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Effects of surfactant on biodesulfurization by *Corynebacterium* sp. ZD-1 in the presence of organic phase*

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Abstract: The effects of surfactants on dibenzothiophene (DBT) degradation by *Corynebacterium* sp. ZD-1 were investigated in hydrocarbon aqueous biphasic (O/W) systems in shake flask. Among Brij-35, Tween-80, Triton-100X and β-cyclodextrin, Tween-80 was a suitable surfactant to improve the desulfurization rate of dibenzothiophene. The amount of 2-hydroxybiphenyl (2-HBP) formed with Tween-80 present was about 50% more than that formed without surfactant. The results demonstrated that Tween-80 could improve the mass transfer of DBT between organic and aqueous phases, and could be used in dibenzothiophene biodesulfurization systems.

Key words: Biodesulfurization, Tween-80, Dibenzothiophene, *Corynebacterium* sp.
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INTRODUCTION

Emission of sulfur oxides by combustion of fossil fuel is an environmental problem. Governments all over the world have recognized the problems and enacted legislations to reduce the emission (Monticello, 1998; Ohshiro and Izumi, 1999).

Hydrodesulfurization (HDS) is a traditional process used in petroleum desulfurization, which requires high pressure (1~20 MPa) and high temperature (290~450 °C) (Monticello, 1998). However, up to 70% of the sulfur in petroleum is in the form of heterocyclic sulfur compounds such as dibenzothiophene (DBT) and substituted DBTs (methylated DBTs and benzo-DBTs) that cannot be completely removed by HDS process. Thus DBT and DBTs are generally considered as the sulfur model compounds for biodesulfurization (Monticello, 2000).

Early researches using model compound like dibenzothiophene in aqueous systems bore little resemblance to the conditions that the biocatalyst would encounter in commercial applications (Ohshiro and Izumi, 1999). Many researches showed that DBT desulfurization rate in two-phase systems was higher than that in aqueous phase. The organic phase might be model oil such as *n*-tetradecane (Noda *et al*., 2003), diesel (Ma *et al*., 2004), hexadecane (Li *et al*., 2005), or the real fossil fuels including middle distillates, diesel oil, and gasoline (Sylvie and Rodolfo, 2003). The results indicated that barely soluble in water, DBT degradation by microorganisms is located at the interface between the organic and the aqueous phases. When an oil-bacteria-water emulsion is formed, the overall biotransformation rate depends not only on biokinetic factors, but also on physicochemical constrains that control the bioavailability. Dibenzothiophene desulfurization by *Rhodococcus* appears to occur intracellularly with DBT uptake from the oil phase possibly occurring after transient adsorption to

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the cell (Oldfield *et al.*, 1997). In the reaction of desulfurization enzyme (*dsz*) recombinants of *Escherichia coli* and *Pseudomonas putida*, DBT appears to partition into the aqueous phase prior to cellular uptake (Gallardo *et al.*, 1997). That means bioavailability depends on the DBT uptake mechanism which occurs either directly by contact between the cells and the organic phase or by DBT solubilization in the aqueous medium.

Surfactants are amphiphilic compounds (containing hydrophobic and hydrophilic portions) that reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface. They have been used industrially for their desirable properties including solubility enhancement, surface tension reduction, wettability and foaming capacity. Only few were reported for surfactant's application in biodesulfurization. Marzona *et al.*(1997) found that the binding of benzothiophene (BT) and DBT with cyclodextrins (CD) could strongly enhance their solubility in water. They also measured the growth kinetics of *Acinetobacter* with β-CD+DBT and observed an inhibitory effect when the concentration of DBT was over 0.13 mmol/L. Jiang *et al*.(2002) found that surfactants could improve the desulfurization rate. The *pseudomonas delafieldii* strain R-8 could remove 72% of the organic sulfur from low sulfur diesel oil (S<300 mg/L) in 72 h at 250 r/min with Tween-80 present.

To select a suitable surfactant for *Corynebacterium* sp. ZD-1 to enhance its desulfurizaion activity in hydrocarbon aqueous biphasic systems, the effects of some surfactants on DBT biodegradation were studied. The solubilization effect of surfactants on DBT and the effect of surfactants on microorganism growth were also evaluated.

MATERIALS AND METHODS

Chemicals

Dibenzothiophene (DBT) (99%), Brij35 (97%) and 2-hydroxybiphenyl (2-HBP) (98%) were obtained from Acros Organics (New Jersey, USA); Trition-100X (98%) was from Sigma Chemical Company. All other chemicals were of analytical grade, commercially available and used without further purification.

