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Biodesulfurization of hydrocarbons and diesel fuels by *Rhodococcus* sp. strain P32C1

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Abstract

The ability of *Rhodococcus* sp. P32C1 for desulfurization of *n*-hexadecane (*n*-C16) containing dibenzothiophene (DBT), and two different diesel oils is evaluated. Cells cultivated in the medium containing DBT of 0.1 mM showed higher desulfurization activity, and the effects of cell concentration on the desulfurization reaction were studied. Three hydrocarbon–aqueous phase ratios of 25, 50 and 75 vol.% were used for desulfurization of *n*-C16 containing 1 and 24 mM of DBT as a sulfur source. The maximum specific production rate of 2-hydroxybiphenyl (2-HBP) in two-phase system was 43.5 mmol/(kg dry cell·h).

Based on the optimum conditions determined for desulfurization of *n*-C16, diesel oils with different sulfur contents were treated by resting cells. The total sulfur content of 303 ppm in the light diesel oil previously processed through hydrodesulfurization (HDS) was reduced by 48.5 wt.%. Another diesel oil with initial sulfur of 1000 ppm was desulfurized by 23.7 wt.%. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Biodesulfurization; *Rhodococcus* sp. P32C1; Hydrocarbon; Two-phase system

1. Introduction

Emission of sulfur-oxides to the atmosphere through combustion of petroleum fractions causes serious environmental problems such as acid rain. To reduce these emissions, the total sulfur content in fuels should be reduced during refining processes. In the next decade, the availability of light crude oils with lower sulfur contents will be decreased [1], and the demands for middle-distillate fractions with very low sulfur contents will be increased [2]. This new situation will force the petroleum refining companies to process sour crude oils and to invest a lot to build and operate the conventional process of hydrodesulfurization (HDS).

Organic sulfur compounds in the middle-distillate fractions, e.g., the diesel and fuel oil range, mainly consist of benzothiophenes and dibenzothiophenes (DBTs), removal of which are considerably difficult by HDS. Therefore, HDS is not effective for deep-desulfurization (reducing the sulfur content from 500 to 50 ppm) of fractions containing heterocyclic sulfur compounds. As an alternative method, microbial desulfurization of petroleum has attracted many attentions during last decade [3].

For commercialization of this new alternative process, biodesulfurization (BDS) must be able to selectively remove sulfur from fuels. Several species which can metabolize DBT via a sulfur specific pathway (4S pathway) have been isolated [4–8], but a few information on the desulfurization of hydrocarbons and diesel oils has been reported. New strain P32C1, which belongs to *Rhodococcus* sp. was separated in our previous work from soils in Iran, and was able to desulfurize DBT during growing phase and in resting cells [9]. In an aqueous phase, the maximum specific production rate of 2-hydroxybiphenyl (2HBP) from DBT was 37 mmol/(kg dry cell h) [9]. In this study, the desulfurization activity of P32C1 in two-phase system of a phosphate buffer and *n*-hexadecane (*n*-C16) containing DBT have been evaluated, and the effects of several parameters on the rate of desulfurization are examined. Based on the optimum conditions determined for desulfurization of *n*-C16, the extend

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of sulfur removal from two samples of diesel oils by resting cells of P32C1 is studied.

2. Materials and methods

2.1. Chemicals

Normal hexadecane, DBT (nacalai tesque, Kyoto, Japan), and 2HBP (Wako Pure Chemical Industries, Osaka, Japan) were used. Two types of diesel fuels were supplied from Japan Energy (Tokyo, Japan). The specifications of these two fuels are shown in Table 1. ATM-diesel with sulfur content of 16,200 ppm was a straight run fraction from a crude distillation unit, and HDS-diesel was hydrodesulfurized diesel with a total sulfur of 303 ppm. Diesel oil containing sulfur of 1000 ppm was prepared by mixing these two samples. All other chemicals were of analytical grade, commercially available and used without further purification.

2.2. Bacterial strains

Rhodococcus sp. P32C1, which formerly was identified as *Corynebacterium* sp. P32C1 by the National Collection of Industrial and Marine Bacteria (NCIMB Japan), was further characterized by the same company (Table 2). P32C1 strain could not metabolize maltose and manitol but its urease test was positive, and thus this coryneform bacterium might be *Rhodococcus equi* or *Rhodococcus maris*. Positive glucosidase screen test of P32C1 increased the probability of *R. maris*, thus, at present it was identified as *Rhodococcus* sp. P32C1.

