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Degradation of 2,6-di-*tert*-butylphenol by an isolated high-efficiency bacterium strain

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Abstract: An aerobic bacterium strain, F-3-4, capable of effectively degrading 2,6-di-*tert*-butylphenol (2,6-DTBP), was isolated and screened out from an acrylic fiber wastewater and the biofilm in the wastewater treatment facilities. This strain was identified as *Alcaligenes* sp. through morphological, physiological and biochemical examinations. After cultivation, the strain was enhanced by 26.3% in its degradation capacity for 2,6-DTBP. Results indicated that the strain was able to utilize 2,6-DTBP, lysine, lactamine, citrate, *n*-utenedioic acid and malic acid as the sole carbon and energy source, alkalinize acetamide, asparagine, L-histidine, acetate, citrate and propionate, but failed to utilize glucose, D-fructose, D-seminose, D-xylose, serine and phenylalanine as the sole carbon and energy source. The optimal growth conditions were determined to be: temperature 37℃, pH 7.0, inoculum size 0.1% and shaker rotary speed 250 r/min. Under the optimal conditions, the degradation kinetics of 2,6-DTBP with an initial concentration of 100 mg/L was studied. Results indicated that 62.4% of 2,6-DTBP was removed after 11 d. The degradation kinetics could be expressed by Eckenfelder equation with a half life of 9.38 d. In addition, the initial concentration of 2,6-DTBP played an important role on the degradation ability of the strain. The maximum initial concentration of 2,6-DTBP was determined to be 200 mg/L. Above this level, the strain was overloaded and exhibited significant inhibition.

Keywords: 2,6-di-*tert*-butylphenol; bacterium strain; *Alcaligenes* sp.; isolation

Introduction

Phenolic compounds are widely used in the industries of metallurgy, pharmacy, petroleum and pesticide. As a consequence, they are major pollutants in the wastewaters produced by these industries. The phenolic pollutants, if discharged without proper treatment, will contaminate surface water, ground water and soils, and eventually pose a serious threat to public health.

Degradation of phenolic compounds by pure biological cultures has been extensively studied during the last few years (Häggblom, 1992). For example, chlorophenols were degraded by *Ralstonia* (Steinle, 1998; Yabuuchi, 1995), *Alcaligenes* (Balfanz, 1991; González, 1996; Hill, 1996), *Pseudomonas* (Dapaah, 1992; Xun, 1996) and *Desulflovibrio* (Christiansen, 1996), phenol by *Pseudomonas* sp. (Wu, 1992), *Fuasrium flowiferum* (Anselmo, 1992) and *Alcaligenes* sp. A7-2 (Menke, 1992). Biodegradation of other phenolic compounds, such as *p*-cresol (O'Reilly, 1989) and aminophenol (Zhao, 2000), has also been reported. Degradation pathways of a few phenolic compounds have been proposed (Zhao, 2000; Bhat, 1994; Nishino, 2000). However, little is known about the biodegradation of 2,6-DTBP.

2,6-DTBP is largely used in the manufacture of pharmaceuticals, chemical fibers, rubbers and fragrances as an intermediate compound. The Ethyl Corporation reported that 2,6-DTBP was acute toxic to freshwater alga, rainbow trout, fathead minnow, daphnia, and gammarus even at low concentrations ($LC_{50} \leq 1.0$ mg/L). This compound could not be degraded under anaerobic condition by an inoculum of primary sludge within 56 d (Walker, 1995). Zhang (Zhang, 1999) reported that 2,6-DTBP is bio-refractory through the study of quantitative structure-activity relationship for biodegradation. So, it is significant to study the biodegradation of 2,6-DTBP.

This study was first to isolate a specific bacterium strain, namely F-3-4, using 2,6-DTBP as the sole carbon and energy source. Thereafter, the optimal growth conditions of this strain were determined and the biodegradation characteristics of 2,6-DTBP were investigated. Since 2,6-DTBP is one of the major pollutants present in the wastewater produced by an acrylic fiber company in Shanghai, the strain F-3-4 is expected to supply a useful bacteria resource for biological treatment of similar industrial wastewaters.

