Journal of Environmental Sciences Vol.16, No.6, pp.1011—1015, 2004

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2004)06-1011-05 **CLC number**: X172 **Document code**: A

Desulfurization of dibenzothiophene by a newly isolated *Corynebacterium* sp. ZD-1 in aqueous phase

WANG Miao-dong, LI Wei*, WANG Da-hui, SHI Yao

(Department of Environmental Engineering, Zhejiang University, Hangzhou 310027, China. E-mail: w_li@zju.edu.cn)

Abstract: Sulfur emission through fuel combustion is a global problem because it is a major cause of acid rain. Crud oil contains many heterocyclic organic sulfur compounds, among which dibenzothiophene (DBT) and DBTs bearing alkyl substitutions usually are representative compounds. A strain was isolated from refinery sludge and identified as *Corynebacterium* ZD-1. The behavior of DBT degradation by ZD-1 in aqueous phase was investigated. *Corynebacterium* ZD-1 could metabolize DBT to 2-hydroxybiphenyl(2-HBP) as the dead-end metabolite through a sulfur-specific pathway. In shake flask culture, ZD-1 had its maximal desulfurization activity in the late exponential growth phase and the specific production rate of 2-HBP was about 0.14(mmol·kg dry cell^{-1}·min^{-1}, mmol·KDC^{-1}·min^{-1}). Active resting cells for desulfurization should be prepared only in this period. 2-HBP inhibited the growth of strain ZD-1, the production of DBT degradation enzymes, and the activity of enzymes. Sulfate inhibited the production of dibenzothiophene(DBT) degradation enzymes but had no effect on the enzymes' activity. The production rates of 2-HBP at lower cell densities were higher and the maximum amount conversion of DBT to 2-HBP(0.067 mmol/L) after 8 h was gained at 9.2 g dry cell/L rather higher cell density. The results indicated that this newly isolated strain could be a promising biocatalyst for DBT desulfurization.

Keywords: biodesulfurization; dibenzothiophene; *Corynebacterium*

Introduction

Emission of sulfur-oxides to the atmosphere through combustion of fossil fuel is a main reason that causes serious environmental problem such as acid rain. As the crisis of energy source become worse, mine of fossil fuel with high sulfur contents is inevitable. But the demand of middle-distiller fraction with very low sulfur content will be increased. This new situation will force the petroleum refining companies to invest technologies of deep-desulfurization before combustion. Because of its gentle reaction conditions and low-cost combining with hydrodesulfurization (HDS), biodesulfurization technology will attract more and more attention in future.

There are more than 200 kinds of sulfur compounds, in which dibenzothiophene (DBT) and its derivatives are regarded as model compounds that are difficult to be degraded to assess the efficiency of desulfurization. It was reported that a strain isolated by Kilbane(Kilbane, 1989) could degrade DBT to 2-hydroxybiphenyl (2-HBP) in a sulfur-specific pathway—"4S" pathway, removing sulfur from DBT without destructing the carbon-bond. Since then many strains that can utilize DBT as sole sulfur source have been isolated, such as *Rhodococcus* sp. UM3 and UM9(Purdy, 1993), *R. erythropolis* sp. D-1(Izumi, 1994) and *Corynebacterium* sp. SY1(Omori, 1992). For *Rhodococcus erythropolis* IGTS8, molecular cloning and characterization of genes responsible for sulfur oxidation have been studied(Denome, 1993; 1994; Piddington, 1995), and the enzymatic system for desulfurization pathway has been elucidated(Ohshiro, 1994; 1995a; 1997; Gray, 1996). Although total sulfur removals of petroleum by biodesulfurization are significant(Premuzic, 1999; Grossman, 1999), the level is insufficient to meet the required sulfur levels for all fuels. Native biocatalysts were slow, their stability was inadequate, and their sulfur selectivity too narrow for commercialization(Beverly, 1999). For biodesulfurization to be commercial, microorganisms with high activity and selectivity are required. A strain, which was isolated from refinery sludge and identified as *Corynebacterium* ZD-1, could metabolize DBT to 2-hydroxybiphenyl(2-HBP) as the dead-end metabolite through a sulfur-specific pathway. Optimal concentration of DBT for ZD-1 growth and DBT degradation was 0.2 mmol/L, and the specific production rate of 2-HBP could reach 0.14 mmol·kg dry cell^{-1}·min^{-1} (Wang, 2004). The behavior of DBT degradation in aqueous phase by ZD-1 was investigated in this study.

