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Deep desulfurization of hydrodesulfurized diesel oil by *Pseudomonas delafieldii* R-8

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Abstract: Biodesulfurization is a promising technology for deep desulfurization. The remaining alkylated DBTs (dibenzothiophenes) in the HDS-treated (hydrodesulfurized-treated) diesel oil could be selectively and efficiently desulfurized by resting cells of *Pseudomonas delafieldii* R-8, a Gram-negative bacterium. The desulfurization activities of resting cells were greatly affected by W/O ratio (the volume ratio of aqueous phase to oil phase) and cell concentration. The desulfurization activity increased with the increase in the W/O ratio. When the W/O ratio and cell concentration were 2 and 25 mg cm^{-3}, the desulfurization activity was as high as 0.41 mg(total sulfur) g^{-1}(dry cell weight, DCW) h^{-1}, ie higher than that reported previously. GC-AED (gas chromatography with an atomic emission detector) analysis showed that the total reductions for all the C1DBTs and C2DBTs were approximately 100%, 94.63% for C3DBT, and 97.09% for C4DBT (designated C*x*DBT, where *x* is the number of alkyl groups attached). The rates of biodesulfurization relate to the number and position of alkyl groups attached to the DBT.

Keywords: biodesulfurization; hydrodesulfurized diesel oil; *Pseudomonas delafieldii*; alkylated dibenzothiophenes

INTRODUCTION

Diesel oil contains large amounts of organosulfur compounds and its combustion generates sulfur oxides, which leads to harmful effects on the environment as acid rain and air pollution. Thus, the regulations for the sulfur level in diesel oil have become increasingly strict.[1] The hydrodesulfurization (HDS) process has been routinely applied in refineries to decrease the sulfur content in diesel oil. The process converts organic sulfur in the feed to hydrogen sulfide in the presence of metallic catalysts and hydrogen gas under extremely high temperature (290–450 °C) and pressure (10–20 MPa). Increasingly more severe and more costly HDS is required to satisfy lower sulfur levels, especially because the hydrodesulfurized catalysts are very expensive.

Biodesulfurization (BDS) is a promising technology and offers an alternative way to reach lower sulfur levels selectively and cost-effectively.[2,3] In particular, BDS can produce some high value-added surfactants such as benzenesulfinase. Thus, BDS has attracted increased attention since the end of the last century. Many bacterial strains such as *Rhodococcus erythropolis* IGTS8,[4,5] *R erythropolis* D-1,[6] *R erythropolis* KA2-5-1,[7] *R erythropolis* ECRD-1[8] and *Gordonia nitida* CYKS1[9] have been reported for use in desulfurization. These reports mainly focused on biodesulfurization of model oil containing dibenzothiophenes (DBTs), benzothiophene and naphthothiophene.[10] In order to be commercially useful, biodesulfurization must be able to remove the sulfur from real fuels. There have been a few reports on the biodesulfurization of fossil fuels, including diesel oil.[11] *Mycobacterium phlei* WU-F1 was applied in biodesulfurization of hydrodesulfurized light gas oils,[12] *R erythropolis* ECRD-1 was used for deep desulfurization of extensively hydrodesulfurized middle distillate oil,[6,13] and *R erythropolis* I-19 desulfurized alkylated dibenzothiophenes from a hydrodesulfurized middle distillate.[4] *Gordona* strain CYKS1 was also used to desulfurize diesel oils,[14,15] and *R erythropolis* IGTS8 was used for desulfurization of crude oils.[16] However, diesel oil is a complicated system, which consists of a variety of aromatics, alkenes and aliphatics. The solvent tolerance of the bacteria is one of the significant factors for biodesulfurization of real fuels. *Pseudomonas delafieldii* R-8, a Gram-negative bacterium, was isolated by our laboratory[17] and had higher solvent tolerance than Gram-positive bacteria.[18] The mechanism of biosulfurization complies with a 4S pathway and could convert DBT to 2-HBP and sulfate.

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In this study, the main objective was to investigate the desulfurization of hydrodesulfurized diesel oil with *P delafieldii* R-8. The conditions (aqueous media, the volume ratio of aqueous phase to oil phase, cells content, styles of organosulfur compounds, kinetics, etc) for biodesulfurization were optimized.