1 Materials and methods

1.1 Substrate

2,6-DTBP was purchased from the Institute of Assistant of Beijing (AR grade).

1.2 Media

The broth medium for microorganism isolation and cultivation was prepared as follows (Zhou, 2000): beef extract 5 g, NaCl 5 g, and peptone 10 g per liter of distilled water (16 g agar powder was added in solid medium), pH 7.2. The Bushnell-Hass base medium (B. H.) for the degradation of 2,6-DTBP was prepared as follows (Bushnell, 1941): $MgSO_4$ 0.2 g, KH_2PO_4 1 g, $(NH_4)_2SO_4$ 1 g, $CaCl_2$ 0.02 g, K_2HPO_4 1 g, and 2 drops of concentrated $FeCl_3$ solution per liter of distilled water with varying pH conditions (16 g agar powder is added in solid medium). All other media for the identification of the strain F-3-4 were prepared

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following the literature (Dong, 2001; BTGIM, 1978; Oberhofer, 1974; 1977).

1.3 Source of inocula

The microbial populations were obtained from the wastewater and the biofilm in the wastewater treatment facilities of an acrylic fiber company in Shanghai.

1.4 Isolation and screening of degrading strains for 2,6-DTBP

Several dilutions at different concentrations were got by diluting the mixture obtained by breaking the biofilm and the samples from wastewater with glass beads sterilized and diluting into normal saline. They were coated the solid broth medium plane tables with 2, 6-DTBP at 30 g/L. After incubated for 3 d, parts of the single colonies were selected and scribed onto tables with 2,6-DTBP at 40 g/L to incubate for 3 d. At last, single colonies, capable to tolerate the toxicity of 2,6-DTBP in high concentrations, were picked out and scribed onto solid broth medium tables without 2,6-DTBP to incubate for 24 h. Culture was picked out to incubate again, and repeated for several times to purify and isolate them. Through the degrading capability test, a pure culture with largest degrading capacity was screened out from several cultures. It was used as the test microorganism.

1.5 Preparation of microorganism suspension

The strain was cultivated on slants at 37℃ for 24 h, inoculated with a loop to 30 ml broth medium contained in 250 ml flasks, and incubated on 37℃ shakers at 250 r/min (revolutions per minute) for another 24 h. After isolation and cleaning with normal saline, the microorganism suspension with a desired concentration was prepared.

1.6 Strain cultivation

An aliquot of 3 ml of strain F-3-4 suspension (3% concentration (w/v) based on the wet weight of cells per liter of normal saline) was inoculated into 30 ml B.H. medium containing 500 mg/L 2,6-DTBP, and incubated on shakers at 37℃ and 250 r/min. After 4 d of cultivation, the culture solution turned obviously turbid. The acclimation procedure was repeated several times before the strain was applied to 2,6-DTBP degradation experiments.

1.7 Strain growth conditions and 2,6-DTBP degradation

During strain cultivation, 2,6-DTBP was spiked into the B. H. medium as the sole carbon source till a final concentration of 100 mg/L. Single-factor-design was adopted to conduct experiments which included temperature 25, 30, 37, 40 and 45℃, initial pH 5.0, 6.0, 7.0, 8.0 and 9.0, inoculum size 0.01%, 0.05%, 0.10%, 0.15% and 0.20%, and shaker rotary speed (directly reflecting aeration capacity) 150, 200, 220, 250 and 300 r/min. The optical density (OD) and the concentration of 2, 6-DTBP of the culture solution at 11 d were analyzed to show the growing conditions effect and determine the optimal growth conditions.

1.8 Degradation of 2,6-DTBP by isolated strain under optimum growth conditions

The degradation of 2,6-DTBP was investigated after the optimal growth conditions for the isolated strain were determined. 2,6-DTBP was spiked into the B.H. medium as the sole carbon source till a final concentration of 100 mg/L. Each flask containing 30 ml of B.H. medium was inoculated with 1 ml microorganism suspension (3% wet cells weight concentration, corresponding to an inoculum size of 0.10%). These flasks, sealed by grinding glass plugs which were further wrapped around by parafilm, were incubated on 37℃ shakers at 250 r/min for 11 d. Blank flasks without inoculum were simultaneously incubated as control. Samples were withdrawn from the incubated medium at pre-selected times to determine the concentration of 2,6-DTBP.

