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Biodesulfurization of hydrodesulfurized diesel oil with *Pseudomonas delafieldii* R-8 from high density culture

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Abstract

Biodesulfurization (BDS) process of hydrodesulfurized diesel oil by *Pseudomonas delafieldii* R-8 was studied. Twenty-five grams (dry cell) l^{-1} of cell density was obtained by production of *P. delafieldii* R-8. The desulfurization activity of cells grown in logarithmic and stationary phases is higher than that of cells grown in other phases. The maximal desulfurization rate can reach 0.33 mg g^{-1} h^{-1} during the first 4 h. The metabolites do not affect desufurization activity of diesel oil. Cell suspension grown at stationary phase was directly mixed with diesel oil, and had a high desulfurization activity as 0.32 mg g^{-1} h^{-1} during the first 4 h. In a 5-l fermentor, 500 ml of diesel oil was added when desulfurized cells grown in a stationary phase and the total sulfur was reduced to 313 from 591 mg l^{-1}. Thus, the BDS process can be simplified by directly mixing cell cultivation suspension with diesel oil.
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Keywords: High cell density; 2-Hydroxybiphenyl (2-HBP); *Pseudomonas delafieldii*; Sulfate

1. Introduction

Regulations for sulfur level in diesel oil have become increasingly strict [20]. In the near future, refiners expect to be faced with a 'no sulfur' specification (less than 10–15 ppm sulfur). As a result, over the past a few years there has been considerable interest in developing technologies to remove sulfur from petroleum products. There are many conventional refinery operations that can be used to remove sulfur and a number of new technologies that have also been developed to satisfy the need. In order to achieve the 'no sulfur' specification, dibenzothiophene (DBT) and substituted DBTs which are the main sulfur compounds in diesel oils and gasolines must be desulfurized [18].

Biodesulfurization (BDS) has the potential benefits of lower capital and operating costs and will produce substantially less greenhouse gases and high valuable byproducts [11,13,19]. Moreover, BDS is to take advantages of the specificity of enzymes, especially for DBT and substituted DBTs. Therefore, BDS offers an alternative way to obtain 'no sulfur' products and has more promising prospects. There have been a number of reports over the past decade on BDS [7,22,25]. Despite considerable progress in BDS, there are still some difficulties to be commercially applied in the industry. Critical aspects of the process include cost of biocatalyst, reactor design and oil–water separation. Mass production of biocatalyst with high desulfurzation activity is an important technique to decrease the cost of biocatalysts, which is carried out by high cell density cultivation. At present, there are only two reports on the high density cultivation of cells for BDS. Honda et al. [10] reported that about 30 g l^{-1} of *Rhodococcus erythropolis* could be obtained by fed-batch culture with pH-stat feeding. Chang et al. [2] further reported production of desulfurization biocatalysts by two-step fermentation, in which the steps of cell growth and the desulfurization activity inducement were separated. After acquiring a high cell density in the first step using an inorganic sulfur source, the desulfurization activity of the cells was induced using DBT as the sulfur source in the second step. Finally, 92.6 g l^{-1} of cell density was obtained.

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However, these reports only focused on increasing cell density and have not referred to the development of BDS process. Most of these microorganisms reported are gram-positive bacteria. Gram-negative bacterium has high solvent tolerance [24] and is more promising for BDS from petroleum products. In our laboratory, a gram-negative bacterium, *Pseudomonas delafieldii* R-8, has been isolated for BDS [15]. In the present study, mass production of R-8 strain was carried out by one step method. The BDS process was developed by studying the effects of metabolites and cultivation conditions on desulfurization activity.

2. Materials and methods

2.1. Chemicals

DBT (dibenzothiophene) and DMSO (dimethyl sulphoxide) were purchased from Acros Organics (USA). 2-HBP (2-hydroxybiphenyl) and *n*-dodecane were purchased from Tokyo Kasei Kogyo Co., Ltd (TCI, Japan). *N*-hexane and methanol were of liquid chromatography grade and commercially available. All other chemicals were of analytical grade. DBT or DMSO (dimethyl sulphoxide), was prepared as 100 mM in ethanol (anhydrous).

2.2. Diesel oil

The hydrodesulfurized diesel oil, containing 591 m l^{-1} of total sulfur, was obtained from SINOPEC Research Institute of Petroleum Processing.