Table 1
Analysis of diesel fuels

Name	HDS-diesel	ATM-diesel
Density (g/l) at 15°C	0.8384	0.8569
Sulfur (ppm)	303	16,200
Viscosity at 30°C	5.699	5.922
Cetane index	64	56
Distillation (D-86) (vol.%)		
IBP	229.0°C	221.0°C
5	262.0°C	265.0°C
10	272.0°C	272.0°C
20	281.0°C	280.5°C
30	288.5°C	288.0°C
50	301.5°C	303.0°C
70	315.5°C	322.0°C
90	337.5°C	351.0°C
95	348.0°C	364.0°C
EP	357.0°C	375.5°C
Aromatics (HPLC)		
Mono-	24.7 wt.%	15.9 wt.%
Di-	1.87 wt.%	11.1 wt.%
2,5-	0.34 wt.%	2.41 wt.%
Tri-	0.16 wt.%	1.98 wt.%
Total	27.1 wt.%	31.4 wt.%

Table 2
Characteristics of P32C1 strain (second phase of identification)

Taxonomic properties	Test result
Oxido-reductase enzyme	−
Pirazinamidase	+
Pyrrolidonyl-arylamidase	−
Alkaline phosphatase	+
β-Glucuronidase	−
β-Galactosidase	−
α-Glucosidase	+
N-acetyl-β-glucosaminidase	−
Screen with glucosidase	+
Urease	+
Solubility in gelatin	−
Carbon source for growth	
Glucose	−
Ribose	−
Xylose	−
Mannitol	−
Maltose	−
Lactose	−
Sucrose	−
Glycogen	

2.3. Media and growth conditions

A sulfur-free Medium A [9] containing 5 g/l glycerol was used, and DBT-ethanol solutions of 27 or 100 mM were added to the sterilized medium. For subculture and to produce cells used for the desulfurization reaction, 500 ml flasks were shaken at 30°C and 150 rpm. In case a large amount of cells were required, cells were cultured in a jar-fermentor with the same type and working condition as in our previous work [9]. Cells were harvested during growth or in the late logarithmic phase by centrifugation at 10,000*g* for 10 min at 4°C and washed twice with a 0.1 M potassium phosphate buffer (pH 7.0). The harvested cells were suspended in the phosphate buffer or mixture of the buffer and hydrocarbon at a desired concentration and were used for the desulfurization reaction.

2.4. DBT desulfurization in aqueous and two-phase systems

The reaction rates with resting cells were measured in test tubes or in 50 ml screw-cap glass bottles at 30°C and under rotary shaking at 150 rpm. For the desulfurization reaction in the phosphate buffer, the DBT-ethanol solution (27 mM DBT) was added at the concentration of 1.0 mM, and degradation of DBT and formation of 2HBP were measured (see Section 2.5). The specific production rate of 2HBP (mmol/(kg dry cell·h)) was calculated.

For two-phase systems, *n*-C16 containing two different concentrations of DBT, 1 and 24 mM, were prepared. The concentration of 24 mM DBT corresponds to the total sulfur content of 1000 ppm which is similar to practical concentration of sulfur in BDS process. Normal hexadecane

containing DBT was mixed with the 0.1 M phosphate buffer suspending harvested cells from late logarithmic phase of growth at different ratios of the hydrocarbon phase of 25, 50 and 75 vol.% and used for measurement of the desulfurization rate. The total volume of the mixture was 20 ml. *n*-C16 was selected as a model hydrocarbon for two-phase studies for its similarity with diesel oil fractions.

2.5. Analytical methods

The concentration of cells was determined from the linear relationship between the optical density at 660 nm (OD_{660}) and dry cell weight [9]. The concentrations of DBT and other components in culture medium and in the reaction mixture were analyzed by High-Performance Liquid Chromatography (HPLC, LC-10 system, Shimadzu, Kyoto, Japan) equipped with a reverse-phase column (Cosmosil 5C18-AR-300, nacalai tesque, Kyoto, Japan) after acidifying the samples to pH 2.0 and extraction with ethyl acetate. A mobile phase was 50% of acetonitrile with a flow rate of 1 ml/min. The absorbance of the effluent solution from the column was continuously measured mainly at 280 nm. The concentrations of DBT and 2HBP in *n*-C16 were also measured by HPLC with minor modifications. The hydrocarbon phase was separated from aqueous phase containing cells by centrifugation at 10,000*g* for 30 min at 30°C. A portion of *n*-C16 was diluted 10 times with ethyl acetate and 10 μl of the diluted samples was injected into the reverse-phase column. To elute adsorbed *n*-C16 from the column after analysis of samples, a washing cycle with pure acetonitrile was run for 10 min. The total sulfur content in diesel fuels was measured by X-ray fluorescence method and in accordance with ASTM standard D-4294.