Microorganism and media

The minimal salt medium (MSM) used in this study was a sulfur-free medium containing 5 g glycerol, 5.0 g $K_2HPO_4{\cdot}3H_2O$, 2.0 g $NaH_2PO_4{\cdot}2H_2O$, 0.2 g $MgCl_2{\cdot}6H_2O$, 5.0 g NH_4Cl, 1 ml of mineral solution per 1000 ml of deionized water. One ml of mineral solution that contained 0.1 mg $CuCl_2{\cdot}2H_2O$, 0.4 mg $CoCl_2{\cdot}6H_2O$, 0.2 mg $ZnCl_2$, 20 mg $CaCl_2$, 0.05 mg H_3BO_3, 0.2 mg $NaMoO_4{\cdot}2H_2O$, 4 mg $FeCl_3{\cdot}7H_2O$, 0.1 mg $AlCl_3{\cdot}6H_2O$ and 0.8 mg $MnCl_2{\cdot}4H_2O$. DBT was dissolved in ethanol (100 mmol/L) and added to a sterilized MSM.

Corynebacterium sp. ZD-1 (Wang *et al*., 2004a; 2004b) was isolated from refinery sludge of Hangzhou Refinery Co. (Hangzhou, China).

Culturing of strain ZD-1

ZD-1 strain was cultured in 250 ml conical flasks containing 100 ml of MSM at 30 °C and 150 r/min in a rotary shaker. The initial DBT concentration was 0.2 mmol/L. Cells were harvested in the late growth phase, washed twice with a 0.1 mol/L potassium phosphate buffer (pH 7.0), and then suspended in phosphate buffer and stored at −20 °C.

Dibenzothiophene desulfurization study

All desulfurization experiments were carried out in duplicate in 25 ml flasks using DBT in *n*-hexadecane as model oil at 30 °C in a rotary shaker.

Solubility experiments

Solubility experiments were performed in 25 ml flasks in the presence of excess DBT at 30 °C and 150 r/min for 72 h in a rotary shaker, analyzed after filtrating to separate the undissolved DBT, and repeated thrice.

Analytical methods

The concentration of cells was calculated from a linear relationship between the optical density at 620 nm (OD_{620}) and dry cell weight. DBT and 2-HBP were determined with GC (Fuli 9790A) equipped with Flame Ionization Detector using SE-54 capillary column (30 m×0.32 mm×0.32 μm). The injector and detector temperature were both 280 °C. The column temperature was 150 °C for 5 min, followed by 5 °C/min to 200 °C for 5 min and followed by 5 °C/min to 280 °C for 5 min. Carrier gas used was N_2.

RESULTS AND DISCUSSION

Effect of O/W ratio on the desulfurization activity

The volume ratio of oil to water (O/W) affects the bioavailability of DBT when biodesulfurization occurs in the interface between the organic and the aqueous phases.

The O/W ratio is also an important factor in determining the reactor productivity and thus the reactor volume.

As shown in Fig.1, the effect of O/W ratio on specific production rate of 2-HBP exhibited similarity to the 2-HBP formed. When the O/W ratio was 1:2, both the specific production rate of 2-HBP and the 2-HBP formed were much higher than others. While under higher oil held-up, with O/W ratio of over 2:1, far less degradation activity was detected. This was similar to the study with *Pseudomonas delafieldii* R-8 in dodecane (Luo *et al*., 2003) with an optimum of O/W ratio at 1:4. Under low concentration, an oil-in-water emulsion was formed with the oil/water interface increasing with the proportion of oil. Meanwhile hexadecane decreased the feed back inhibition of the biocatalyst due to the by-products accumulation in water phase. Thus, The specific production rate of 2-hydroxybiphenyl (2-HBP) in media with 1:2 O/W ratio was about 1.72 times that in aqueous phase. Because water was necessary for enzyme activity, too high concentration of hexadecane resulted in low desulfurization rate.

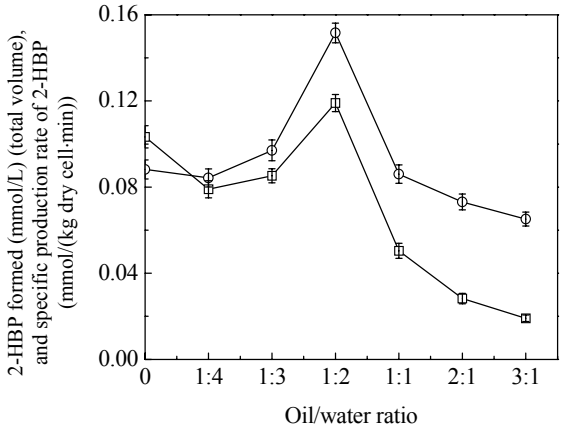

Fig.1 Effect of O/W ratio

Reactions were performed at 30 °C, 200 r/min, 10 ml total volume, 0.5 mmol/L DBT, and lasted for 9 h. ○: Specific production rate of 2-HBP; □: 2-HBP formed

Effects of rotation rate on the desulfurization

The shaker rotation rate was another important factor that could influence the biodesulfurization activity. The concentration of the 2-HBP formed is shown in Fig.2. At lower rotation rate, increasing shake rate could improve the mixture of the two phases, accelerate the transfer of DBT to the water phase, and enhance the transport of oxygen as well. Thus, the concentration of 2-HBP increased with the increasing rotation rate before it reached 250 r/min. At higher rotation rate, the mass-transfer limited desulfurization had turned to reaction controlled. Moreover, too high rotation rate would be harmful to the bacteria. Therefore, the rotation rate of 250 r/min was preferred.

