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ORIGINAL PAPER

Improvement of biodesulfurization activity of alginate immobilized cells in biphasic systems

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Abstract The immobilization of *Pseudomonas delafieldii* R-8 in calcium alginate beads has been studied in order to improve biodesulfurization activity in oil/water (O/W) biphasic systems. A gas jet extrusion technique was performed to produce immobilized beads. The specific desulfurization rate of 1.5 mm diameter beads was 1.4-fold higher than that of 4.0 mm. Some nonionic surfactants can significantly increase the activity of immobilized cells. The desulfurization rate with the addition of 0.5% Span 80 increased 1.8-fold compared with that of the untreated beads. The rate of biodesulfurization was markedly enhanced by decreasing the size of alginate beads and adding the surfactant Span 80, most likely resulting from the increasing mass transfer of substrate to gel matrix.

Keywords Biodesulfurization · Immobilization · Alginate · Dibenzothiophene · *Pseudomonas delafieldii* · O/W systems

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Introduction

With stricter environment regulations and steady increases in the average sulfur content of petroleum, refiners are facing major challenges to developing new design approaches to ultra-low sulfur diesel fuels [24]. Presently, biodesulfurization (BDS) has attracted intensive interest due to its benefits of cost-effective, mild reaction conditions, and low impact on the environment [5, 8, 11, 19]. Dibenzothiophene (DBT) is usually considered as the model compound for biodesulfurization research.

There are numerous reports on the treatment of diesel oils or model oil mixtures by using suspensions of growing or resting cells [3, 15, 18, 20, 27]. Nevertheless, the treatment of oils using free cells has some limitations such as high cost of the biocatalyst and low volumetric ratio between the organic phase and the aqueous one. Also, separation of product oil from oil–water-biocatalyst emulsion is very troublesome [4, 14]. From the point of industrial application of BDS, cell immobilization was considered to be one of the most promising approaches [22].

So far, very few published papers are available on BDS by immobilized cells [2, 7, 9]. Calcium alginate is currently one of the most widely used entrapment carriers for immobilization of enzymes and whole cells for its advantages of biocompatibility, cheapness and simplicity [10, 16]. However, the entrapment technique often leads to a decrease of biocatalytic activity, mostly caused by diffusional limitations and steric hindrance [6, 7, 13]. The existence of diffusional limitations reduces the catalytic efficiency of immobilized biocatalysts and therefore should be minimized. Herein, immobilization of resting cells of *Pseudomonas delafieldii* R-8 was conducted by entrapment in calcium alginate. The kinetics of biodesulfurization by

the alginate immobilized cells was analyzed. In the present study, a simple cell-immobilization technology was developed, aiming to increase the biodesulfurization rate of immobilized biocatalysts in oil/water biphasic systems. It was achieved by decreasing the size of alginate beads and adding Span 80 in the bead-forming process. Improvements in the rate of biodesulfurization were possibly due to the improvement of mass transfer rate of substrate and reduced product inhibition.

Materials and methods

Chemicals

Dibenzothiophene (DBT) and Span 80 (Sorbitan monooleate) were purchased from Acros Organics (US). 2-Hydroxybiphenyl (2-HBP) and *n*-dodecane were purchased from Tokyo Kasei Kogyo Co., Ltd. (TCI; Japan). Tween 20 (Polyoxyethylene sorbitan monolaurate) was purchased from Fluka Chemika (Switzerland). Sodium alginate, Span 20 (Sorbitan monolaurate), Tween 80 (Polyoxyethylene sorbitan monostearate) were purchased from Beijing Chemical Reagents Company (China). Methanol was liquid chromatography grade. Sodium dodecyl sulfate (SDS), Cetyl trimethyl ammonium bromide (CTAB), Triton X-100 (polyethylene glycol *p-tert*-octylphenyl ether) and other materials were analytical grade and were available commercially.

Bacterial strain and cultivation

Pseudomonas delafieldii R-8 (CGMCC No. 0570) was isolated from the sewage pool of Shengli oil field of China, and was capable of desulfurizing DBT to 2-HBP [7, 23]. The cells were incubated in 500 mL flasks containing 120 mL basal salt medium (BSM) supplemented with 0.2 mmol/L DBT as the source of sulfur. Cell cultivation was carried out at 30 °C on a rotary shaker operated at 180 rpm (THZ-C rotary shaker, Taicang city, Jiangsu province, China). The inoculum size was 2% (v/v). The BSM was composed of KH_2PO_4 2.44 g, $Na_2HPO_4{\cdot}12H_2O$ 12.03 g, NH_4Cl 2.0 g, $MgCl_2{\cdot}6H_2O$ 0.4 g, $CaCl_2$ 0.75 mg, $FeCl_3{\cdot}6H_2O$ 1 mg, $MnCl_2{\cdot}4H_2O$ 4 mg and glycerol 10 g in one liter of deionized water.

