β-Cyclodextrin and its derivatives-enhanced solubility and biodegradation of 2-nitrobiphenyl

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Abstract: This paper investigated the effects of β-cyclodextrins (β-CD) and its two derivatives, hydroxypropyl-β-cyclodextrin (HPCD) and carboxymethyl-β-cyclodextrin (CMCD), on the solubility and biodegradation of 2-nitrophenyl by an *Acinetbacter* sp. Results showed that β-CD, HPCD and CMCD could not be utilized by *Acinetbacter* sp. as sole carbon source and none of the CDs had toxic effects on the growth of the bacteria in the experiments; all the CDs could enhance the apparent solubility and accelerate the biodegradation of 2-nitrobipheny. It showed that biodegradation-accelerating effects of CDs on 2-nitrobiphenyl were correlated with their solubility-enhancing effects. Among three CDs investigated, CMCD had the most obvious effects both on the apparent solubility and the biodegradation, followed by β-CD and HPCD.

Keywords: β-CD; HPCD; CMCD; 2-nitrophenyl; biodegradation

Introduction

Cyclodextrins are cyclic, non-reducing cyclic maltooligosaccharides produced from the enzymatic degradation of starch and related compounds by certain bacteria that contain the cyclodextin glycosyltransferase (Wang *et al.*, 1998; Lynn *et al.*, 2000). The most pertinent property of cyclodextrins is that they have a hydrophilic shell and a toroidal-shaped, apolar (hydrophobic) cavity. Thus, cyclodextrins have the ability to form water-soluble inclusion complexes by incorporating suitable sized low-polarity molecules in their cavities (Szejtli, 1982). In this way, the aqueous solubility of several organic compounds is increased by cyclodextins through their dynamic equilibrum exchange with guest molecules that then dissociate from the cycodextrin complex and become available for catabolism (Bardi *et al.*, 2000). The inclusion of cyclodextrins found may also reduce biotoxicities of some low-plariy compounds (Song *et al.*, 1999).

The most three types of cyclodextrins are designated α-, β- and γ-cyclodextrins (Lynn *et al.*, 2000). β-Cyclodextrin (β-CD) is the least expensive among these three cyclodextrin homologues. However, it has only a low water solubility, which limits its application. Thus, β-CD is often chemically modified to enhance its water solubility. Hydroxy-propyl-β-cyclodextrin (HPCD) and carboxymethyl-β-cyclodextrin (CMCD) are two typical derivatives of β-cyclodextrin with relatively higher water solubility.

In recent years, the function of cyclodextrins have been noticed and applied to enhancing biodegradation of some hydrocarbons pollutants. For example, Wang *et al.* (1998) demonstrated that HPCD can enhance biodegradation of phenanthrene; Bardi and Mattei *et al.* (2000) found that β-CD can accelerate the biodegradation of dodecane, naphthalene, tetracosane and anthracene; Schwartz and Bar (1995) founded

β-CD can enhance the biodegradation of toluene and *p*-toluic; Wang *et al.* (2004) found that HPCD could accelerate the biodegradation of nitrobenzene.

2-Nitrobiphenyl is a suspicious carcinogen with a relative low solubility (<20 mg/L), which was mainly used as dyeing intermediate, plasticizers etc. in the industry. To the best of our knowledge, comparison of the influence of cyclodextrins and their derivatives on the biodegradation of 2-nitrobiphenyl has not been reported. The objective of this study was to evaluate the effect of β-CD and its two typical derivatives— CMCD and HPCD on the biodegradation of 2-nitrobiphenyl.

1 Materials and methods

1.1 Chemicals

All of the chemicals used were analytical grade (≥99% pure) or purity ≥97%. All the water used was double-distilled water. β-CD was obtained from the food and Fermetation Institute of Jiangsu Province, HPCD from Fluka (Buches, Switzerland) and 2-nitrobiphenyl from Aldrich Corporation (USA); CMCD was synthesized by our laboratory; methanol (HPLC grade), used as a carrier for HPLC analysis, was from Tedia Company, Inc. (USA). Other chemicals used were from School of the Chemistry and Chemical Engineering, Nanjing University, Nanjing, China.

