°C for 24 h with shaking, as described in Fig. 4. Filled triangle, resting cells; filled square, immobilized cells.

between 5 and 8.5, with an apparent pH optimum of 7 (80.9 mg per kg of DCW⁻¹ h⁻¹) (Fig. 7). The activity of the resting cells or free cells also decreased sharply at a lower pH. In contrast, the immobilized cells were more stable, especially at a pH between 7 and 8.5, meaning that changing the initial pH from 7 to 8.5 did not affect the activity of the cells immobilized in the PVA beads.

Li *et al.* [20] also reported similar results, where cells immobilized in PVA exhibited higher activities within a pH range of 5.4 to 7.7. When using different pH values, the degradation ability of immobilized cells remained consistently higher than that of suspended or resting cells. When changing the pH from 5 to 8.5, the degradation rate of immobilized cells was about 72–75%, whereas the degradation ability of resting cells was only 30–34%. Furthermore, the resting cells lost more than 40% of their activity when the pH was 5.

The effect of pH on the degradation of linear alkyl benzene sulfonate (LAS) also shows that suspended cells in wastewater have low activities in acidic or alkali solutions, whereas immobilized cells have high activities. Thus, as the anti-poison ability of immobilized cells increases, the pH range within which they can grow also increases [22]. It has already been reported that immobilized cells can tolerate changes of medium pH and are less affected by such changes when compared with resting or free cells.

Heat Resistance of Immobilized Cells

The heat resistance of the immobilized and resting cells, and their desulfurization activity were compared at 27°C, 32°C, and 37°C on a rotary shaker at 160 rpm, respectively.

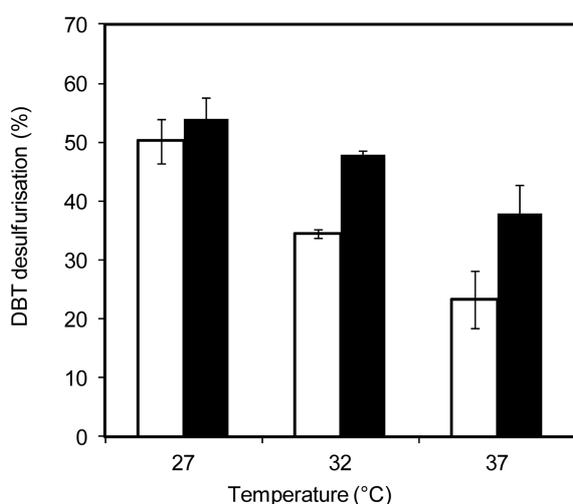


Fig. 8. Effect of temperature on desulfurization activity of resting and immobilized cells of *S. subarctica* T7b.

The reactions were carried out at 27°C, 32°C, and 34°C for 24 h, pH 7, with shaking, as described in Fig. 4. White columns, resting cells; black columns, immobilized cells.

Fig. 8 shows the desulfurization rate of the immobilized and resting cells at different temperatures, and reveals that for both cell types their desulfurization activity was dependent on the temperature. However, the resting cells lost more desulfurization activity at a high temperature, as the heat resistance of the cells apparently increased when the cells were entrapped in the PVA beads. Yet, for both cell types, the optimal temperature was determined as 27°C. Dursun and Tepe [3] also reported that resting cells were more sensitive to temperature than immobilized cells, and that immobilization increased the thermal stability of the cells [33]. Thus, temperature is an important factor affecting cell performance.

Comparison of Consecutive Desulfurization When Using Resting and Immobilized Cells as Biocatalysts

The DBT degradation activities of the resting and immobilized cells were compared in an experiment that lasted 24 h. After the first degradation cycle, the beads were recovered from the flask and transferred into a fresh medium to start a new biodesulfurization assay. Table 4 shows the results of three consecutive assays using the immobilized and resting cells. The PVA-T7b biocatalysts were able to degrade the DBT in successive degradation cycles, where the degradation rates (after 24 h) for the first, second, and third cycles were 72%, 56.6%, and 36.7%, respectively. Thus, the degradation data for the second cycle showed a 15.4% decrease when compared with the first cycle. However, the difference in the operational stability between the resting and immobilized cells was more evident when comparing the first and third biodegradation cycles (Table 4). The immobilized cells exhibited a better stability and activity when compared with the resting cells, as demonstrated by the decreasing activity level of the PVA-T7b. Thus, based on the above characteristics, immobilized cells are easier to apply to petroleum biodesulfurization than resting cells.

In conclusion, the optimal biodesulfurization conditions for the resting cells of *S. subarctica* T7b were strongly dependent on the cell concentration, aqueous-to-oil phase ratio, cell age, and sulfur source for growth. Moreover, the

Table 4. Comparison of consecutive biodesulfurization when using resting cells and PVA-*S. subarctica* T7b beads as biocatalysts.

Repeated batch	Desulfurization activity (%)	
	Resting cells	Immobilized cells
1 st	68.6	72.0
2 nd	51.0	56.6
3 rd	25.4	36.7

The biodesulfurization was carried out as described in Fig. 4 (the amounts of resting cells and immobilized cells were the same and each batch cycle was only 24 h of incubation). At the end of each cycle, the beads were separated from the supernatant and inoculated into fresh medium. The data given represent the average of three independent experiments.

use of immobilized cells of *S. subarctica* T7b based on entrapping with polyvinyl alcohol was an effective method that could be applied to the biodesulfurization of aromatic sulfur compounds in fossil oil. The stability of the immobilized cells was better than that of the resting cells at different initial pHs and higher temperatures. The decrease in the level of biodesulfurization activity of the immobilized cells was lower than that of the resting cells when performed consecutively without activation. The immobilized cells exhibited a high DBT desulfurization activity, including repeated use without loss of biodesulfurization activity. In addition, they were easily separated from the oil and water phases after the desulfurization process. Therefore, the use of immobilized cells is a promising method with great potential for application to oil biodesulfurization.

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