Cell immobilization in calcium alginate beads

Cells were harvested in the late exponential phase by centrifugation at 5,000 × g for 5 min. The harvested cell pellets were washed twice with deionized water and suspended in it. Sodium alginate was dissolved in deionized water (4%, w/v) and mixed well with equal volume of cell suspension. Certain amounts of surfactant such as Tween 20, Tween 80, Span 80, etc. were added respectively into the mixture. The target concentration of these surfactants was set at 0.5% (w/v).

We used conventional methods to yield 2–5 mm beads by extruding the alginate as drops into a calcium salt solution for gelation. The gas jet extrusion technique, which was modified from Seifert and Phillips [21], was employed to produce smaller alginate beads. The bead fabrication procedure with a novel instrument is illustrated in Fig. 1. The resultant slurry was extruded through a cone-shaped needle into a stirred 0.1 M $CaCl_2$ gelling solution. The slurry is intruded as discrete droplets so as to form calcium-alginate beads with normal size (2.5 and 4 mm in diameter). To prepare smaller beads than 2 mm diameter, nitrogen gas was introduced around the tip of the needle to blow off the droplets. By adjusting the gas flow rate to 0.5 L/min, the size of the beads can be controlled at 1.5 mm in diameter.

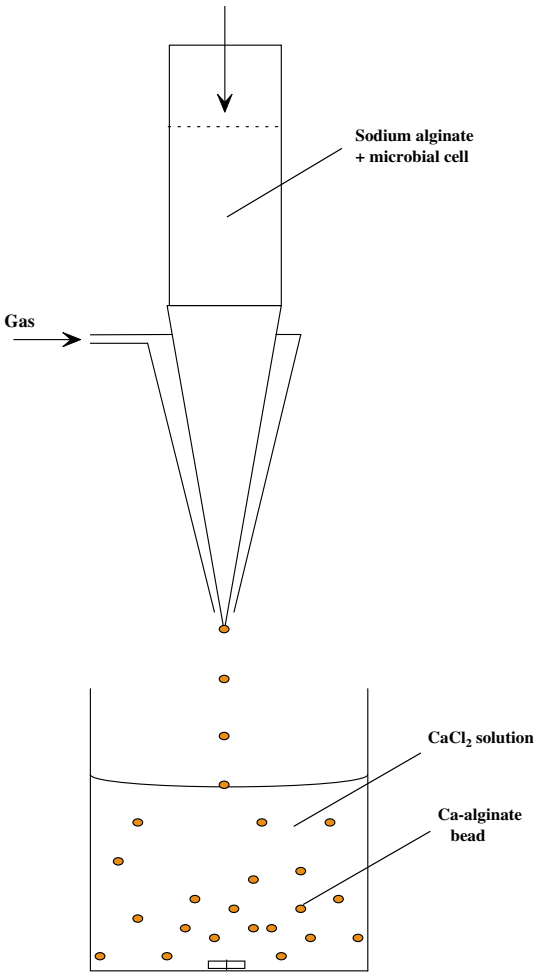

Fig. 1 Procedure for preparation of calcium-alginate beads

The beads were left in calcium chloride solution about 2 h for stabilization. Then, the beads were washed with deionized water to remove the residual calcium ions and then kept in saline at about 4 °C. Before being used, immobilized cells were activated for 1 day in modified BSM (MBSM) supplemented with 0.1 mmol/L DBT. Activation was carried out at 30 °C on a 180 rpm rotary shaker (THZ-C rotary shaker, Taicang city, Jiangsu province, China). To avoid the dissolution of alginate-immobilized cells in the BSM, MBSM was used as the aqueous phase for the activation of the immobilized cells. The phosphate components of MBSM were changed to KH_2PO_4 0.24 g and $Na_2HPO_4{\cdot}12H_2O$ 1.20 g. Other components were the same as those in BSM.

Biodesulfurization in model oil

We prepared model oil consisting of 1 mmol/L DBT in *n*-dodecane. The reaction solution contained alginate-immobilized cells (6 g beads, containing 0.15 g of dry cells), 5 mL of model oil and 10 mL of MBSM medium. Unless otherwise stated, the size of beads used in experiments was 2.5 mm. Biodesulfurization was carried out in 100 mL flasks at 30 °C on a rotary shaker at 180 rpm (THZ-C rotary shaker, Taicang city, Jiangsu province, China). The time course of DBT utilization and 2-HBP productions were obtained by sampling at defined time intervals for analyzing with HPLC. After reaction, the beads were separated from the model oil by a stainless steel sieve and regenerated in MBSM medium for recycle desulfurization. The regeneration procedures were the same as the activation described before. All experiments were conducted in triplicate.