2.3. Bacterial strain and cultivation

P. delafieldii R-8 (CGMCC 0570) was isolated by our laboratory [15]. Minimal salt medium (MSM) contained the following compounds per liter 2.44 g of KH_2PO_4, 12.03 g of $Na_2HPO_4{\cdot}12H_2O$, 2.0 g of NH_4Cl, 0.4 g of $MgCl_2{\cdot}6H_2O$, 0.75 mg of $CaCl_2$, 1 mg of $FeCl_3{\cdot}6H_2O$ and 4 mg of $MnCl_2{\cdot}4H_2O$. Sodium sulfate was prepared as 100 mM in water. MSM supplemented with 1% (w/v) glycerol as the carbon source and 0.1 mM DBT as a sulfur source was used in flask cultures. The flask cultures were carried out in 500-ml flasks containing 120 ml medium for 36 h at 30 °C and 170 rpm (rotates per min) in a rotary shaker. High cell density cultivation was carried out at 30 °C in a 5-l jar fermentor (Bioflo 3000, NBS Inc., Edison, USA) with working volume of 3 l. The inoculum size was 5% (v/v). The nutrient concentrations were two-fold higher than the original MSM composition. 20 g l^{-1} of glycerol was used as the carbon source. Both 0.1 mM DBT and 1.0 mM DMSO were used as the sulfur source. 0.2 g l^{-1} of yeast extract was supplemented to enhance cell growth. 0.3 g l^{-1} of antifoam agent was added to avoid foaming. The pH was maintained at 7.0 by adding 25% NH_4OH solution. The dissolved oxygen concentration was maintained above 20% air saturation by manipulating agitation speed. The aeration rate was 0.7 vvm (air volume/medium volume/min). After 36 h cultivation, 0.1 mM DBT, 4 mM DMSO, 15 g l^{-1} glycerol, 2 g l^{-1} NH_4Cl, 15 ml ethanol, 14.64 g l^{-1} KH_2PO_4 and 72.18 g l^{-1} $Na_2HPO_4{\cdot}12H_2O$ were supplemented.

2.4. Cell preparation

Cells grown in the fermentor were collected by centrifuging at 5000 × *g* and 4 °C for 6 min (Avanti J-E, Beckman, USA). The collected cells were washed twice with a brine solution (0.85% NaCl), and then were re-suspended into the brine solution. The cell re-suspension was kept at 4 °C until being used.

2.5. Biodesulfurization

Biodesulfurization was practised by mixing cell culture suspension or re-suspension with diesel oil. The volume ratio of oil phase to aqueous phase was 1:2 (or 1:3). The reaction was carried out in flasks at 30 °C on a rotary shaker at 170 rpm.

2.6. Determination of cell concentration

Cell concentration was obtained by measuring its optical density at 600 nm (OD_{600}). Set the Spectrophotometer to 600 nm and zero the optical density with the supernatant fluid after cultivation medium being centrifugated. One unit of optical density was equal to 0.43 mg (dry cell weight) ml^{-1}.

2.7. HPLC analysis

The culture broth was acidified to pH 2.0 with 1 M HCl and extracted on a vortex mixer for 2 min with an equal volume of *n*-hexane. The *n*-hexane phase was used for quantification.

High performance liquid chromatography (HPLC) analysis was used for the quantitative assay of DBT and 2-HBP. HPLC analysis was performed on a Hewlett Packard 1100 (HP1100, Agilent, USA) liquid chromatography equipped with an autosampler, a reversed-phase Zorbax SB-C18 column (4.6 mm × 150 mm; 3.5 μm) and a diode array detector (set at 280 nm). The mobile phase was 90% of methanol in water (v, %) with a flow rate of 1.0 ml min^{-1}.

2.8. The analysis of total sulfur content of diesel oil

The total sulfur content (by weight) was determined in triplicate for each sample by combustion of samples and measurement of the released sulfur dioxide using a microcoulomb analyzer (RPA-200, JiangHuan Electroanalysis, China). The desulfurization activity was expressed as the amount of total sulfur (mg) consumed by 1 kg of dry cells (DCW) per hour (mg kg^{-1} h^{-1}).