1.2 Bacteria

A strain of *Acinetobacter* sp. proven to have the ability to degradate 2-nitrobiphenyl was initially isolated from the activated sludge collected at Nanjing Chemistry Factory (Nanjing, China) by selective enrichment with nitrobenzene and 2-nitrobiphenyl for several months. The enrichment and isolation procedure was described in detail in the thesis of Cai (2003). The strain was maintained on nutrient agar slants at 4°C, which contained 100 mg nitrobenzene, 10 mg 2-nitrobiphenyl and 2 g agar/L in a basal

mineral medium (BMM; BMM consisted of 3.8 g Na₂HPO₄, 1.0 g KH₂PO₄, 3.0 g KCl, 0.28 g MgSO₄ in 1 L of distilled water).

1.3 Inoculum preparation

The strain was transferred from slants to sterile 150 ml conical flasks bearing 50 ml autoclaved BMM with nitrobenzene and 2-nitrobiphenyl provided as the carbon and energy source. The conical flasks were closed with cotton wool stoppers and then incubated with shaking (120 r/min) at 30℃ in an orbital shaker for about 24 h. After incubating, the cell suspension was centrifuged at 10000 r/min for 10 min and washed twice with autoclaved BMM, then resuspended in BMM to optical density at λ 600 nm (OD₆₀₀) about 0.20—0.25. OD₆₀₀ was tested by a 2201 UV-Vis spectrophotometry (Shimadzu Corp., Japan). OD₆₀₀ was used as an index to evaluate the growth condition of the bacteria. The liquid culture was stored at 4℃ and used as inoculum for the following biodegradation experiments.

1.4 Solubility of 2-nitrobiphenyl

1.4.1 Absorbance curve of 2-nitrobiphenyl

The relationship between the concentration of 2-nitrobiphenyl and its absorbance was determined first. For the measurement, quantified 2-nitrobiphenyl was added in the 50 ml volumetric flask and then dissolved by methanol to volume 50 ml. Then 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ml solution was taken from the flask respectively and diluted with 50:50 methanol/water solution in 10-ml volumetric flasks. Absorbance was then analyzed by 2201 UV-Vis spectrophotometry. The maximum absorption wavelength of 2-nitrobiphenyl was 230 nm.

1.4.2 Effects of CDs on the solubility of 2-nitrobiphenyl

For the measurement of solubility of 2-nitrobiphenyl in cyclodextrins solution, 10 ml solutions containing various amounts of CDs were added to 20-ml corex centrifuge tubes respectively. Quantified 2-nitrobiphenyl was then added to each tube in quantities in excess of the solubility limit. Duplicate tubes were prepared for each cyclodextrin concentration. Blanks were prepared in an identical manner with the exception that no cyclodextrin was added. All the samples were equilibrated on a reciprocating shaker for at least 2 d at 30℃. After equilibration, the samples were centrifuged at 10000 r/min for 20 min. A 1-ml aliquot of the supernatant was then withdrawn and diluted with 50:50 methanol/water in 10-ml volumetric flasks and absorbance of the samples was then analyzed by 2201 UV-Vis spectrophotometry. The role of methanol was to inhibit the formation of cyclodextrin-solute complexes, thereby keeping the UV spectrum unchanged (Wang *et al.*, 1993). The wavelength for UV detection was 230 nm. According to the

absorbance curve of 2-nitrobiphenyl obtained above, concentration of 2-nitrobipheny in CDs equilibrated solution could be determined.