3. Results and discussion

3.1. Effects of DBT concentration on growth and desulfurization activity of cells

Fig. 1(A) shows growth curves of P32C1 strain in flasks containing 150 ml of Medium A, supplemented with different concentrations of DBT. Growth behavior of P32C1 for DBT of 0.05–0.5 mM was generally the same. Cells were harvested in the late exponential phase of growth and resuspended in the phosphate buffer containing 0.5 mM DBT to a cell density of 30 g dry cell/l. The desulfurization activity of the cells was measured as the specific production rate of 2HBP (mmol/(kg dry cell·h)) and shown in Fig. 1(B). The cells harvested from the medium containing

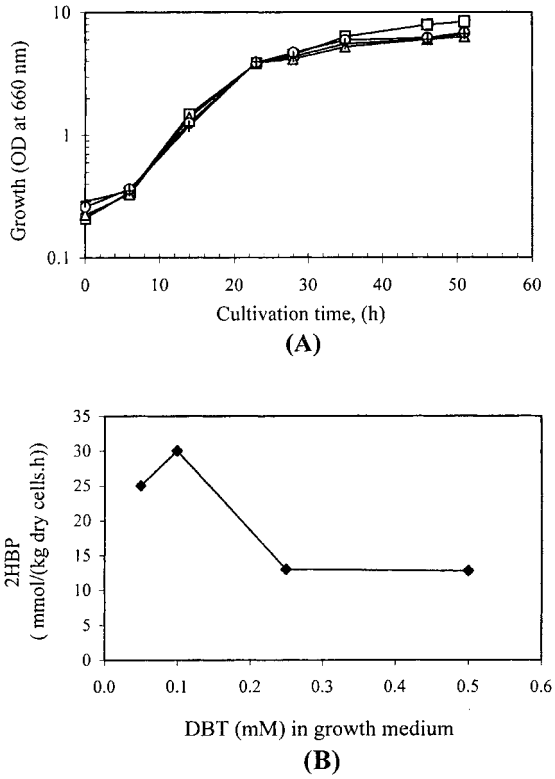

Fig. 1. Growth behavior of P32C1 strain with different amount of DBT (A), specific production rate of 2HBP with the resting cells (B). Symbols: (□) 0.05; (△) 0.1; (O) 0.25; (+) 0.5 mM.

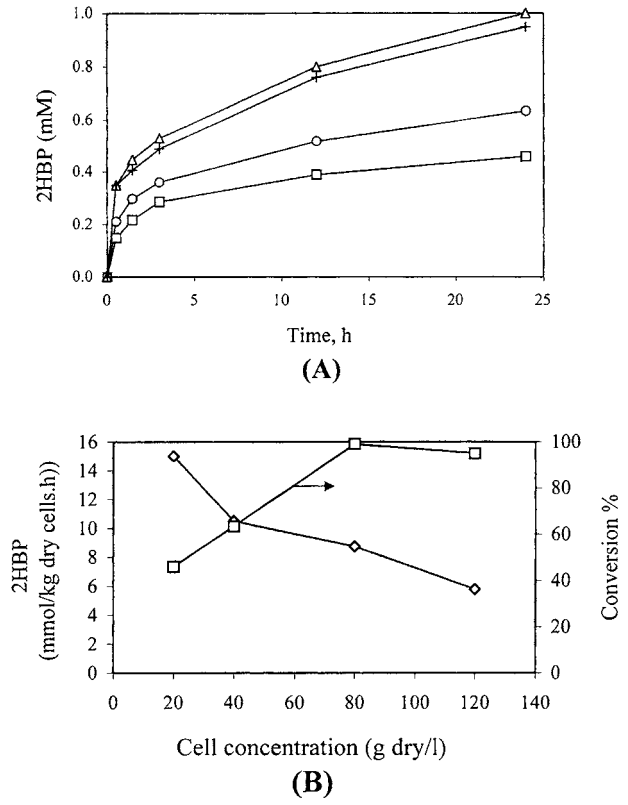

Fig. 2. 2HBP production with different cell amount (A), specific production rate and maximum conversion of 2HBP for 24 h (B). DBT concentration in hydrocarbon phase and phase ratio were 1 mM and 50 vol.%, respectively. Symbols: (□) 20; (O) 40; (△) 80; (+) 120 g dry cell/l.