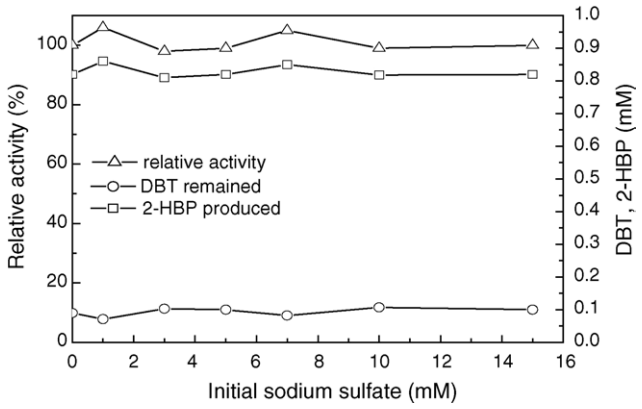

Fig. 1. Effect of sodium sulfate on the desulfurization activity of resting cells of *P. delafieldii* R-8. Note: BDS was carried out in a 100-ml flask containing 5 ml model oil and 15 ml cell re-suspension that cell content was 25 mg ml^{-1}.

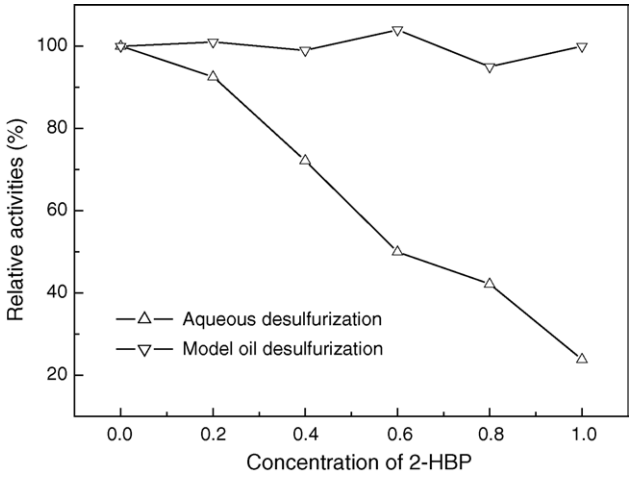

Fig. 2. Effect of 2-HBP on the desulfurization activity by resting cells of *P. delafieldii* R-8. Note: (1) BDS from aqueous phase: BDS was carried out in a 100-ml flask containing 15 ml cell re-suspension that cell content was 25 mg ml^{-1}, initial concentration of DBT was 1.0 mmol l^{-1}. (2) BDS from model oil: BDS was carried out in a 100-ml flask containing 5 ml model oil and 15 ml cell re-suspension that cell content was 25 mg ml^{-1}.

3. Results

3.1. Effect of metabolites on desulfurization activity

In order to study effects of metabolites on desulfurization activity, model oil was used to avoid the disturbance of other components such as aromatics and olefins in diesel oil. The model oil consists of 1.0 mM of DBT in *n*-dodecane. According to BDS mechanism [16], the final metabolites of DBT desulfurized by R-8 cells were mainly sulfate and 2-HBP. BDS reaction was carried out by mixing model oil with cell re-suspension. Firstly, various concentrations of sodium sulfate were respectively added into the reaction system. The effects of sodium sulfate on the desulfurization activity (the amount of DBT (mmol) consumed by one kilogram of dry cells (DCW) per hour) of R-8 cells were shown in Fig. 1. The desulfurization activity for DBT was not affected by sodium sulfate even with its concentration as highly as 15.0 mM. Secondly, the effect of 2-HBP on desulfurization activity was tested. Various concentration of 2-HBP in ethanol was added into a cell re-suspension containing 1.0 mM of DBT. With the increase of initial concentrations of 2-HBP, the relative activity decreased (Fig. 2). The desulfurization activity of the bacterium reduced to about 20% of that with no addition of 2-HBP when initial concentration of 2-HBP added was 1.0 mM. It means that toxicity of 2-HBP decreases the activities of desulfurization enzymes. However, when various concentration of 2-HBP was added into a reaction system consisting of oil, 2-HBP did not inhibit the desulfurization activity for model oil (Fig. 2).