Analytical methods

Cell density was calculated from the absorbance at 600 nm, with reference to a calibration curve constructed with scalar dilutions of a cell suspension of known density. One optical unit for the R-8 cells was equivalent to 0.395 g dry weight/L. The cell density within Ca-alginate beads was determined after dissolving the beads in a 0.5 mmol/L EDTA solution with continuous stirring. To determine the average size of the alginate beads, ten individual beads were measured with vernier calipers.

High-performance liquid chromatography (HPLC) was used for the quantitative assay of DBT (retention time = 5.94 min) and 2-HBP (retention time = 3.45 min) in the dodecane phase. HPLC was performed on a Hewlett Packard 1100 (HP1100, Agilent, USA) liquid chromatography equipped with an autosampler, a reversed-phase Kromasil-C18 column (250 × 4.6 mm; 5 μm) and a diode array detector (set at 254 nm). The mobile phase was composed of methanol–water (90:10, v/v) with flow rate of 1 mL min^{-1}. The specific desulfurization activity was expressed as the production rate of 2-HBP, which is the end product of the DBT degradation pathway.

The morphology of alginate-immobilized cells was determined using a scanning electron microscope (SEM) (JSM-6700F, JEOL, Japan).

Results

Effect of the size of immobilized beads on BDS

Desulfurization of DBT was carried out with different sizes of alginate-immobilized beads in the presence of *n*-dodecane. Table 1 shows the results of DBT remaining and 2-HBP produced in 24 h of reaction. The mean diameters of the immobilized beads were 1.5, 2.5 and 4.0 mm. The results demonstrated that the desulfurization of DBT by alginate-immobilized cells was affected by the size of the immobilized beads. The specific desulfurization rate of 1.5 mm diameter beads was 1.4-fold higher than that of 4.0 mm. Klein et al. [12] reported that a small microbead size is important for minimizing the mass-transfer resistance problem normally associated with immobilized cell culture. The desulfurization rate was improved with decreasing bead size, most probably due to the minimizing of the mass transfe barrier *r*.

Influence of the addition of surfactants on desulfurization rate

The influence of surfactants on the immobilized cells was evaluated by comparing the conversion of DBT into 2-HBP in model oil systems. Figure 2 compares 2-HBP production by immobilized cells treated with seven different kinds of surfactants, while an immobilization procedure with no surfactant served as the control. The production of 2-HBP mostly occurred in the first day. The addition of non-toxic and non-ionic surfactants including Span 20, Span 80,

Table 1 Effect of bead size (diameter) on biodesulfurization rate by alginate-immobilized cells of *P. delafieldii* R-8

Bead size (mm)	2-HBP production (mmol/L)	DBT residue (mmol/L)	Specific desulfurization rate (mmol L^{-1} h^{-1})
1.5	0.327	0.409	1.36×10^{-2}
2.5	0.286	0.445	1.19×10^{-2}
4.0	0.237	0.510	0.99×10^{-2}

* Reaction conditions: 6 g beads. Volume ratio of oil-to-water was 0.5, 24 h of reaction

Tween 20 and Tween 80 greatly enhanced the desulfurization rate compared to the control. However, the addition of CTAB and SDS decreased the production of 2-HBP. As shown in Fig. 2, Span 80 showed the highest effect on desulfurization activity.

Selection of optimal concentration of Span 80

In order to select the optimal concentration of Span 80 with respect to the desulfurization rate, further experiments were carried out. Calcium-alginate beads prepared with different Span 80 concentrations were used for biodesulfurization in oil-water systems. Figure 3 shows the influence of Span 80 on 2-HBP production. It can be observed that the 2-HBP production increased with Span 80 concentration. Optimal desulfurizing capability was obtained when the Span 80 concentration was controlled between 0.4 and 0.7%. In 24 h, the desulfurization rate with the addition of 0.5% Span 80 was 1.8-fold higher than that of without Span 80.

