Journal of Environmental Sciences Vol. 18, No. 2, pp. 369—372, 2006

ISSN 1001 -0742
CN 11- 2629/X

Article ID: 1001-0742(2006)02-0369-04 **CLC number**: X172 **Document code**: A

Biodegradation of 2,6-ditert-butylphenol by immobilized microorganism strains

ZHANG Ya-lei[1], ZHANG Zhi-gang[1], XU De-qiang[2], QIANG Zhi-min[3], LI Guang-ming[4], ZHAO Jian-fu[4]

(1. Key Laboratory of Yangtze Water Environment of Ministry of Education, Tongji University, Shanghai 200092, China. E-mail: zhangyalei@mail.tongji.edu.cn; 2. School of Life Science, Fudan University, Shanghai 200433, China; 3. Department of Civil, Architectural and Environmental Engineering, University of Missouri-Rolla, Rolla, MO 65409, USA; 4. State Key Laboratory of Pollution Control and Resource Reuse, Tongji University, Shanghai 200092, China)

Abstract: 2,6-Ditert-butylphenol (2,6-DTBP) is a major organic contaminant presenting in acrylic fiber manufacturing wastewaters. This compound is of high bio-resistance due to its complex structure which consists of one phenol group and two highly branched tert-butyl groups. This research attempted to improve the biodegradation efficiency of 2,6-DTBP through various strain immobilization methods. The stratified immobilization can settle oxygen transmission in the single microorganism immobilization, and can realize two-process reaction in the single device by choosing two symbiotic microorganisms. Two effective strains, named F-1-4 and F-3-4, which were screened out in our previous work, were used to degrade 2,6-DTBP after being immobilized in calcium alginate gel. Results indicate that the substrate removal efficiency of various immobilization methods follows the order: stratified > single F-3-4 > mixed ≈single F-1-4. The immobilized biodegradation capacity was higher than the free one. After an incubation time of 12 d, 91% of 2,6-DTBP could be degraded by the stratified immobilization method, compared to 79% achieved by the mixed immobilization method with an initial 2,6-DTBP concentration of 100 mg/L. The stratified immobilization satisfies the oxygen demand nature of the aerobic F-3-4 and the facultative F-1-4, thus yielding the highest degradation efficiency. Both the outer layer strain F-3-4 and the inner layer strain F-1-4 can grow actively on the substrate of 2,6-DTBP, as illustrated by SEM images. This study shows that the highly bio-refractory compound, 2,6-DTBP, can be effectively degraded using appropriately immobilized microorganism strains.

Keywords: biodegradation; 2,6-ditert-butylphenol; immobilization; microorganism strains

Introduction

Phenolic compounds are extensively used for synthesis of antiseptics, antirusts, bactericides and pesticides, and thus are major pollutants presenting in wastewaters of these industries. 2,6-Ditert-butylphenol (2,6-DTBP) is typically present in wastewaters of acrylic fiber manufacturing industry. Its chemical structure consists of one phenol group and two tert-butyl groups. Phenol is commonly considered as a bio-toxin, while the highly branched tert-butyl group is hardly biodegradable. Therefore, 2,6-DTBP is highly resistant to most microorganisms in the environment due to its complex chemical structure. Immobilization technology has been utilized to enhance the biodegradability of refractory organic compounds. Immobilization restricts microorganism mobility within a confined space. It possesses such advantages such as providing high microorganism concentrations, minimizing microorganism washout problems at high influent flow rates, eliminating microorganism recovery and recycle, and improving genetic stability (Belkis and Fazilet, 1998). The most common application is immobilization of a single type of strain. However, for a significantly bio-refractory compound, co-metabolism in a microcosm composed of two or more types of microorganism strains may greatly enhance the biodegradation efficiency. Beunink and Rehm (1988, 1990) reported an effective degradation of DDT and 4-chloro-2-nitrophenol by integrating the concept of oxygen grads into the immobilization technology.

In our previous works(Zhang *et al.*, 2003, 2005), two microorganism strains capable of effectively degrading 2,6-DTBP (named F-1-4 and F-3-4) were isolated from an acrylic fiber wastewater. The aim of this study was to compare the effects of various immobilization methods, namely, immobilizations of single, homogeneously mixed and stratified strains of F-1-4 and F-3-4, on the degradation efficiency of 2,6-DTBP.