0.1 mM DBT showed highest activity which was 30 mmol/(kg dry cell·h).

3.2. Effects of cell density on desulfurization in two-phase systems

To study the effects of cell density on desulfurization of *n*-C16, cell suspensions of 20, 40, 80 and 120 g dry cell/l in the phosphate buffer were mixed with the same volume of *n*-hexadecane contained 1 mM DBT, and the 2HBP production rate was measured in the hydrocarbon phase (Fig. 2(A)). The specific production rate of 2HBP calculated from initial rate of reaction and the maximum conversion of DBT to 2HBP after 24 h are shown in Fig. 2(B). The production rates of 2HBP at higher cell concentrations were lower, probably due to mass transfer limitation, specially oxygen transfer needed for the oxidation reaction of DBT, but the maximum conversion of DBT were higher. The results of biodesulfurization of *n*-C16 at the higher sulfur content (24 mM DBT) are shown in Fig. 3. General behavior was similar but the maximum rate of 2HBP production was higher (36 mmol/(kg dry cell·h)), and the maximum conversion (26 mol%) was lower.

3.3. Effects of phase ratio on desulfurization in two-phase systems

In Fig. 4, the initial rate (30 min) of 2HBP production and DBT conversion after 24 h for three different hydrocarbon fractions of 25, 50, and 75 vol.% are shown. Two different cell concentrations of 40 and 120 g dry cell/l of aqueous phase were used. The maximum rate and conversion were calculated based on total volume of the two-phase systems. For the initial DBT concentration of 1 mM the specific production rate of 2HBP was higher for the fraction of 75%, and the maximum conversion were generally the same due to the low DBT concentration (Fig. 4(A)). Effects of the hydrocarbon fraction were also investigated for higher DBT concentration of 24 mM (Fig. 4(B)). The specific production rates increased with increase in the DBT concentration and the hydrocarbon fraction.

3.4. Biodesulfurization of diesel fuels

Two samples of diesel fuels with sulfur content of 303 and 1000 ppm were used for biodesulfurization with P32C1 strain. A hydrocarbon phase ratio of 25% and a cell

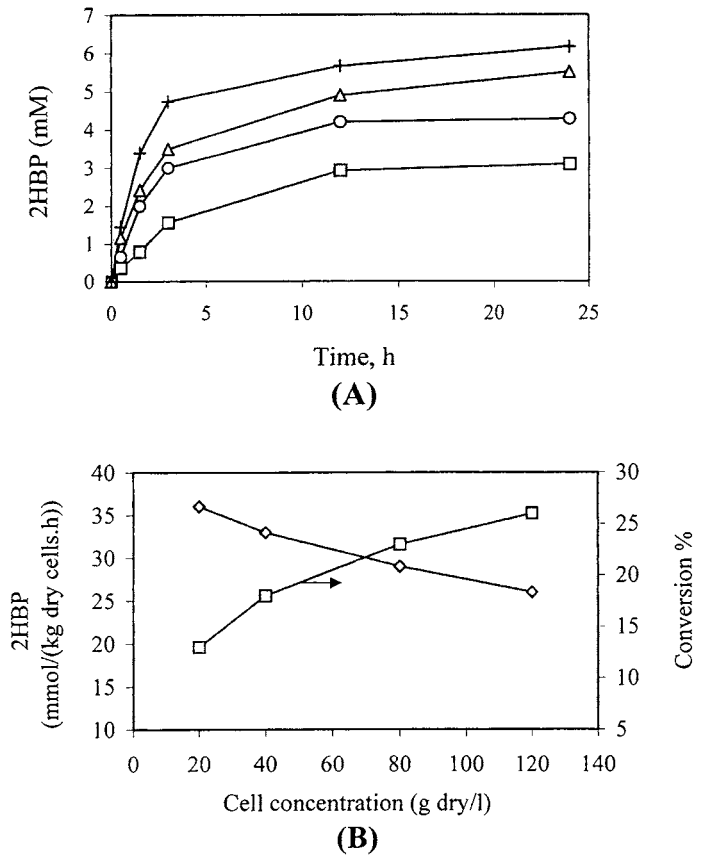

Fig. 3. 2HBP production with different cell amount (A), specific production rate and maximum conversion of 2HBP for 24 h (B). DBT concentration in hydrocarbon phase and phase ratio were 24 mM and 50 vol.%, respectively. Symbols: (□) 20; (O) 40; (△) 80; (+) 120 g dry cell/l.