1 Materials and methods

1.1 Chemicals

DBT and 2-HBP were purchased from Fine Chemical Co.(USA). All other chemicals were of analytical grade, commercially available and used without further purification.

1.2 Microorganism and media

The minimal salt medium(MSM) used in this study was a sulfur-free medium containing 2 g of glycerol, 5.0 g of $K_2HPO_4 \cdot 3H_2O$, 2.0 g of $NaH_2PO_4 \cdot 2H_2O$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 2.0 g of NH_4Cl, 1 ml of mineral solution per 1 L of deionized water. 1 ml of mineral solution contained 0.1 mg of $CuCl_2 \cdot 2H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 0.2 mg of $ZnCl_2$, 20 mg of $CaCl_2$, 0.05 mg of H_3BO_3, 0.2 mg of $NaMoO_4 \cdot 2H_2O$, 4 mg of $FeCl_3 \cdot 7H_2O$, 0.1 mg of $AlCl_3 \cdot 6H_2O$ and 0.8 mg of $MnCl_2 \cdot 4H_2O$. DBT was dissolved in ethanol(50

Foundation item: The National Natural Science Foundation of China(No.20276068); * Corresponding author

mmol/L) and added to a sterilized MSM.

Strain ZD-1 was isolated from refinery sludge of Hangzhou Refinery Co.(Hangzhou, China). One gram of the sludge was suspended in 10 ml of MSM, supplemented with 0.2 mmol/L DBT as the sulfur source and sharked in the rotary shaker for 48 h at 30℃. After being centrifuged at 1000 r/min for 2 min to separate the solid, the culture was transferred into the fresh medium. After 4 d cultivation, single-colony isolation was repeated on the plate of the same medium containing 1.5% agar, and finally, isolated colonies were streaked on the plate. Selection of ZD-1 strain among 5 isolated strains was based on ability to grow in the presence of DBT and production of 2-HBP from DBT.

Flask cultures of ZD-1 were performed in 250 ml conical flasks containing 100 ml of MSM at 30℃ and 150 r/min in a rotary shaker. The initial DBT concentration was set to 0.2 mmol/L. To prepare resting cells, cells were harvested in the late logarithmic phase by centrifugation at 4800 r/min for 15 min and washed twice with a 0.1 mol/L potassium phosphate buffer(pH 7.0). The harvested cells were suspended in the phosphate buffer at a desired concentration and stored at -20℃.

1.3 Analytical methods

The concentration of cells was determined from the linear relationship between the optical density at 620 nm (OD_{620}) and dry cell weight. The metabolic products of DBT in culture medium were analyzed by GC-MS after acidifying the samples to pH ≤ 2.0 and extracting with ethyl acetate. Sulfate was qualitatively analyzed by reacting with $BaCl_2$ in HNO_3 solution. DBT and 2-HBP in reaction mixture were analyzed by UV-VIS spectrophotometer. DBT has two characteristic absorption peaks at 311 nm and 325.5 nm (Setti, 1992). Liquid samples were acidified to pH ≤ 2.0 and extracted with the same volume of *n*-hexylane. The concentration of DBT was determined from the linear relationship between the optical density at 311 nm and concentration of DBT. The extraction rate was more than 99%. In basic conditions, 2,6-dichloro-quinoneimine(Gibbs reagent) reacted with 2-HBP to form blue compound, whose concentration was proportionate with optical density at maximum absorption wave of 610 nm in definite range(Wang, 1984). Liquid sample was centrifuged at 4800 r/min for 10 min and the supernatant was adjusted to pH ≥ 9.0 with 20% Na_2CO_3 solution and then diluted to 5 ml with damping fluid of H_3BO_3 (pH = 9.0). 0.06 ml of Gibbs reagent was added to and reacted with the dilution at 30℃ for 30 min before determination.