1.5 Metalbolizability of CDs by the bacteria and effects of CDs on bacterial growth

The ability of the bacteria to use CDs as the sole carbon and energy source was tested through shaken-flask experiments, which were performed in 500 ml conical flasks containing 200 ml autoclaved BMM. Three kinds of CDs were added to the cultures at the initial concentration of 0, 0.5, 1.0, 2.0, 5.0 g/L respectively. All flasks were inoculated with microbial suspension (2%, v/v) and incubated at a reciprocating shaker at 30℃ with 120 r/min. Duplicated samples were taken from time to time for the OD₆₀₀ measurements. All the flasks and solution were sterilized before bacteria-related experiments and they would not mentioned in the following assays.

Different amounts of CDs were added to 2-nitrobiphenyl contained BMM (50 ml) in 100 ml conical flasks and incubated with the bacteria suspension (2%, v/v). Duplicated samples were taken from time to time for the OD₆₀₀ measurements in order to find whether the CDs had toxic or positive effects on the bacteria growth.

1.6 Biodegradation study

The biodegradation was quantified by direct measurement of 2-nitrobiphenyl loss, used to determine substrate utilization. For the substrate utilization experiment, each 50 ml of BMM in conical flask was supplemented with 7.2% w/v 2-nitrobiphenyl; β-CD, HPCD and CMCD were added alternatively to the flasks to initial concentration of 5 g/L. All the samples were equilibrated on a reciprocating shaker for 2 d at 30℃. After equilibration, each flask was inoculated with the bacteria suspension (2%, v/v) except for control ones. The incubation was done on a reciprocating shaker at 30℃ with 120 r/min. Periodically, enough methanol was added directly to a set of samples and each flask was washed 3 times with methanol and the solution was mixed in another volumetric flask, and the final volume was made to 250 ml. Methanol was used to dissolve 2-nitrobiphenyl. The recycle ratio of 2-nitrobiphenyl extracted by methanol was reliable and repeatable (Shao, 2004). 10 ml mixed solution was withdrawn and centrifuged at 10000 g for 20 min. Supernatant was analyzed by high-performance liquid charomatography (HPLC, 1100 series, Agilent Corp., USA) to measure the concentration of 2-nitrobiphenyl. The assays were conducted in triplicate. According to the concentration measured and the volume, we could caculate the amount of remaining 2-nitrobiphenyl in the solution.

HPLC was performed on a XDB C8 column (5 μm, 4.6 × 150 mm; Agilent Corp., USA) with

methanol-water (70 : 30) as the mobile phase at a rate of 1.0 ml/min. 2-Nitrobiphenyl was detected by its UV A_{230} with an HP1040 diode array detector.

2 Results

2.1 Absorbcance curve of 2-nitrobiphenyl

The relationship between the concentration of 2-nitrobiphenyl and its absorbance is shown in Fig.1. It showed a linear relationship between the concentration of 2-nitrobiphenyl and its absorbance.
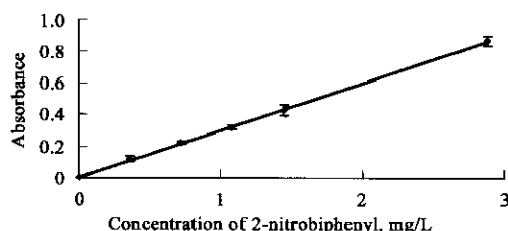

Fig.1 Relationship between the absorbance and the concentration of 2-nitrobiphenyl