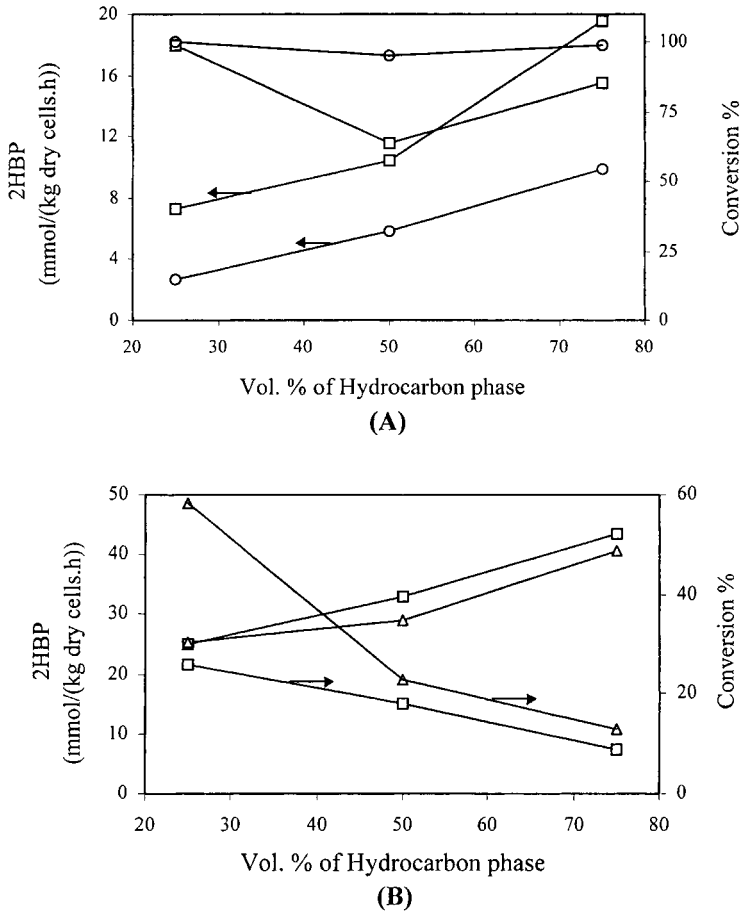

Fig. 4. Specific production rate of 2HBP and maximum DBT conversion after 24 h with different cell concentrations and DBT concentration of 1 mM (A) and 24 mM (B) in *n*-C16 (the basis for reaction rate was total volume). Symbols: (□) 40; (△) 80; (O) 120 g dry cell/l.

concentration of 80 g dry cell/l were selected for a higher rate of reaction and conversion. Final sulfur content of two samples after 24 h of reaction at 30°C were 156 and 763 ppm which are corresponding to 48.5 and 23.7% of desulfurization, respectively.

Rhodococcus sp. IGTS8 (ATCC 53968) in a two-phase system of *n*-C16 and a buffer with a phase ratio of 3.0 has shown the activity between 1 and 5 mg 2HBP produced/(g dry of biocatalyst·h) [10]. The resting cells of *Gordona* strain CYKS1 could decreased the total sulfur in a diesel fuel from 0.15 to 0.06%. The volume fraction of the oil phase was 10% in the reaction mixture [8]. *Paenibacillus* sp. strains A11-1 and A11-2 could grew in presence of oil and the sulfur content in the oil was decreased from 800 to 720 ppm [7]. The desulfurization ability of *Rhodococcus* sp. P32C1 in the two-phase system was comparable with these strains and showed more activity.

4. Conclusion

Newly isolated strain *Rhodococcus* sp. strain P32C1 showed high activities for desulfurization of organic sulfur compounds in *n*-C16 and middle-distillate fractions like diesel oils. Its desulfurization ability was higher than those of other strains in two-phase systems. The solubility of DBT in water is in the order of 0.005 mM, although this may increase a little by surfactant produced by cells. Since cells were suspended in the aqueous phase, the increased rates in higher hydrocarbon fractions might suggest transfer of DBT through the interface between the aqueous and hydrocarbon phase or adsorption of cells at the interface.

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