MATERIALS AND METHODS

Bacterial strain and medium

P delafieldii R-8 (CGMCC 0570) was isolated from the sewage pool of Shengli oil field(China).[17] The culture medium was composed of 2.44 g KH_2PO_4, 12.03 g $Na_2HPO_4.12H_2O$, 2.0 g NH_4Cl, 0.4 g $MgCl_2.6H_2O$, 0.75 mg $CaCl_2$, 1 mg $FeCl_3.6H_2O$, 4 mg $MnCl_2.4H_2O$ and 10 g glycerol in 1 dm^3 of deionized water. DBT was prepared as 100 mmol dm^{-3} in ethanol; 0.1 mmol dm^{-3} DBT acted as the source of sulfur. Cells were cultivated in 500 cm^3 flasks containing 150 cm^3 culture medium. Cell cultivation was carried out in flasks in a reciprocal shaking incubator at 30 °C at 180 rpm.

Diesel oil

Hydrodesulfurized diesel oil, containing 591 mg(total sulfur) dm^{-3}, was obtained from the Research Institute of Petroleum Processing (Beijing, China).

Biodesulfurization of oil

Biodesulfurization of diesel oil was performed by resting cells of *P delafieldii* R-8. In preparing resting cells, the cells in the late logarithmic phase were harvested from the culture broth by centrifugation at 5000 × *g* and 4 °C for 6 min (Avanti J-E, Beckman, America). The cells were washed twice with brine solution. The resulting resting cells were kept in 0.1 mol dm^{-3} phosphate buffer (pH 7.0) at 4 °C.

The reaction mixture consisted of diesel oil and aqueous medium containing resting cells. The reaction for desulfurization was carried out in flasks in a reciprocal shaking incubator at 30 °C at 180 rpm.

Analytical methods

The reaction mixture was centrifuged at 10 000 × *g* for 1 min at 20 °C. The oil phase was collected as the upper fraction. The total sulfur content (by weight) was determined in triplicate for each sample by combustion of samples and measurement of released sulfur dioxide with a model RPA-200 microcoulomb analyzer (JiangHuan Electroanalysis, China). The distribution of sulfur compounds in diesel oil and the treated oil was analyzed by gas chromatography with an atomic emission detector (GC-AED, G2350A; Agilent, USA).

RESULTS

Effects of aqueous media on the desulfurization activity for diesel oil

Sulfur-free culture medium, 0.1 M phosphate buffer (pH 7.0) and brine (8.5 g dm^{-3} sodium chloride) were, respectively, selected as the aqueous medium for desulfurization of diesel oil. The volume ratio of aqueous to oil phase (W/O) was 5:1 and the volume of oil was 5 cm^3. The cell concentration was 30 mg(DCW, dry cell weight) cm^{-3} of aqueous phase. The results showed that the desulfurization rates in culture medium, phosphate buffer and brine were 0.398 mg(total sulfur) g^{-1}(DCW) h^{-1}, 0.362 mg(total sulfur) g^{-1}(DCW) h^{-1} and 0.379 mg(total sulfur) g^{-1}(DCW) h^{-1}, respectively. The biodesulfurization of diesel oil by resting cells of R-8 could proceed in brine and the phosphate buffer as efficiently as in the growth medium. The possible reason was that the desulfurization activity was not affected by the medium after the desulfurization enzymes had formed in the cells.[19] Brine was selected preferentially as aqueous medium for biodesulfurization in the following experiments.