1.4 DBT desulfurization in aqueous phase system

The reaction rates with resting cells were measured in 50 ml screw-cap flask at 30℃ and under rotary shaking at 180 r/min. The cells were suspended in the phosphate buffer solution. After the DBT-ethanol solution (50 mmol/L) was added at desired concentration, the degradation of DBT and formation of 2-HBP were measured and the specific production rate of 2-HBP (mmol · KDC^{-1} · min^{-1}) was calculated.

2 Results and discussion

2.1 Identification of DBT degradation product

The GC-MS of DBT and DBT-sulfone degradation products are shown in Fig.1. The compound with retention time of 9.13 min in (a) and 5.52 min in (b) was identified as 2-HBP. The compounds with retention time of 10.18 min in (a) and 8.85 min in (b) were identified as DBT and DBT-sulfone respectively. Since concentration of 2-HBP did not decrease in all later period of culture, 2-HBP was thought to be the dead-end metabolite of DBT and DBT-sulfone. At the same time the degradation rate of DBT-sulfone was as twice as that of DBT. Some of the culture media were centrifuged at 4800 r/min for 15 min and the supernatants were reacted with $BaCl_2$ in HNO_3 solution. The white precipitation suggested that there were sulfate to have been formed. It was reported that *Corynebacterium* sp. SY1 could metabolize DBT to 2-hydroxybiphenyl(2-HBP) as the dead-end metabolite through a sulfur-specific pathway (Toshio, 1992). SY1 oxidized DBT to DBT sulfoxide and then to DBT sulfone. This reaction may be followed by hydrolytic cleavage to form sulfonic acid and subsequent release of sulfite or sulfate. The DBT degradation pathway of *Corynebacterium* sp. ZD-1 might be the same with that of *Corynebacterium* sp. SY1.

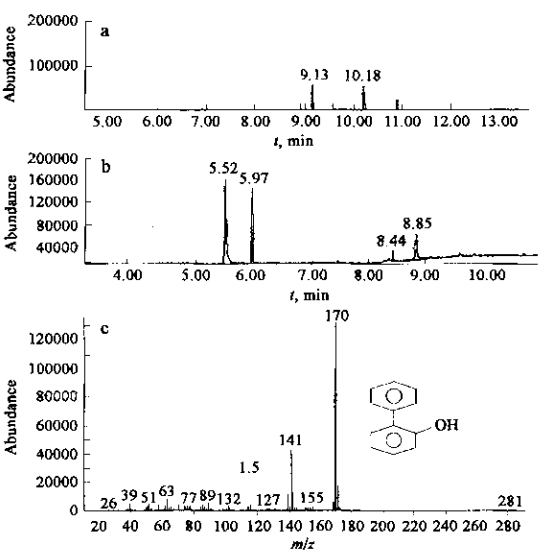

Fig.1 GC-MS of DBT and DBT-sulfone degradation products
a. GC of DBT; b. GC of DBT-sulfone; c. MS of 2-HBP

2.2 Identification of the isolated strain ZD-1

Isolated strain was identified on the basis of its morphological and physiological properties by Zhejiang Institute of Microorganism(Table 1). An isolate, ZD-1, from the colonies was an aerobic, nonmotile, nonspore-forming,

and gram-positive bacterium. ZD-1 bacteria were rod shaped, as observed by a light microscope. The isolate was catalase positive. The colony was transparence, ridgy in central, slippy, lustrous, wet, fleshcolor and became brick red after culturing on bevel for 3 days. Thus the strain was identified as *Corynebacterium* strain ZD-1.