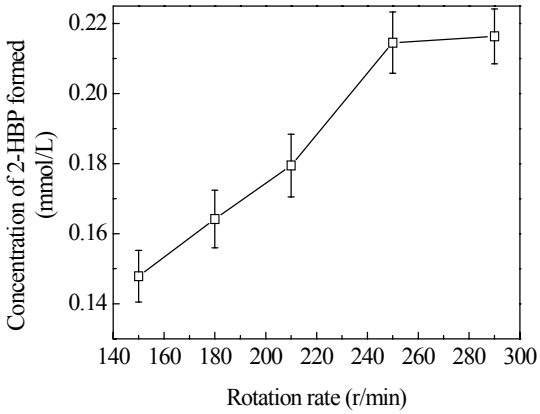

Fig.2 Effects of rotation rates

Reactions were performed at 1:2 O/W ratio, 30 °C, 1 mmol/L DBT, and lasted for 2 h

Enzyme stability in organic media

The longevity of desulfurization enzymes was dependent on the microorganisms used for bioconversion and the media they were in. As shown in Fig.3, the concentration of 2-HBP remained constant after 6 h. This indicated the desulfurization enzymes were inactivated. It was found that the desulfurization activity of ZD-1 resting cells in aqueous phase remained for eight hours in the former research (Wang *et al*., 2004a; 2004b).

In this case, the presence of hexadecane exerted a little unstable effect on the stability of the resting cells, like some other strains that had been reported (Samir *et al*., 2003).

Solubilization of DBT

Fig.4 shows the solubilition of DBT in deionized

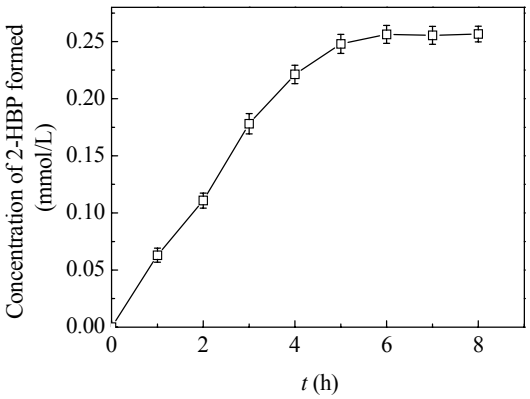

Fig.3 Desulfurization curve
Reactions were performed at 1:2 O/W ratio, 1 mmol/L DBT, 30 °C and 250 r/min

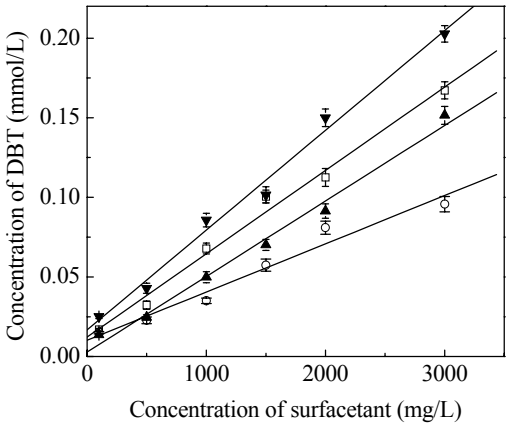

Fig.4 The solubility of DBT at concentrations of surfactants
Solubility experiments were performed in flasks in the presence of excess DBT at 30 °C and 150 r/min for 72 h, and analyzed after filtrating to separate the undissolved DBT. □: Tween-80; ○: β-cyclodextrin; ▲: Brij; ▼: Triton-100X

water vs the concentration of the four kinds of surfactants. The solubility of DBT in water was greatly enhanced linearly by each of the four surfactants.

Solubility enhancement efficiencies of surfactants above the CMC followed the order Triton-100X>Tween-80>Brij 35>cyclodextrin. Defining S_w^* as the DBT concentration in the presence of surfactant, S_w as the DBT concentration in water, S_w^*/S_w means the enhancement factor of the surfactant for DBT. As S_w was about 5.7×10^{-6} mol/L, enhancement factors in this order were about 35.6, 29.3, 26.6 and 16.8 when the concentration of surfactants reached 3000 mg/L.