1.9 Effect of initial concentration of 2,6-DTBP

The effect of initial concentration of 2, 6-DTBP was investigated under optimal growth conditions, which included 41, 72, 106, 153, 200, 404, 595 and 1007 mg/L.

1.10 Sample pretreatment and analysis

Samples were first pretreated following the procedures: add 10 ml of *n*-hexane into each flask, vigorously hand-shake for 2 min, immediately transfer the mixture into a centrifuge tube, centrifuge (Sorvall Super T21 superspeed tabletop refrigerated centrifuge, Sorvall, USA) at 10000 r/min and 4℃ for 5 min, and withdraw and dilute the supernatant to an appropriate concentration for analysis. An ultraviolet-visual spectrophotometer (UV-2201, Shimadzu, Japan) was utilized to determine the concentration of 2,6-DTBP at 276 nm. For the purpose of comparison, the concentration of 2, 6-DTBP was alternatively analyzed by HPLC (model 1050, Hewlet Packard, USA) coupled with a KR100-5 C_{18} reverse phase column and an ultraviolet detector. It was found that the two analytical methods yielded similar results, which means the separation using HPLC was not necessary for our samples. Due to the analytical convenience, the UV-2201 was adopted for the determination of 2,6-DTBP concentrations. The optical density (OD) of the culture solution was examined by a visual spectrophotometer (SP-1150, China) at 640 nm to determine the amount of bacteria growth.

1.11 Strain identification

Through examinations of its morphological, physiological and biochemical characteristics and reference to the literature (Krieg, 1984; Holt, 1994; Dong, 2001; Bacterial, 1978; Oberhofer, 1974; 1977), the isolated strain (F-3-4) was identified.

2 Results and discussion

2.1 Strain isolation and identification

A strain, being able to tolerate the toxicity of high concentration 2,6-DTBP and capable to degrade 2,6-DTBP, was isolated out from the acrylic fiber wastewater and biofilm in its treatment facilities, and named F-3-4.

The morphological characteristics of the strain F-3-4 were determined as follows: Gram-negative, rod, short rod or approximate cocci shape, motile with one to five peritrichous flagella, 0.5—0.6 μm wide and 0.6—2.0 μm long. The morphological characteristics of the colony include: round shape, straw yellow color at the broth agar surface, 0.5—1.0 mm in diameter, smooth and moist surface, low umbos, cracked-leaf likely fringe, and emission of fruit odor. The physiological and biochemical characteristics of the strain were detailed in Table 1. Results indicated that the characteristics of the strain F-3-4 closely matched those of *Alcaligenes* described in the references (Krieg, 1984; Holt,

1994). Therefore, the strain F-3-4 was identified as *Alcaligenes* sp.

2.2 Effect of strain cultivation on 2, 6-DTBP degradation

Strains with no cultivation, cultivation for two times, and cultivation for four times were prepared for microorganism suspensions, inoculated into the B. H. medium with 100 mg/L 2,6-DTBP, and incubated. The culture solutions were withdrawn at pre-selected time to analyze the concentration of 2, 6-DTBP. Fig. 1 shows that through cultivation, the degrading ability of the strain F-3-4 was enhanced. At an initial substrate concentration of 100 mg/L, the cultivated strain (for four times) degraded 26.3% more of 2,6-DTBP than the non-cultivated strain.