Table 1 Characteristics of ZD-1 strain

Taxonomic properties	Test result
Urease	-
Solubility in gelatin	-
V.P.	-
Methyl red	-
Benzazole	-
Litmus-milk	-
Oxidation fermentation of glucose	-
Oxidation fermentation of glycerol	-
Monose	-
Amino acid	-
No nitrogen source	-
Alcohol	-
NH_4-N	+
NO_3-N	+

2.3 Glowth of *Corynebacterium* ZD-1 and DBT degradation in shaking flask

Fig.2 shows the growth of ZD-1 strain in 500 ml flask containing 150 ml of MSM, as well as the concentration of 2-HBP, pH and the specific production rate of 2-HBP in different period. The strain showed the maximum(1.80 g dry cell·L^{-1}) at 45.5 h of cultivation. The pH decreased from 6.62 to final 5.34. The 2-HBP concentrations did not decrease during stationary phase. 2-HBP could accumulate up to a concentration of 0.109 mmol/L in the culture medium and was not used as a carbon source for cell growth. ZD-1 had its maximal desulfurization activity in the late exponential growth phase and the specific production rate of 2-HBP was about 0.14 mmol·kg dry $cell^{-1}$·min^{-1}, thus, active resting cells for desulfurization should be prepared in this period.

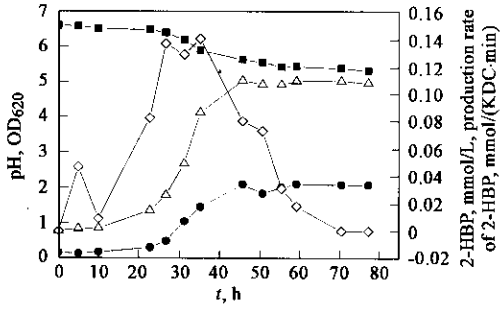

Fig.2 Desulfurizaiton of DBT by *Corynebacterium* strain ZD-1
△ formation of 2-HBP; ■ pH change during growth; ◇ specific production rate of 2-HBP; ● optical density of ZD-1 at 620 nm

2.4 Effects of sulfate and 2-HBP

As one metabolite of DBT degradation, sulfate might have effects both on the production and the activity of DBT degradation enzymes.

Cells that cultured with Na_2SO_4 as sulfur source did not have DBT degradation enzymes. These cells were respectively inoculated into 5 ml of MSM with different concentration of Na_2SO_4 and cultured at 30℃ for 24 h, and then the concentrations of 2-HBP were analyzed. As shown in Fig.3, there were almost no 2-HBP to form when the concentration of Na_2SO_4 was above 0.2 mmol/L, suggesting the sulfate inhibited the production of DBT degradation enzymes strongly.

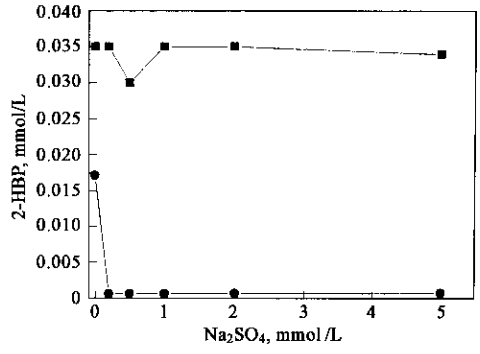

Fig. 3 Production of 2-HBP by ZD-1 in different concentration of Na_2SO_4
■ resting cells reacted for 1 h; ● cells cultured with Na_2SO_4 reacted in MSM for 24 h

Resting cells cultured with DBT as sole sulfur source were in dormancy, but they already had all kinds of enzymes. 5 ml of resting cells were reacted with 0.2 mmol/L DBT in different amount of Na_2SO_4 for 1 h and then the formations of 2-HBP were measured. The amount of 2-HBP did not differentiate very much(Fig.3), which indicated that sulfate had no effect on activity of DBT degradation enzymes.

2-HBP affected desulfurization activity of ZD-1 on the two aspects, enzymes' production and activity(Fig.4). The amounts of the degradation of DBT (0.2 mmol/L) were 27.4% and 62.4% respectively when the initial concentration of 2-HBP was 0 mmol/L. As shown in Fig.4, almost no DBT were degraded at 1.0 mmol/L of 2-HBP and the amount of DBT degradation decreased quickly with increasing of 2-HBP amounts. These results indicated that the production and the activity of DBT degradation enzymes were both inhibited by 2-HBP strongly. It was shown that strain ZD-1 had no growth at 0.1 mmol/L 2-HBP(Fig.5) when the effect of 2-HBP on the growth of strain ZD-1 was studied. However, 2-HBP did not affect the growth of ZD-1 with Na_2SO_4 although 1.0 mmol/L of 2-HBP had toxicity on it. Considering the inhibitory effect of 2-HBP on desulfurization of DBT, which would cause the lack of sulfur supply, it was easy to understand.