Scanning morphology of calcium-alginate beads

In order to understand cells distribution in calcium-alginate beads with Span 80, the sections of the beads after being repeatedly used for three times were observed by SEM. The results are shown in Fig. 4. A few *P. delafieldii* R-8 cells were observed on the surface of gel beads (Fig. 4a) and a highly macroporous structure was found in the beads which may favor of diffusion of substrates and dissolved gas. Large numbers of cells were evident within the

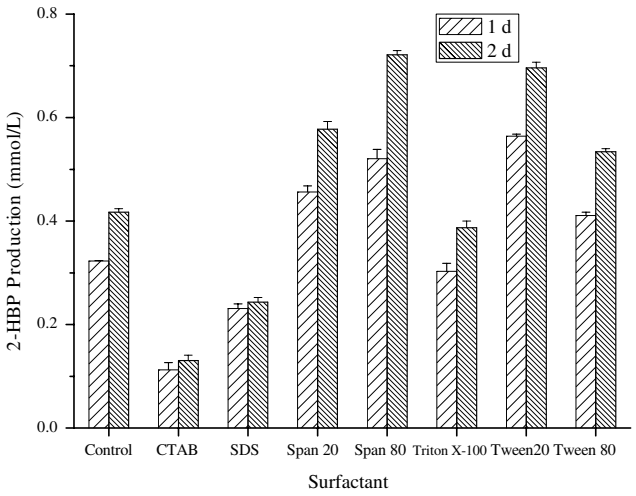

Fig. 2 Effect of different surfactants on the desulfurization activity of immobilized beads. Reaction conditions: 2.5 mm bead diameter, model oil (1 mmol/L DBT in *n*-dodecane), O/W (v/v) = 1/2, 2 days of reaction

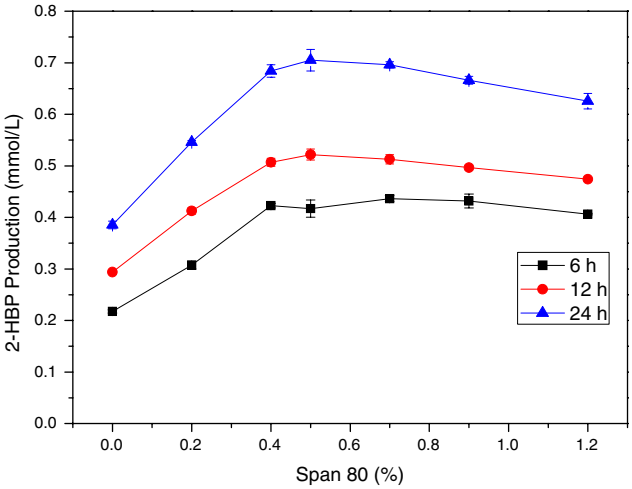

Fig. 3 Desulfurization activity of alginate beads treated with different concentrations of Span 80

Internet structures of the beads (Fig. 4b). *Pseudomonas delafieldii* R-8 cells appeared to be entrapped and maintained their structural integrity within the gel matrix.

Kinetics of biodesulfurization by alginate immobilized cells

Many kinetic analyses of biodesulfurization have been performed using free cells [26]. However, there are few reports on the kinetics of biodesulfurization by immobilized cells, especially, alginate-immobilized cells. In this work, we compared the kinetic parameters of desulfurization using alginate immobilized R-8 cells with or without addition of Span 80. The reaction systems were consisted of 6 g new activated immobilized beads (or 0.15 g dry cells), 20 mL MBSM medium and 10 mL model oil. The initial desulfurization rate was studied with various DBT concentrations in model oil (Fig. 5). The results could be represented by the Michaelis–Menten equation, as follows:

$$\upsilon = -\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{V_{\max} S}{K_m + S}$$

where S, concentration of DBT (mmol L^{-1}); $V_{\max}$, the limiting maximal velocity (mmol L^{-1} h^{-1}, or μmol/g DCW/h); K_m, Michaelis constant (mmol L^{-1}).

As shown in Fig. 5, both the K_m values of the free and immobilized cells were equivalent and were found to be 1.65 mmol L^{-1}. The $V_{\max}$ value of immobilized cells was less than the value of free cells. The $V_{\max}$ value of the immobilized cells with Span 80 was 0.34 mmol L^{-1} h^{-1}, and that of the control was 0.22 mmol L^{-1} h^{-1}. The $V_{\max}$ value of the free cells was 0.42 mmol L^{-1} h^{-1}. The Michaelis constants, an intrinsic parameter of cells, are not