1 Materials and methods

1.1 Microorganism strains

Bacteria were originally collected from the biofilm of a biological tower filter and the sedimentation tank of a petrochemical wastewater treatment plant in Shanghai. Two microorganism strains, which have demonstrated effective degradation ability for 2,6-DTBP, were isolated in our previous work (Zhang *et al.*, 2003, 2005). One was characterized as acrobic (F-3-4) and the other as facultative(F-1-4).

1.2 Strain immobilization

The procedures for immobilizing mixed microorganism strains are described as follows: (1)

Foundation item: The National Natural Science Foundation of China(No. 50008011), the Hi-Tech Research and Development Program(863) of China (No. 2004AA649310), the Key Technologies Research and Development Program of China (No. 2003BA808A17) and the Program of STCSM (No. 05QMX1453)

cultivate the individual strain suspensions of F-3-4 and F-1-4 for 20 h on broth media (Zhou and Gao, 2000); (2) centrifuge the two strain suspensions at 8000 r/min and 4℃ for 20 min to separate wet cells; (3) wash the wet cells with normal saline solution three times; (4) homogeneously mix the two strains together with 2% of sodium alginate glue at a weight ratio of 1:1.6 and temperature 30—40℃; (5) drop the mixture into 4% of $CaCl_2$ solution; (6) calcify for approximately one hour to form the mixed bacteria grains of similar sizes. The bacteria grains were washed three times with normal saline solution before use. The immobilization of single microorganism strain was completed in the same manner.

The procedures for immobilizing stratified microorganism strains are described as follows: (1) cultivate the individual strain suspensions of F-3-4 and F-1-4 for 20 h on broth media; (2) centrifuge the two strain suspensions at 8000 r/min and 4℃ (for 20 min) to separate wet cells; (3) individually mix the wet cells of F-1-4 and F-3-4 with 2% of sodium alginate glue at a weight ratio 1:1.6 and temperature 30—40℃; (4) inject the F-1-4 mixture dropwisely into 2% of $CaCl_2$ solution and calcify for one hour to form bacteria grains; (5) wash the F-1-4 grains with normal saline solution three times; (6) coat the surface of F-1-4 grains with a layer of the F-3-4 and sodium alginate mixture; (7) drop into 4% of $CaCl_2$ solution; (8) calcify for about one hour to form the stratified bacteria grains of similar sizes. The stratified strains were thus composed of an outer layer of the aerobic F-3-4 and an inner layer of the facultative F-1-4. They were washed three times with normal saline solution before use.

1.3 Sample analysis

2,6-DTBP was analyzed by light absorbance after sample pretreatment (i.e., solvent extraction and centrifugation). The detailed procedures are depicted as follows: (1) add 10 ml of *n*-hexane to each sample flask and vigorously shake for 2 min by hands; (2) centrifuge the mixture at 10000 r/min and 4℃ for 8 min; (3) withdraw the supernatant and make appropriate dilution; (4) determine the concentration of 2,6-DTBP with UV-Vis spectrophotometry(UV-2201, Shimadzu, Japan) at 276 nm.

1.4 Biodegradation experiments

2,6-DTBP was first spiked into 30 ml of pH 7.0 B.H. medium in a series of incubation flasks as the sole carbon source. To each flask 2.4 g of immobilized bacteria grains were added, corresponding to an inoculum size of 0.10%. The flasks were then closed with ground glass stoppers. Around the stoppers parafilm was further wrapped to prevent potential evaporative leakage of 2,6-DTBP. These flasks were incubated on shakers with a constant temperature of 25℃ at 250 r/min. Samples were withdrawn from the incubated media at pre-selected times to analyze the concentration of 2,6-DTBP.

1.5 SEM images

The immobilized bacteria grains were washed with normal saline solution three times, marinated successively in 0.06 mol/L hexamethylene diamine solution for one hour and in 10% of glutaric dialdehyde solution for 10 min, successively dehydrated in 40%, 60%, 80% and 100% ethanol solutions for 20 min each, and vacuumized in an ion paint sprayer(Eiko IB, Eiko, Japan). Once the vacuum degree reached 0.1 Torr, the sample was further sprayed golden for 5 min at a current of 6 mA. The SEM(Model S520, Hitachi, Japan) images were taken thereafter.