Besides the inhibitory effects of 2-HBP and sulfate were the main limiting factors of the desulfuriation of DBT, the resistance of strain ZD-1 to inhibition by 2-HBP was similar to that of many other reported strains(Izumi, 1994; Rhee, 1998; Kayser, 1993).

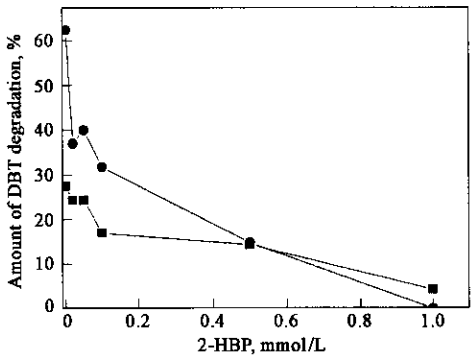

Fig. 4 The amount of DBT degradation in different concentrations of 2-HBP by ZD-1
■ resting cells reacted for 2 h; ● Cells cultured with Na_2SO_4 reacted in MSM for 48 h

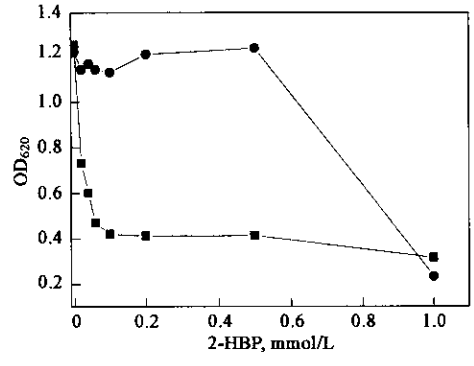

Fig. 5 Optical densities of ZD-1 cultured in different concentration of 2-HBP with different sulfur source
■ DBT; ● Na_2SO_4

2.5 Desulfurization by resting cells and effect of cell concentration

DBT desulfurization by resting cells is shown in Fig.6. DBT was degraded fast at first, but the reaction stopped at 8 h. It might because the deactivation of DBT degradation enzymes. It was found that the amount of 2-HBP produced was a little less than the amount of DBT decreased. But no other intermediate and dead-end metabolites were found by GC-MS. It was assumed that some portion of the substrate might dissolve into cell membrane or bind to inert surface (Honda, 1998; Wang, 1996).

To study the effect of cell concentration on desulfurization, cell suspensions with varied concentration were added to the 0.5 mmol/L DBT solution and reacted at 30℃ for 8 h. It was difficult to measure the DBT concentration accurately at high cell concentration, therefore, the activity of the resting cells was expressed as 2-HBP production rather DBT degradation (Izumi, 1994; Ohshiro, 1997). As shown in Fig.7, the production rates of 2-HBP at lower cell concentration were higher and the maximum conversion of DBT to 2-HBP(0.067 mmol/L) was gained at 9.2(g dry cell/L) rather higher cell concentration. This was probably due to the mass transfer limitation, especially oxygen transfer needed for the oxidation reaction of DBT

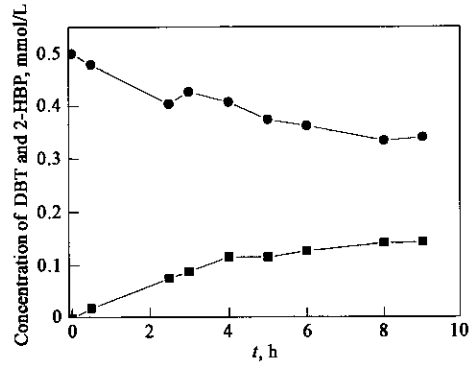

Fig.6 DBT desulfurization by resting cell of ZD-1
■ 2-HBP; ● DBT

(Maghsoudi, 2000; Ohshiro, 1995b).