Table 1 Physiological and biochemical characters of strain F-3-4

Positive	Negative
Aerobic growth	$NO_3^- \rightarrow NO_2^-$
Catalase	Gas production from NO_3^-
Oxidase	Alkalinization from:
Oxidative metabolism (alkalinization)	Nicotinamide
Gas production from NO_2^-	Itaconate
Alkalinization from:	Utilization as sole carbon and energy source:
Acetamide	Glucose
Asparagine	D-fructose
L-histidine	D-seminose
Acetate	D-xylose
Citrate	Serine
Propionate	Phenylalanine
Utilization as sole carbon and energy source:	Acid production from carbohydrates:
Lysine (weekly)	Glucose
Lactamine	D-fructose
Citrate	D-mannitol
n-utenedioic acid	D-xylose
Malic acid	Gelatin hydrolysis
Growth at 20—37℃	Cellulose hydrolysis
	Aesculin hydrolysis
	Indole production
	Oxidizing ethanol to acetic acid
	NaCl for its growth

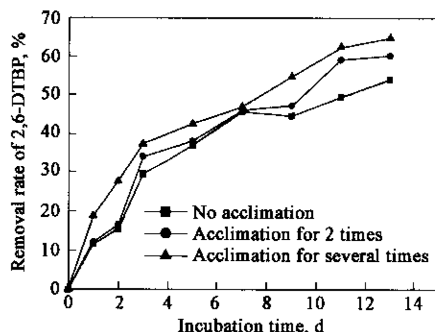

Fig. 1 2,6-DTBP degradation by cultivated strain F-3-4

2.3 Effects of growth conditions on strain growth and 2,6-DTBP degradations

Using 2,6-DTBP as the sole carbon source, the effects of various growth conditions including temperature, initial pH, inoculum size and shaker rotary speed were examined by monitoring the concentration of 2,6-DTBP and the OD of the culture solution.

Fig. 2 shows that the cultivation temperature exerted a significant effect on strain growth as reflected by the OD. Below 30℃, no strain growth was observed and correspondingly, there was low or no removal of 2,6-DTBP. Above 30℃, both strain growth and 2,6-DTBP degradation amounts increased rapidly with raised temperature till the optimal temperature of 37℃. Further increase of temperature led to a rapid drop in both strain growth and 2, 6-DTBP degradation amounts. The phenomena, no growth below 30℃, results from the small solubility of 2,6-DTBP at B.H. base medium. In water with pH 7.0 and temperature 25℃, its solubility is only 4.11 mg/L(Walker, 1995). The melting point of 2,6-DTBP varies between 34 to 38℃ with its purity. In this study, its melting point is 37℃ approximately. At low temperature, especially below 30℃, small solubility resulted in lack of carbon and energy source because 2,6-DTBP was used as sole carbon and energy source. And most 2,6-DTBP was presented as solid grains resulting in affect the effective touch and combination between 2, 6-DTBP grains and degrading enzymes. Above 30℃, however, its solubility increased with raised temperature, leading to increase in strain growth and corresponding 2, 6-DTBP degradation amount. While above 37℃, the degrading enzymes were deactivated and strain was killed by high temperature.

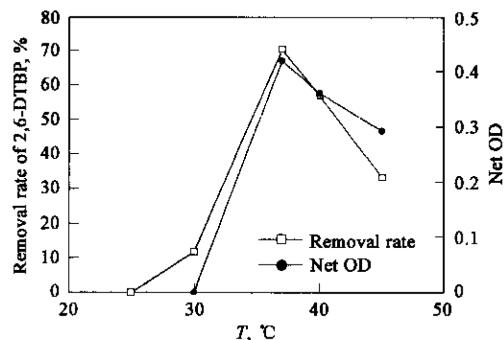

Fig. 2 Effect of temperature on strain growth and 2,6-DTBP degradation

The optimal temperature for strain growth was 37℃ (Fig. 2), higher than those of other phenol-degrading bacteria reported in the literature (Steinle, 1998; Zhao, 2000; Tirrola, 2002; Hu, 1994). The strain F-3-4 showed the higher temperature dependence. Coincidentally, the wastewater temperature in the biological tower filter is around 37℃ which is equal to the optimal temperature for strain growth. The coincidence in temperature may result from the fact that the strain F-3-4 originated from the wastewater and biofilm.

Fig. 3 shows that below an inoculum size of 0.1%, both strain growth and 2, 6-DTBP degradation amounts increased with increasing inoculum size. Above 0.1%, the strain growth amount dropped rapidly while the 2, 6-DTBP removal increased slightly with increasing inoculum size. The reason might be that great accumulation of metabolite produced by great number of microorganisms, above an inoculum size of 0.1%, in obturated system resulted in great toxicity to them.