2 Results and discussion

2.1 Biodegradation with immobilized single microorganism strain

The biodegradation of 2,6-DTBP was first investigated using immobilized single microorganism strain, i.e., F-1-4 and F-3-4 individually, at various initial substrate concentrations. Results indicate that the bio-refractory compound, 2,6-DTBP, can be effectively degraded in the initial period of reaction; thereafter the reaction rate slows down (after 4 d) and further becomes insignificant (after 10 d), as depicted in Fig.1. Within the first 2 d, the biodegradation rate increases with increasing initial substrate concentration, reaching approximately 16.2, 28.5 and 33.8 mg/ (L·d) at an initial substrate concentration of 50, 100 and 150 mg/L, respectively. The decelerated reaction rate is probably due to the formation of organic metabolites which pose toxicity to microorganism strains. In biological processes, it is well known that metabolites are usually more difficult to biodegrade than parent compounds. After an incubation time of 12 d, the removal efficiencies of 2,6-DTBP reach 86%, 86% and 79% corresponding to an initial substrate concentration of 50, 100 and 150 mg/L, respectively. Results also indicate that the immobilized F-3-4 strain can degrade 2,6-DTBP more effectively than the immobilized F-1-4 strain. After 12 d, only 81% of 2,6-DTBP is degraded by the F-1-4 strain at an initial substrate concentration of 100 mg/L (Fig.1). The F-3-4 strain is aerobic, while the F-1-4 strain is facultative. Under an aerobic condition, F-1-4 may need time to acclimate.

2.2 Biodegradation with immobilized mixed microorganism strains

The microorganism strains of F-1-4 and F-3-4 were homogeneously mixed in calcified grains at a weight ratio of 1.5:1. Results indicate that the biodegradation of 2,6-DTBP exhibits a similar trend as observed in biodegradation with single microorganism strains: the reaction rate is high in the first 4

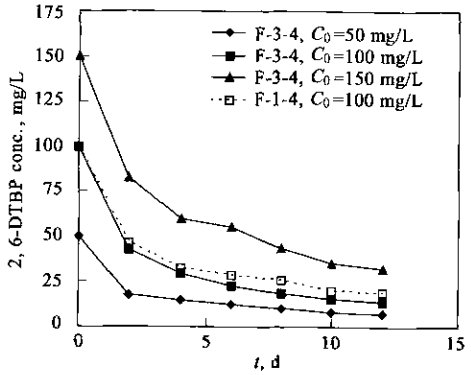

Fig.1 Biodegradation of 2,6-DTBP with immobilized single microorganism strains

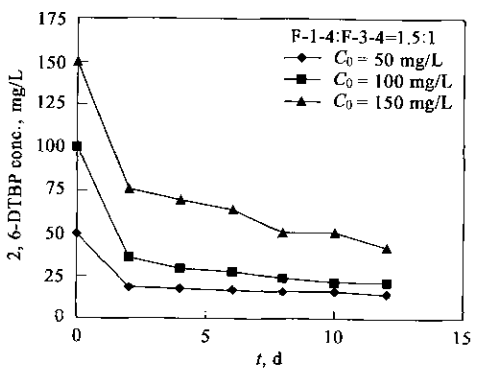

Fig.2 Biodegradation of 2,6-DTBP with immobilized mixed microorganism strains(F-1-4 : F-3-4=1.5 : 1)

d and slows down thereafter, as shown in Fig. 2. Since both individual strains of F-1-4 and F-3-4 are inhibited by the metabolites of 2,6-DTBP, it is not surprising that the mixed strains will also be inhibited. After incubating for 12 d, the removal efficiency of 2,6-DTBP reaches 70%—80% as the initial substrate concentration ranges from 50 to 150 mg/L.

The effect of various mixing ratios of F-1-4 to F-3-4 on 2,6-DTBP degradation was also investigated. Results indicate that although biodegradation rate increases with increasing F-1-4 percentage in the majority of reaction time, similar removal efficiencies (about 80%) are achieved at the end of reaction(Fig. 3). It is mentioned above that the immobilized individual strain of F-3-4 degrades the substrate more effectively than the F-1-4 strain. However, for the immobilized mixed microorganism strains, increasing the percentage of F-3-4 will conversely decrease the initial degradation rate of 2,6-DTBP. The mechanism of co-metabolism between F-1-4 and F-3-4 strains which leads to this phenomenon is unclear at present. Results also demonstrate a severe inhibition of metabolites on 2,6-DTBP degradation. If the initial degradation rate is higher, the successive degradation will become slower due to more metabolites generated during the initial period of reaction. Therefore, three mixing ratios approach approximately the same substrate removal efficiency at the end of reaction. When the incubation time is long enough, the mixing ratio of two strains will not affect the degradation efficiency of 2,6-DTBP.