Effects of W/O ratio on the desulfurization of diesel oil

The volume ratio of W/O is an important factor in determining the reactor volume required and the reactor productivity. The desulfurization reaction solution contained 10 cm^3 diesel oil, 0.60 g(DCW) resting cells and brine, with the volume of brine ranging from 0 to 100 cm^3. The corresponding W/O ratios were 0, 0.5, 1.0, 3, 7 and 10, respectively. As shown in Fig 1, the desulfurization rates increased with the increase in the W/O ratio. When the W/O ratio was over 2, the desulfurization activity increased very slowly. The maximal desulfurization rate was 0.41 mg(total sulfur) $g(DCW)^{-1}\,h^{-1}$. The possible reason might be due to the formation of an oil-in-aqueous phase emulsion. The interfacial area increased and resulted in the increase in the desulfurization rate. When the volume of oil is a fixed value, the maximal interfacial area does not change with the further increase in the water volume. Thus, the desulfurization rate also did not change when the W/O ratio was over 2. However, when the W/O

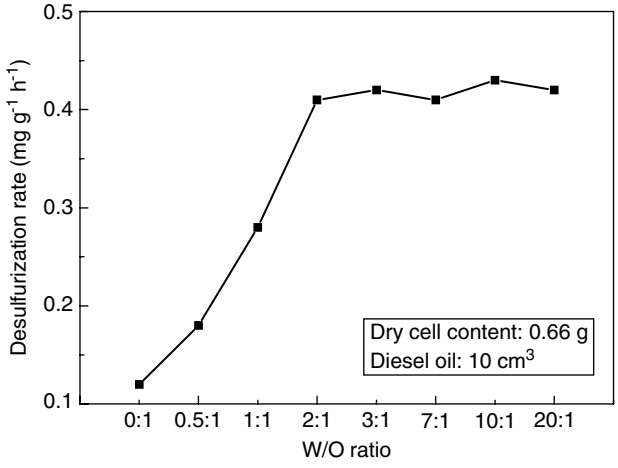

Figure 1. Effects of W/O ratio on the desulfurization rates for total sulfur of diesel oil by resting cells of *P delafieldii* R-8.

ratio was 0, the total sulfur content decreased very slowly. The desulfurization activity was very low: this is because the desulfurization enzymes have not their catalysis activities without an aqueous medium.

Effects of cell contents on the desulfurization of diesel oil

To study the effect of cell concentration on the desulfurization rate, the reaction solution containing 40 cm^3 brine, 10 cm^3 diesel oil and a known amount of resting cells were used. The cell concentration in the aqueous phase was changed from 5 to 35 mg(DCW) cm^{-3}. The maximal desulfurization rate for the first 4 h was 0.43 mg(total sulfur) $g(DCW)^{-1}\,h^{-1}$ with the cell concentration of 25 mg cm^{-3}. As shown in Fig 2, the specific desulfurization rate of total sulfur decreased when the cell concentration exceeded 25 mg cm^{-3}. The reaction solution was centrifuged to separate the oil from the aqueous phase after the end of the reaction. It was shown that almost all cell pastes collected at the organic–aqueous interface after centrifugation when the cell concentration was 25 mg cm^{-3}. However, at higher cell concentrations, some of the cells collected at the organic–aqueous interface while others precipitated at the bottom of the cuvette. This implied that the cells which precipitated at the bottom did not contact well with the diesel oil phase to react with sulfur compounds. From such phenomena, we speculated that the desulfurization process might be limited by the rate of surface renewal as new biocatalyst interacts with the organic–aqueous interface, as found by Kaufman *et al*.[16] This indicated that the mass-transfer resistance was probably the controlling factor in the reaction. The biodesulfurization rate in the aqueous phase is higher than that in the two oil–aqueous phases. If the other conditions were kept, the desulfurization rate was 0.39 mg(total sulfur) $g(DCW)^{-1}\,h^{-1}$ with an agitated vessel reactor, and 0.35 mg(total sulfur) $g(DCW)^{-1}\,h^{-1}$ with a reciprocal shaker. However, both total reductions are similar, about 69%. All these experiments, including the effects of W/O ratio, prove the same thing, ie the mass-transfer process might the controlling step of the BDS reaction.