2.2 Solubilization effects of cyclodextrins to 2-nitrobiphenyl

The solubilization effects of β-CD, HPCD and CMCD on 2-nitrobiphenyl is shown as Fig.2. It showed a linear relationship between the apparent solubility of 2-nitrobiphenyl and the concentration of β-CD, HPCD and CMCD (concentration of CDs from 0 to 10 g/L). Apparent solubility was defined as the amount of 2-nitrobiphenyl in solution in the presence of cyclodextrins. It indicated that β-CD and its two derivatives significantly increased the apparent solubility of 2-nitrobiphenyl. For example, the apparent solubility of 2-nitrobiphenyl in a 8 g/L β-CD solution was 59.9 mg/L; in HPCD was 40.4 mg/L and in CMCD was 80.0 mg/L. Compared to the measured aqueous solubility of 14.2 mg/L when with no cyclodextrins, the apparent solubility of 2-nitrobiphenyl was increased by 2.8, 4.2, and 5.6 times respectively for β-CD, HPCD and CMCD. In the experimental concentration range of CDs, among β-CD, HPCD and CMCD, CMCD has the most obvious solubility-enhancing effects, β-CD the second, HPCD the last when their initial concentrations were the same.
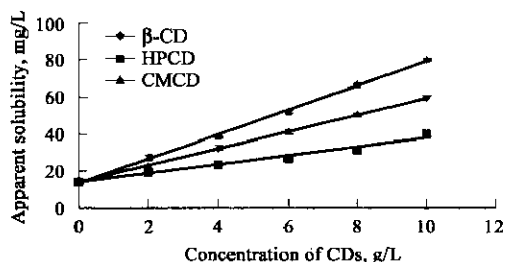

Fig.2 Relationship between the apparent solubility of 2-nitrobiphenyl and the concentration of CDs

2.3 Metabolizability of CDs by *Acinetobater* sp. and effects of CDs on the bacterial growth

Fig.3 shows that a common phenomenon: when no 2-nitrobiphyl was added, the OD did not increase in spite of concentration of CDs changes, which showed that neither of the cyclodextrins could serve as the sole carbon and energy source to support the growth of the bacteria. When CDs were added in the 2-nitrobiphenyl contained solution, bacteria growth became better than that in the solution without CDs and by the enhancement of the concentration of CDs, the positive effects became more obvious; when no CDs but 2-nitrobiphyl was added in the solution, the OD was relatively lower contrasted with CDs contained solution. Combined with the result, we could draw a conclusion that these CDs do not have inhibitory or toxic effects on the bacteria in the experimental concentration range of CDs.

2.4 Biodegradation of 2-nitrobiphenyl

The impact of 0.5% β-CD, HPCD and CMCD on the biodegradation of 2-nitrobiphyl is shown in Fig.4. Fig.4 can clearly found that all the cyclodextrins could enhance the biodegradation rate of 2-nitrobiphyl. Among β-CD, HPCD and CMCD, when their initial concentrations were the same, CMCD had the most pronounced effects on the biodegradation of 2-nitrobiphenyl, followed by β-CD and HPCD. For instance, in the biodegradation study, the time for degradation of 2-nitrobiphenyl in the solution with CMCD was about 120 h, while with β-CD, HPCD were about 168 h and 192 h respectively and if no CDs added, after 192 h, the degradation ratio of 2-nitrobiphenyl was only about 32.5%. For control samples that contained 2-nitrobiphenyl but no
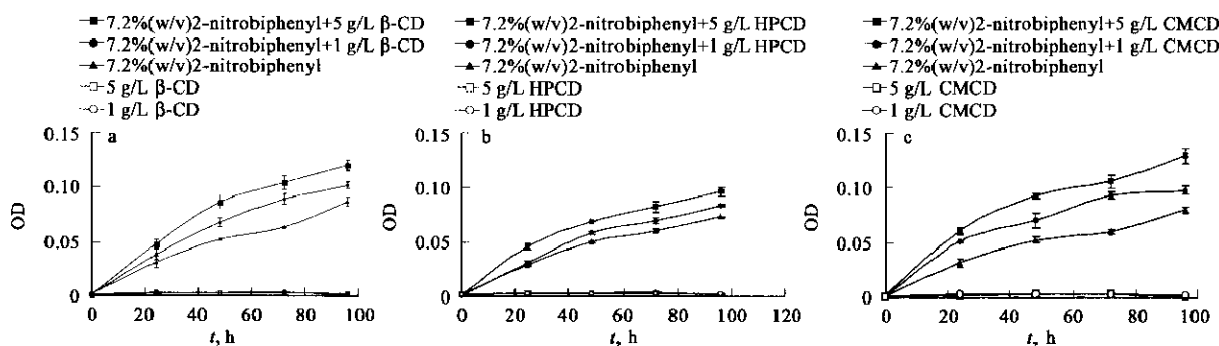