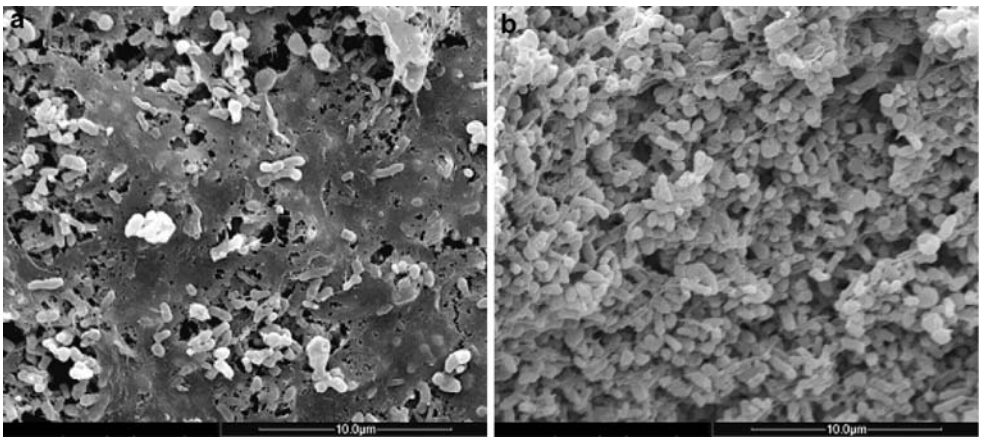

Fig. 4 SEM of alginate-immobilized cells of R-8 after 150 h of desulfurization of model oil **a** Surface of alginate-immobilized cells; **b** Cross section of alginate-immobilized cells

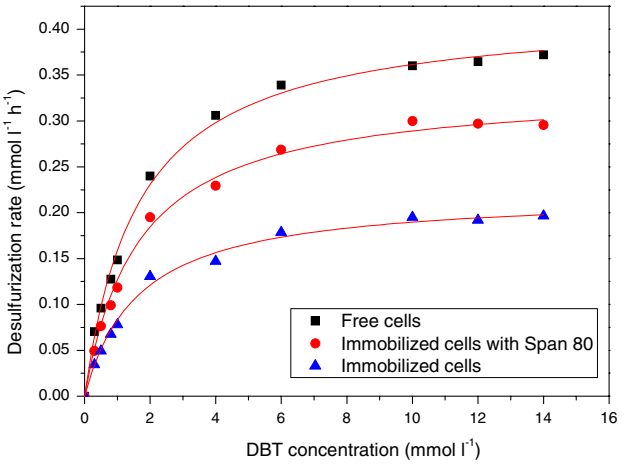

Fig. 5 Kinetics of desulfurization of DBT by the free and immobilized R-8 cells. The reaction was performed in O/W biphasic systems

affected by the immobilization process, while the maximal desulfurization rates are affected.

Discussion

Immobilized enzymes and whole cells as biocatalysts provide advantages such as enhanced stability, repeated or continuous use, easy separation from the reaction mixture and possible modulation of catalytic properties. Immobilization has become an indispensable part of industrial biotransformations [17]. The study of immobilized whole cells would give a solution to the problem in petroleum biodesulfurization, such as the troublesome process of recovering desulfurized oil and the short life of biocatalyst.

Hou et al. [9] reported that the maximal desulfurization rate of immobilized cells was lower than that of non-immobilized cells. The main advantages of immobilized cells could be repeated and convenient operations. As with most immobilization systems, the diffusional rate of substrates and products within the bead often limits productivity. Mass transfer involved in diffusion of a substrate to a reaction site and in removal of inhibitory or toxic products from the environment may be impeded. With the gas jet extrusion technique, we successfully prepared alginate-immobilized beads for biodesulfurization. This method was rapid and simple. As we have proved, the obvious approach to minimize these effects is to minimize the diffusional distance through a reduction in bead size [1].

Song et al [25] have employed Tween 20 to improve the permeability of the entrapment–encapsulation hybrid membrane. According to Ref. [25], the immobilized beads without Tween 20 would rupture because of the formation of CO_2 and N_2 as result of respiration and denitrification. In our study, we found that Span 80 and Tween 20 can significantly increase the desulfurization activity of immobilized cells. The specific desulfurization activity of various cultures was described in the literature [11]. But there is no report on the activity of immobilized cells. In the Michaelis–Menten equation, the change of V_{max} may result from the differences of nutriments, substrates and metabolites diffusion between the immobilized beads with or without addition of Span 80. Therefore, the V_{max} value of control (15 μmol/g DCW/h) was less than the V_{max} value of Span 80 (23 μmol/g DCW/h). Thus, the use of Span 80 in cell immobilization is beneficial, increasing permeability and mass transfer and resulting in a higher biocatalytic activity.

To improve biodesulfurization rate of immobilized cells is a key to industrialize biodesulfurization technology. We employed a simple way by decreasing the beads size and adding Span 80 to achieve the purpose. The encouraging results of this work may be generally applicable to other area of biocatalytic and biotransformation processes.

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