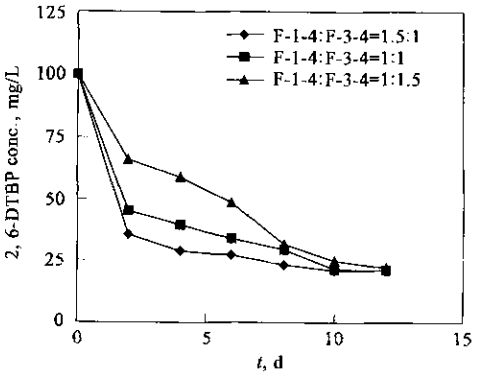

Fig.3 Effect of mixing ratio of immobilized microorganism strains on biodegradation of 2,6-DTBP

2.3 Biodegradation with immobilized stratified microorganism strains

To better fit the oxygen demand nature of the two microorganism stains, the aerobic F-3-4 was immobilized in the outer layer while the facultative F-1-4 was immobilized in the inner layer of the stratified bacteria grains, purposely. Through this stratification it is expected that the outer layer F-3-4 would utilize readily accessible dissolved oxygen and the inner layer F-1-4 would remain under an ideal oxygen-lacking condition, to more effectively degrade 2,6-DTBP. The substrate degradation curves of immobilized single, mixed and stratified strains are compiled in Fig.4. Results indicate that the substrate removal efficiency is improved by stratification, reaching 91% after 12 d with an initial substrate concentration of 100 mg/L. For all immobilization methods tested, the performance follows the order: stratified > single F-3-4 > mixed ≈single F-1-4.

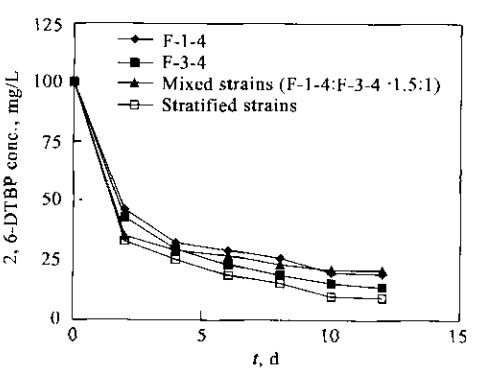

Fig.4 Biodegradation of 2,6-DTBP with immobilized stratified microorganism strains

2.4 Microstructures of immobilized mixed and stratified microorganism strains

The SEM image of the immobilized mixed strains is shown in Fig.5. The SEM images of the outer and inner layers of the immobilized stratified

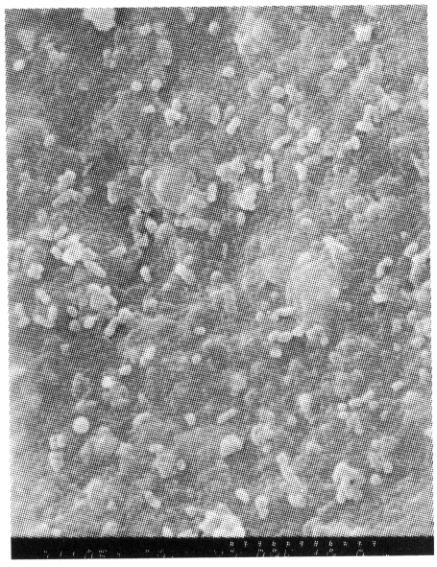

Fig.5 SEM image of immobilized mixed microorganism strains

strains are shown in Fig.6, respectively. These images demonstrate that the strains are abundant and active through being immobilized in calcium alginate. In particular, the F-1-4 strains embedded in the inner layer of the stratified grains can grow well. Since 2,6-DTBP was used as the sole carbon source, the F-1-4 strains would account for partial substrate degradation.