3.2. Effect of cultivation time on desulfurization activity

P. delafieldii R-8 was cultivated in 5-l fermentor with 3 l of culture medium. The cell concentration in cultural medium was indicated by the optical density at 600 nm (OD_{600}). The cells at different growth phases were collected and washed to remove the residual sulfur source. For BDS, 5 ml of diesel oil mixed with 15 ml of re-suspension including 25 mg l^{-1} of dry cells. Fig. 3 showed the growth curve of cells in the fermentor and the desulfurization rate of resting cells at different growth phases, respectively. Supplemental medium was added in 36 h of cultivation. After the supplement, cell concentration was apparently increased, and logarithmic and stationary phases appeared again. After the 85 h, the cell growth started to decline. Fig. 3 shows that the desulfurization activity of cells grown in the logarithmic and stationary phases was higher than that of cells grown in other phases. The maximal desulfurization rate of total sulfur for the first 4 h was 0.33 mg (total sulfur) g^{-1} (dry cells) h^{-1}.

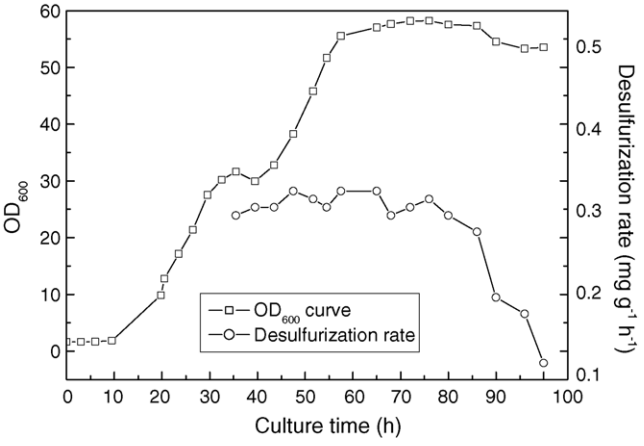

Fig. 3. Growth curves of cells and the desulfurization rate of total sulfur by resting cells of *P. delafieldii* R-8. Note: BDS was carried out in a 100-ml flask containing 5 ml diesel oil and 15 ml cell re-suspension that cell content was 25 mg l^{-1}.

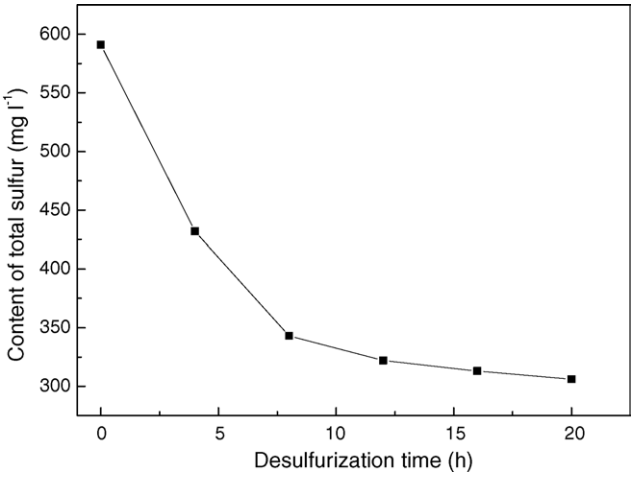

Fig. 4. Time courses of desulfurization of total sulfur using culture suspension of *P. delafieldii* R-8. Note: when cells grew for 65 h in a 5-l fermentor and OD600 was 56, 500 ml of diesel oil was added into the fermentor. The stirred rate was 600 rpm.

3.3. Biodesulfurization for hydrodesulfurized diesel oil

Thirty milliliters of cell suspension (OD_{600} = 55) were taken out from the fermentor after the sulfur source being consumed completely, and were averagely divided two portions. A portion of suspension was collected by centrifugation and the upper solution was moved away. The collected cells were re-suspended into 15 ml of brine solution (0.85% NaCl), and mixed with 5 ml of diesel oil. Another portion of suspension mixed directly with 5 ml of diesel oil. These two processes had similar time courses of BDS and the same BDS rate for the first 4 h, about 0.32 mg (total sulfur) g^{-1} (dry cells) h^{-1}.

After R-8 cells being cultivating for 65 h in a 5-l fermentor with 2.5 l of culture medium (OD_{600} of cell suspension was 56), 500 ml of diesel oil was added into the fermentor. Time courses of the BDS were shown in Fig. 4. The desulfurization rate for the first 4 h was about 0.28 mg (total sulfur) g^{-1} (dry cells) h^{-1}. After 16 h, the total sulfur was reduced to 313 from 591 mg l^{-1}. The 47% of total sulfur was removed. The treated diesel oil was separated by centrifugation. Thus, resting cells or cell suspension can be applied for diesel desulfurization untill about 12 h. After 12 h, their activity of desulfurization decreased and cells started to autolyze.