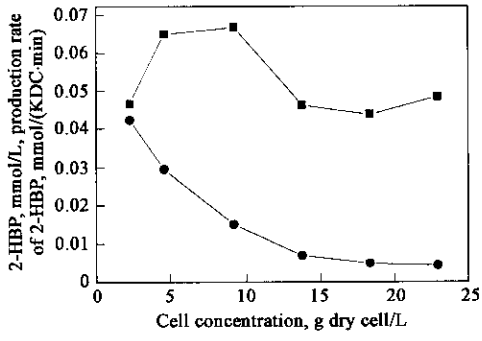

Fig. 7 Specific production rate of 2-HBP and 2-HBP formation with different cell amount
■ 2-HBP; ● specific production rate of 2-HBP

3 Conclusions

Newly isolated strain *Corynebacterium* strain ZD-1 could utilize DBT as sole sulfur source and degrade DBT to 2-HBP as dead-end metabolite. It had good desulfurization ability and its maximal specific production rate of 2-HBP was about 0.14 mmol · KDC^{-1} · min^{-1}. ZD-1 also had the ability to desulfurize DBT in the presence of hydrocarbon like *n*-hexadecane(the paper under preparation).

References:

Beverly L M, 1999. Biodesulfurization[J]. Curr Microbiol, 2: 257—264.

Denome S A, Olson E S, Young K D, 1993. Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp. strain IGTS8[J]. Appl Environ Microbiol, 59: 2837—2843.

Denome S A, Oldfield C, Nash I. J *et al*., 1994. Characterization of the desulfurization genes form *Rhodococcus* sp. strain IGTS8[J]. J Bacteriol, 176: 6707—6716.

Gray K A, Progrebinsky O S, Mrachko G T *et al*., 1996. Molecular mechanisms of biocatalytic desulfurization of fossil fuels[J]. Nature Biotechnol, 178: 6409—6418.

Grossman M J, Lee M K, Prince R C *et al*., 1999. Microbial desulfurization of a crude oil middle-distillate fraction: analysis of the extent of sulfur removal and the effect of removal in remaining sulfur[J]. Appl Environ Microbiol, 65: 181—188.

Honda H, Sugiyama H, Saito I *et al*., 1998. High cell density culture of *Rhidiciccus rhdochrous* by pH-stat feeding and dibenzothiophene degradation [J]. J Ferment Bioeng, 85: 334—338.

Izumi Y, Ohshiro T, Ogino H *et al*., 1994. Selective desulfuization of dibenzothiophene by *Rhodococcus erythropolis* D-1 [J]. Appl Environ Microbiol, 60: 223—226.

Kayser K J, Bielaga-Jones B A, Jackowski K *et al*., 1993. Utilization of organosulfur compounds by axenic and mixed culture of *Rhidicicus rhdochrous* IGTS8[J]. J General Microbiol, 139: 3123—3129.

Kilbane J J, 1989. Desulfurization of coal: the microbial solution[J]. Trend in Biotechnol, 7: 97—101.

Maghsoudi S, Kheirolomoom A, Vossoughi M *et al*., 2000. Selective desulfurization of dibenzothiophene by newly isolated *Corynebacterium* sp. strain P32C1[J]. Biochem Eng, 5: 11—16.

Ohshiro T, Hine Y, Izumi Y, 1994. Enzymatic desulfurization of dibenzothiophene by a cell-free system of *Rhodococcus erythropolis* D-1[J]. FEMS Microbiol Lett, 118: 341—344.

Ohshiro T, Kanabayashi Y, Hine Y *et al*., 1995a. Involvemint of flavin coenzyme in dibenzothiophene degrading enzyme system from *Rhodococcus erythropolis* D-1[J]. Biosce Biotechnol Biochem, 59: 1349—1351.

Ohshiro T, Hirata T, Izumi Y, 1995b. Microbial desulfurization of dibenzothiophene in the presence of hydrocarbon [J]. Appl Microbiol Biotechnol, 44: 249—252.