Biodesulfurization of diesel oil with free R-8 cells

According to the aforementioned results, experiments using a reaction solution containing 80 cm^3 brine, 10 cm^3 diesel oil and 2.0 g(DCW) of resting cells were carried out. To investigate the time course of biodesulfurization of total sulfur in diesel oil with free R-8 cells, the content of total sulfur was analyzed each 2 h. In order to further decrease the sulfur content, the treated oil was recovered and brought again into contact with fresh cells. The results are shown in Fig 3. Curve 1 represents the first BDS-treatment and curve 2 the second BDS-treatment. It resulted in a 90.5% reduction of total sulfur from 591 mg dm^{-3} to 56 mg dm^{-3} by the two consecutive treatments. It has been reported previously that in the desulfurization of diesel oils, the total sulfur content of a middle- distillate unit feed (MDUF) was decreased from 0.15% (w/w) to 0.06% by resting cells of *Gordona* sp CYKS1. The light gas oil (LGO) decreased from 0.3% (w/w) to 0.15%.[12] *R erythropolis* I-19, a genetically engineered bacterium of *R erythropolis* IGTS8, reduced the sulfur in oxidized MDUF from 1850 to 615 ppm, a 67% reduction.[4] We found that the free R-8 cells could maintain good desulfurization activities in the first 6 h. Then the activity decreased substantially, as shown in Fig 3. This indicated that the desulfurization enzymes of resting R-8 cells could maintain their activities for about 6 h in the two oil–aqueous phases. In the first treatment, the desulfurization rate for total sulfur was about 0.36 mg g^{-1}(DCW) h^{-1} for the first 4 h. Comparable desulfurization rates have been reported previously for desulfurization of diesel oils by resting cells of *Gordona* sp CYKS1, and the desulfurization rates of MDUF and LGO were 0.17 and 0.15 mg(total sulfur) $g(DCW)^{-1}h^{-1}$,[12] respectively. The desulfurization rate of MDUF was 0.15 mg(total sulfur) $g(DCW)^{-1}h^{-1}$ by *R erythropolis* I-19.[4]

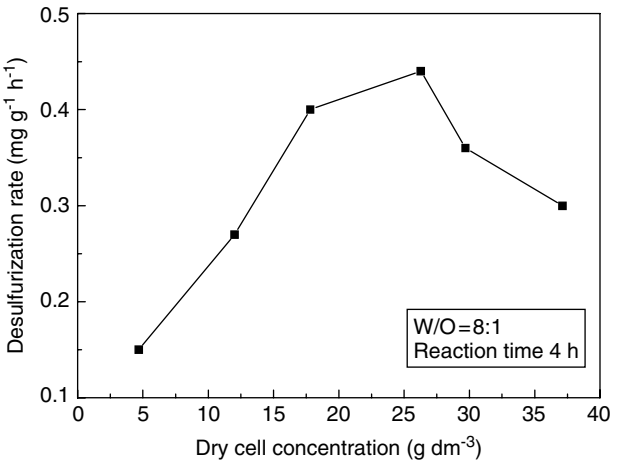

Figure 2. Effects of cell concentrations on the desulfurization rates for total sulfur of diesel oil by resting cells of *P delafieldii* R-8.

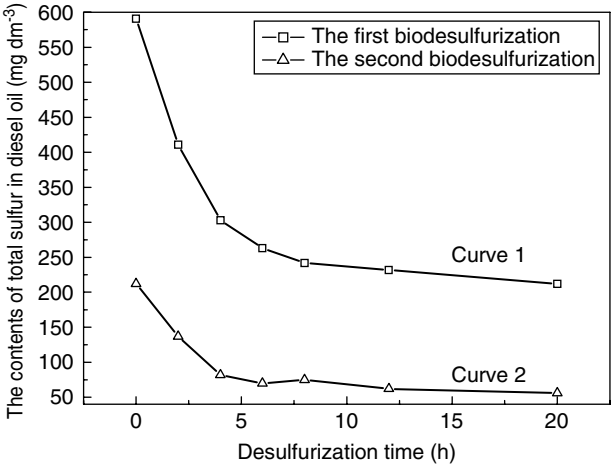

Figure 3. Time courses of two consecutive biodesulfurizations by resting cells of *P delafieldii* R-8.