4. Discussion

Studies have shown that gram-negative *Pseudomonas* strains have higher solvent tolerance than gram-positive bacteria [26], so it is more promising to utilize gram-negative bacteria for BDS of diesel oil. The *Dsz* genes for desulfurization have been cloned from *R. erythropolis* IGTS8 and expressed in a wide range of bacterial species, including *Pseudomonas* strains [4]. *Pseudomonas* bacteria were selected to carry these *Dsz* genes for two reasons. Firstly, they have a high tolerance against the diesel oil in which the sulfur compounds and hydrocarbons are present. Secondly, they could produce biosurfactants to increase the contact between oil and water, which can reduce mass transfer limitations and speeds up BDS rate. Up to now, most of the reported *Pseudomonas* strains metabolize DBT via a pathway with a cleavage of carbon–carbon bonds while leaving the carbon–sulfur (C–S) bond intact [17,5]. Except for *Pseudomonas* sp. CB1, the wild-type gram-negative bacterium with a sulfur-specific pathway was seldom reported [21]. The CB1 strain was capable of destroying the C–S bond of DBT and producing 2,2′-dihydroxybiphenyl [12], but was claimed to be unstable and lost afterward. *P. delafieldii* R-8, a gram-negative strain, was isolated by our group. It had the capability of converting DBT to 2-HBP and sulfate, following a '4S' pathway [15]. The strain can produce biosurfactant, which favors the uptake of DBTs into the cells and resulted in increase of BDS activity. Therefore, the strain isolated has a high value for BDS application. According the '4S' pathway, BDS reaction is catalyzed by four *Dsz* (desulfurization) enzymes and some co-factors such as NADH [21]. The expression of these *Dsz* enzymes activities [16] is induced by some sulfur compounds that include DBT and its analog (dibenzothiophene 5,5-dioxide, DBTO2) and derivative (4,6-dimethyl dibenzothiophene, 4,6-DMDBT). The desulfurization activities of cells have not been affected by one of the final products, sulfate, as shown in Fig. 1. However, it is shown in Fig. 2 that 2-HBP apparently decreased the desulfurization activities of cells. It may be the result of toxicity of 2-HBP, as a kind of phenol, results in the inactivity of the *Dsz* enzymes and various other enzymes. Thus, it decreased the desulfurization activity. According to Fig. 2, the existence of 2-HBP in BDS did not affect the activities of the *Dsz* enzymes during BDS process in oil-water phases. It is because the distribution coefficient of 2-HBP to oil and water was 30, and 2-HBP stayed in oil phase and have no harm the cells.

According to these results, it is feasible that high cell density suspension containing the metabolites of sulfur source was directly used for BDS of diesel oil. The hypothesis was proved by the result of Fig. 4. Five hundred milliliters of diesel oil was treated by the simple process. The desulfurization rate for the first 4 h was about 0.28 mg (total sulfur) g^{-1} (dry cells) h^{-1}. Though the BDS of gas oil [6] and diesel oil [3] has been studied extensively, only 1 ml [1,8], 2 ml [6] and 20 ml [9] of oil were treated. The BDS process was apparently simplified, which had the advantages of cost saving and convenience. The cell–oil mixture can subsequently be separated by using demulsifying agents. Thus, the simple process is convenient to treat a mass of petroleum products, and a continuous operation might be applied.

Though DBT induces the activity of *Dsz* enzymes, 2-HBP of its metabolite inhibits the cell growth [14,19,23]. It is difficult to obtain cells of high density using DBT as the only sulfur source. Chang et al. [2] reported that a two-step method for cell culture was adopted in order to overcome these difficulties, and 92 g l^{-1} of cell density was obtained. However, in our study, a new method experimented in high cell density

cultivation by one step, which the multiple sources of DMSO and DBT were used (Fig. 3). The function of the small quantity of DBT was the inducement of *Dsz* enzymes. Though cell density was less than that reported by Chang et al. [2], it results from different type and characteristic of microorganism. Thus, it offers an alternative way for high cell density cultivation of desulfurizing biocatalysts.

Acknowledgments

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