Ohshiro T, Suzuki K, Izumi Y, 1997. Dibenzothiophene(DBT) degrading enzyme responsible for the first step of DBT deulfurization by *Rhodococcus erythropolis* D-1: purification and characterization[J]. J Ferment Bioeng, 83: 233—237.

Omori T, Monna L, Saiki Y *et al*., 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1[J]. Appl Environ Microbiol, 58: 911—915.

Piddington C S, Kovavevich B R, Rambosek J, 1995. Sequence and molecular characterization of a DNA region encoding the dibenzothiophene desulfurization operon of *Rhodococcus* sp. strain IGTS8[J]. Appl Environ Microbiol, 61: 468—475.

Premuzic E T, Lin M S, Bohenek M *et al*., 1999. Bioconversion reactions in asphaltenes and heavy crude oils[J]. Energy and Fuels, 13: 297—304.

Purdy R F, Lepo J E, Ward B, 1993. Biodesulfurization of organic-sulfur compounds[J]. Curr Microbiol, 27: 219—222.

Rhee S K, Chang J H, Chang Y K *et al*., 1998. Desulfurization of dibenzothiophene and diesel oils by a newly isolated *Gordona* strain CYKS1 [J]. Appl Environ Microbiol, 64: 2327—2331.

Setti L, Rossi M, Lanzarini G *et al*., 1992. The effect on *n*-alkanes in the degradation of dibenzothiophene and of organic sulfur compounds in heavy oil by a *Pseudomonas* sp.[J]. Biotechnol Lett, 14: 515—520.

Toshio O, Lisa M, Yuko S *et al*., 1992. Desulfurization of dibenzothiophene by *Corynebacterium* sp. strain SY1[J]. Appl Environ Microbiol, 58: 911—915.

Wang M D, Li W, Shi Y *et al*., 2004. Optimization of the growth conditions of *Corynebacterium* sp. ZD-1, a sulfur-degradation bacterium[J]. Journal of Zhejiang University (in press).

Wang B Y, Wang X K, You J R *et al*., 1984. Organic reactions named with their discoverer(3)[M]. Beijing: Chemical Industry Press.

Wang P, Krawiec S, 1996. Kinetic analysis of desulfurization of dibenzothiophene by *Rhodococcus erythropolis* in batch and fed - bath cultures[J]. Appl Environ Microbiol, 62: 1670—1675.

(Received for review November 14, 2003. Accepted January 4, 2004)

Take actions against Persistent Organic Pollutants (POPs)

China is facing a great challenge to implement the Stockholm Convention, the participants said at the Sino-US Workshop on Science-Based Decision Making-Implementing the Stockholm Convention on Persistent Organic Pollutants (POPs) in Beijing.

POPs are a small group of persistent, bio-accumulative, long-range transported and toxic chemicals. In May 2001, the Stockholm Convention, a legally binding treaty on Persistent Organic Pollutants, was signed. It entered into force on 27 May, 2004. China signed the Convention on the first day opened for signature.

The actions for control over POPs involve scientists, decision-makers, industry, NGOs and other stakeholders. There is a need for setting up an information platform that links the scientific research and decision-making. For this purpose, the workshop goal are to 1) increase the communication between decision-makers for POPs management and scientists conducting POPs-related research, 2) provide scientific support and guidance for making POPs pollution control policies, and 3) improve China's capability of meeting the obligations of international POPs conventions.

Jointly sponsored by State Environmental Protection Administration of China (SEPA), The Chinese Academy of Sciences (CAS), US National Academy of Sciences (NAS), US National Environmental Protection Agency (EPA), Scientific Committee on Problems of the Environment (SCOPE), China Association for Science and Technology (CAST), and Inter-Academy Panel (IAP), the workshop was held on June 7—10, 2004 in Beijing, China. The workshop was co-organized by Prof. Yonglong Lu, and Prof. John Giesy, with the help of SCOPE CHINA secretariat.

Over 80 officials and experts from environmental protection agencies, academies, industry, NGOs and other organizations attended the meeting. During the three-day session, the following major themes were discussed: Policy instruments for POPs management, POPs exposure, risk assessment, reduction and alternatives, Linkage between scientists and decision-makers.