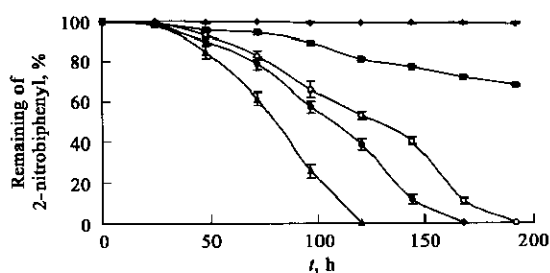Fig.3 Effects of β-CD (a), HPCD (b) and CMCD (c) on the bacterial growth

Fig.4 Remaining of 2-nitrobiphenyl (%) during the biodegradation
■ without CDs; ● β-CD; ▲ CMCD; ○ HPCD; ◆ control

degraders, the added 2-nitrobipheny was nearly completed recovered even after 8 d.

3 Discussion

The apparent solubility of organic compounds in aqueous solution containing cyclodextrin has been observed to increase linearly with the concentration of cyclodextrins and this phenomenon is attributed to the formation of 1 : 1 inclusion complexes, as described by Equation(1) (Wang *et al.*, 1993):

$$S+CD \longleftrightarrow CD-S \qquad (1)$$

For low solubility organic compounds, the relationship between the apparent solubility (S_t) and the aqueous solubility (S_0) can be expressed as Equation(2)(Wang *et al.*, 1993), which clearly explains the phenomenon that apparent solubility of organic compounds increased linearly with the concentration of cyclodextrins.

$$S_t = S_0 (1+ K_s C_0) \qquad (2)$$

Where K_s is the stability constant of the complex which can be determined by $K_s = [CD-S]/[S][CD]$.

If organic compounds did not form 1:1 inclusion complexes, the apparent solubility of organic compounds can not be observed to increase linearly with the concentration of cyclodextrins. This phenomenon can be found from the research of Gao *et al.* (1998).

According to the theory above and former related research, and combined with the results of the solubility experiments that the apparent solubility of 2-nitrobiphenyl increased linearly with the cyclodextrins, we could hypothesize that β-CD, HPCD and CMCD all formed 1:1 complexes with 2-nitrobiphenyl and increased its apparent solubility in the solution.

Compared with each other, different CDs had different effects on the solubility of 2-nitrobiphenyl, this might because that the solubility effects of CDs to hydrocarbons was related closely to the self-solubility of cyclodextrins, the character of the inclusion complexes and environmental factors such as pH, acidity and so on.

The efficiency of biodegradation of hydrocarbons was often seriously hindered by two major factors: (1) low bioavailability of hydrophobic organic compounds to the microorganism and (2) toxicity effects

of substrates exerted on the microorganisms (Schwartz and Bar, 1995). As far as our research was concerned, the solubility of 2-nitrobiphenyl is very low, on the other hand, 2-nitrobiphenyl did not have toxic effects on the bacteria, so the main obstacle of the biodegradation of 2-nitrobiphenyl should come from its low bioavailability. It was hypothesized that cyclodenxtrins could increase the apparent solubilty of 2-nitrobiphenyl by forming inclusion complexes, thereby enhancing bioavailaibility of 2-nitrobiphenyl. This hypothesis was supported by our research. The biodegradation effects of CDs were consistent with their solubility effects on 2-nitrobiphenyl. The larger apparent solubility means more substrate was directly available in solution for supporting the bacterial activity.

Although the concentration changes of β-CD, HPCD and CMCD were not monitored during the biodegradation, we assumed that the *Acinetbacter* sp. would not consume them because the degraders could use none of the CDs as the sole carbon source which had been tested in the research. Not being metabolized by microorganisms, they would maintain their complex or incorporative ability in biodegradation processes (Shao *et al.*, 2003), this might do some help to the potential recycling of CDs when they were applied in bioremediation.

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