3 Conclusions

This study investigated the biodegradation efficiency of a bio-refractory compound, 2,6-DTBP, with various strain immobilization methods. Through immobilizations of single, mixed and stratified strains of F-1-4 and F-3-4, results indicate that the stratified immobilization yields the highest substrate removal efficiency. After an incubation time of 12 d, 91% of 2,6-DTBP can be biodegraded. Considering the significant bio-resistance of 2,6-DTBP, this removal

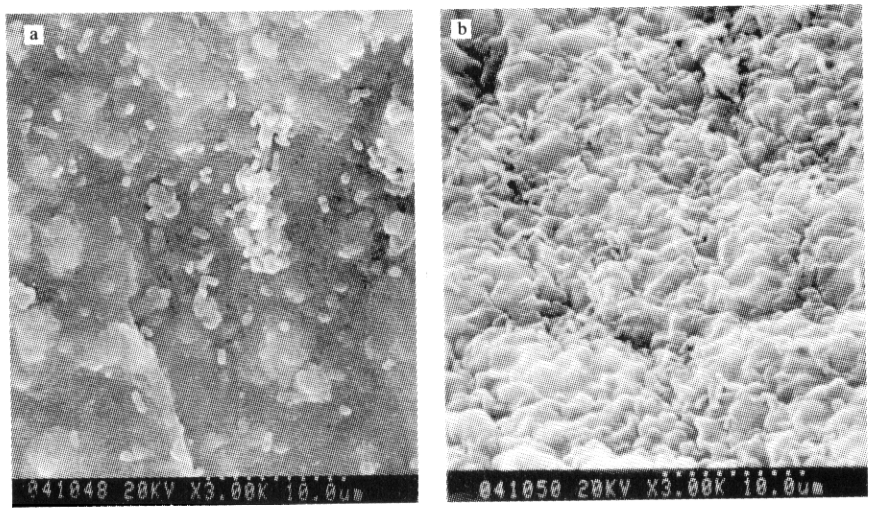

Fig.6 SEM image of immobilized stratified microorganism strains
a. inner layer(F-1-4); b. out layer(F-3-4)

efficiency is plausible. For all immobilization methods tested, the degradation rate is initially high and slows down thereafter. This implies a significant inhibition of the metabolites on further degradation of parent compound. The SEM images demonstrate that the immobilized strains can grow actively on the sole carbon source of 2,6-DTBP.

References:

Bandhyopadhyay K, Das D, Bhattacharyya P *et al.*, 2001. Reaction engineering studies on biodegradation of phenol by *Pseudomonas putida* MTCC 1194 immobilized on calcium alginate [J]. Biochem Eng J, 8(3): 179—186.

Belkis C, Fazilet V S, 1998. Comparison of different production processes for bioethanol[J]. Turk J Chem, 22(4): 351—359.

Beunink J, Rehm H J, 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system[J]. Appl Microbiol Biot, 29(1): 72—80.

Beunink J, Rehm H J, 1990. Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system[J]. Appl Microbiol Biot, 34(1): 108—115.

Bushnell L D, Hass H F, 1941. The utilization of certain hydrocarbons by microorganisms[J]. J Bacteriol, 41(5): 653—673.

Lu C J, Lee C M, Huang C Z, 1996. Biodegradation of chlorophenols by immobilized pure-culture microorganisms[J]. Water Sci Technol, 34(10): 67—72.

Pai S L, Hsu Y L, Chong N M *et al.*, 1995. Continuous degradation of phenol by *Rhodococcus* sp. immobilized on granular activated carbon and in calcium alginate [J]. Bioresource Technol, 51(1): 37—42.

Torres L G, Sanchez-de-la-Vega A, Beltran N A *et al.*, 1998. Production and characterization of a Ca-alginate biocatalyst for removal of phenol and chlorophenols from wastewaters[J]. Process Biochem. 33(6): 625—634.

Zhang Y L, Xu D Q, Fang Z W *et al.*, 2005. Degradation of 2,6-ditert-butylphenol by an isolated high-efficiency bacterium strain[J]. J Environ Sci, 17(2): 272—276.

Zhang Y L, Zhao J F, Gu G W, 2003. Biodegradation kinetic of organic compounds of acrylic fiber wastewater in biofilm [J]. J Environ Sci, 15(6): 757—761.

Zhou Q Y, Gao T Y, 2000. Environmental engineering microbiology [M]. 2nd ed. Beijing: Higher Education Publishing Company. 330.

(Received for review June 27, 2005. Accepted August 31, 2005)