Selection of surfactant

The effects of surfactants on DBT degradation are given in Fig.5. Both Brij and Triton-100X inhibited DBT degradation. β-cyclodextrin produced almost no effect. Tween-80 increased the desulfurization efficiency. In the cell growth study, similar effects were found as above (not shown in Fig.5). When surfactants were added as carbon sources, only Tween-80 could sustain cells growth. As mentioned before, all surfactants investigated here could greatly enhance the solubility of DBT. It was found in the experiments that all four surfactants could promote the organic phase dispersing in aqueous phase, which would improve the mass transfer of DBT between organic and aqueous phases. Thus, their influence on the bacteria might be the main causative reason for the different effects on DBT degradation. Brij and Triton-100X could inhibit the growth of the ZD-1, due to their toxicities to the bacteria. Although β-cyclodextrin did not inhibit cells growth, its ability of solubilization was much less than Tween-80. Therefore, Tween-80 was the optimal surfactant to be selected for further study.

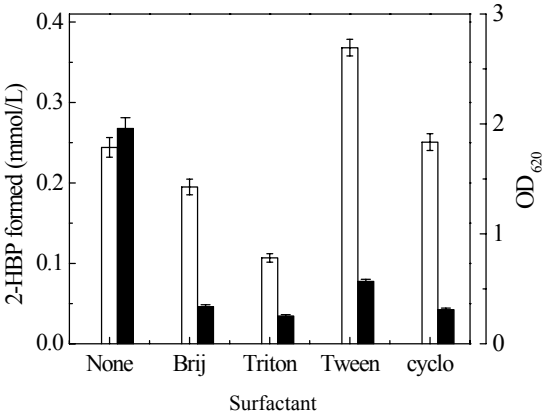

Fig.5 Effects of surfactants on DBT degradation and surfactants acted as carbon source for ZD-1 growth
Degradations were performed at 1:2 O/W ratio, 1 mmol/L DBT, 1 g/L surfactant, 30 °C, 250 r/min, and reacted for 24 h. Growth conditions were 10 ml no-glycerol (expect the none) MSM, 10% inoculation, 0.2 mmol/L DBT, 5 g/L surfactant, 30 °C, 150 r/min, and cultured for 48 h. ■: OD_{620}, □: 2-HBP formed

Optimum concentration of Tween-80

Fig.6 shows that Tween-80 favored ZD-1 growth, probably because Tween-80 increased the absorption and the degradation of the bacteria to DBT,

and thus supplied the bacteria with enough sulfur-source necessary for growth. In another research, the resting cells cultivated in the presence of Tween-80, however, showed no desulfurization activity. This further proved that Tween-80 changed the ways of DBT transferring from aqueous phase to cells. The optimum value of Tween-80 concentration for desulfurization was about 0.5 g/L. The amount of 2-HBP formed with 0.5 g/L Tween-80 present was about 50% more than that formed without surfactant. This was better than the effect of cyclodextrine on IGTS8 (Setti *et al*., 2003).

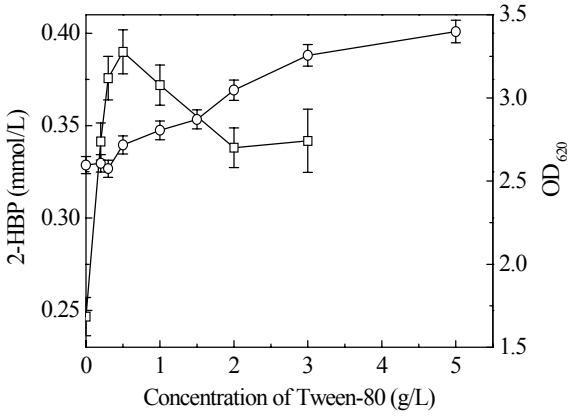

Fig.6 Influence of concentration of Tween-80 on ZD-1 growth and DBT degradation

Reactions were performed at 10 ml MSM, 10% inoculation, 0.2 mmol/L DBT, 30 °C, and 150 r/min for 48 h. Degradations were performed at 1:2 O/W ratio, 1 mmol/L DBT, 30 °C, 250 r/min, and reacted for 6 h. ○: OD_{620}; □: 2-HBP formed

CONCLUSION

Surfactants could strongly enhance the solubility of DBT in water and improve the mass transfer of DBT between organic and aqueous phases. Because of their different influences on strains, they affected the DBT biodesulfurization differently. For *Corynebacterium* sp. ZD-1, Tween-80 could both enhance cell growth and biodesulfurization activity. The results suggested that suitable surfactants could enhance the biodesulfurization rate in hydrocarbon aqueous biphasic (O/W) systems and thus has a potential application in industrial BDS. The experimental methods used in the study could also be applied to choose suitable surfactants for other strains in BDS.

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