Table 1. GC-AED analysis of diesel oil before/after being biodesulfurized by resting cells of *P delafieldii* R-8

Compound	Amount before biodesulfurization ($mg\,dm^{-3}$)	Amount after the first biodesulfurization ($mg\,dm^{-3}$)	Amount after the second biodesulfurization ($mg\,dm^{-3}$)	Total reductions of sulfur compounds (%)
C1DBT	9.04	—	—	100
C2DBT	69.27	8.61	—	100
1 or 3-C2DBT	11.15	—	—	100
C3DBT	50.99	8.06	2.74	94.63
C4DBT	111.81	21.05	3.25	97.09
C5 or C6DBT	32.56	48.03	31.21	4.15
2,6-diCH_3DBT	8.75	—	—	100
1,7-diCH_3DBT	31.22	—	—	100
2,4,6-triCH_3DBT	3.33	2.56	—	100
Total	328.12	88.31	37.2	88.66

Folsom *et al*[4] reported that *R erythropolis* I-19, a Gram-positive bacterium, preferentially desulfurized DBT and C1DBTs, followed by the more highly alkylated C*x*DBTs(where *x* is the number of alkyl groups attached, eg C2DBT includes all dimethyl and monoethyl substituted DBTs). To analyze the desulfurization activity of the Gram-negative bacterium against different alkylated C*x*DBTs in diesel oil, GC-AED analysis was performed. Most of the mercaptan and sulfide components of the total sulfur were eliminated by HDS treatment, leaving primarily thiophenic sulfur compounds. There are the only alkylated DBTs remaining in the HDS-treated diesel oil which are harder to remove than DBTs by conventional HDS treatment.[20] The alkylated DBTs could probably be desulfurized by the most advanced HDS, but it is very costly. Table 1 shows that the types of organosulfur compounds in the hydrodesulfurized diesel oil before biodesulfurization were mainly C*x*DBTs and included 34.08% C4DBT, 24.51% C2DBT, 15.54% C3DBT, 9.92% C5 or C6DBT, 9.51% 1,7-diCH_3DBT, 2.76% C1DBT, 2.67% 2,6-diCH_3DBT and 1.01% 2,4,6-triCH_3DBT. Except for C5 or C6DBT and 2,4,6-triCH_3DBT, the contents of other alkylated DBTs significantly decreased after biodesulfurization. This indicated that C*x*DBTs reacted with R-8 cells with different degrees of desulfurization activities, depending on the extent of alkyl group substitution (ie the *x* value). The total reductions by the two consecutive reactions for all the C1DBTs and C2DBTs were approximately 100%, 94.63% for C3DBT, and 97.09% for C4DBT. However, C5 or C6DBT were hardly attacked by R-8. Firstly, the number of alkyl groups adjacent to the sulfur center in C*x*DBTs increased as the *x* value increased, so the sulfur atom might be sterically hindered. Secondly, the corresponding hydrophobicity might increase with increasing values of *x*. Hydrophobic compounds tend to be expelled from the aqueous environment and thus hardly contact with the cell surface. This resulted in lower reactivities of cells for C5 or C6DBTs and the increasing number of alkyl groups decreased the reactivity. Thus, the BDS process of Gram-negative bacteria is similar to that of Gram-positive bacteria.

CONCLUSIONS

The remaining alkylated DBTs in the hydrodesulfurized diesel oil could be efficiently desulfurized by resting cells of *P delafieldii* R-8, a Gram-negative bacterium. The desulfurization activities of resting cells were almost the same in the non-growth media (such as brine and phosphate buffer) and in the growth medium. The desulfurization activity for diesel oil strongly depended on the W/O ratio and cell concentration. When the W/O ratio and cell concentration were 2 and $25\,mg\,cm^{-3}$, respectively, the desulfurization rate reached its maximal value and was 0.41 mg(total sulfur) g^{-1}(DCW) h^{-1}, a rate higher than that reported previously. Free R-8 resting cells could result in a 90.5% reduction of total sulfur from $591\,mg\,dm^{-3}$ to $56\,mg\,dm^{-3}$ by two consecutive biodesulfurizations. GC-AED analysis showed that the reductions for all the C*x*DBTs remaining in the diesel oil were: approximately 100% for C1DBT and C2DBT, 94.63% for C3DBT, and 97.09% for C4DBT; however, C5 or C6DBT were almost unaffected by free R-8 cells. This result is similar to that reported for of Gram-positive bacteria. Therefore, the use of Gram-negative *P delafieldii* R-8 is promising in the microbial deep desulfurization for diesel oil.

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