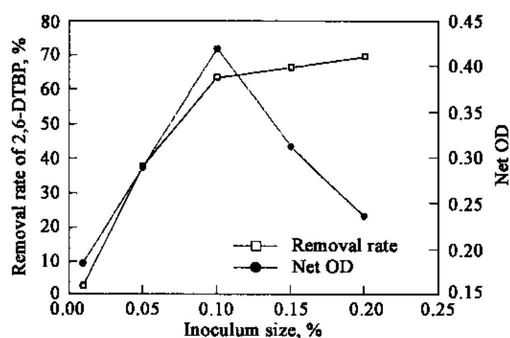

Fig. 3 Effect of inoculum size on strain growth and 2,6-DTBP degradation

The optimal pH was determined to be pH 7.0, as shown in Fig. 4. Results also indicated that the neutral or slightly basic pH condition is preferred by the strain F-3-4, compared with the acidic conditions, agreeing with the pH requirement for most bacteria (Steinle, 1998; Gonzólez, 1996; Hill, 1996; Menke, 1992; Tirrola, 2002) and matching the characteristics of *Alcaligenes* (Bushnell, 1941) as well.

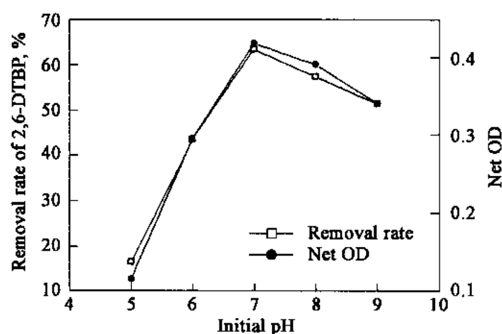

Fig. 4 Effect of initial pH on strain growth and 2,6-DTBP degradations

Results in Fig. 5 indicate that both strain growth and 2,6-DTBP removal increased with increasing shaker rotary speed. However, above 250 r/min the increase in 2,6-DTBP removal efficiency was insignificant.

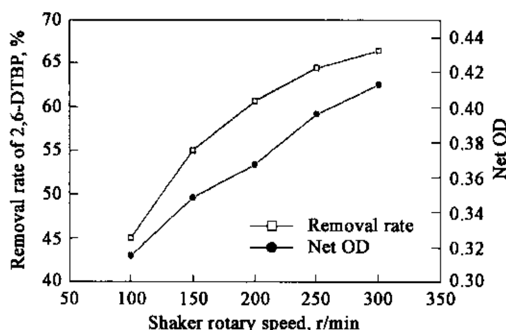

Fig. 5 Effect of shaker rotary speed on strain growth and 2, 6-DTBP degradation

Taking all the substrate removal, strain growth and economical feasibility in to consideration, the optimal growth conditions for the strain F-3-4 were determined as follows: temperature 37℃, initial pH 7.0, inoculum size 0.1%, and shaker rotary speed 250 r/min.

2.4 Degradation of 2, 6-DTBP under optimal growth conditions

Under the optimal growth conditions determined above, the strain F-3-4 exhibited a considerably significant degradation ability for 2,6-DTBP whose initial concentration was 100 mg/L (Fig. 6). The removal efficiency of 2,6-DTBP reached 62.4% after 11 d. Eckenfelder kinetic expression was found to be suitable for describing 2,6-DTBP degradation at relatively low initial substrate concentrations (Fig. 6). The kinetic equation, after fitting experimental data, could be described as follows (R^2 = 0.96, half life = 9.38 d):

$$S = e^{-0.0739t+4.4677}, \quad (1)$$

where, S represents the concentration of 2,6-DTBP (mg/L), and t is the incubation time (d). Although 2,6-DTBP could be biodegraded in this study, it was proved there were difficulties to degrade 2,6-DTBP because in comparison with other phenol-degrading bacteria, strain F-3-4 exhibited a relatively long half time to degrade 2, 6-DTBP due to the higher toxicity of 2,6-DTBP.

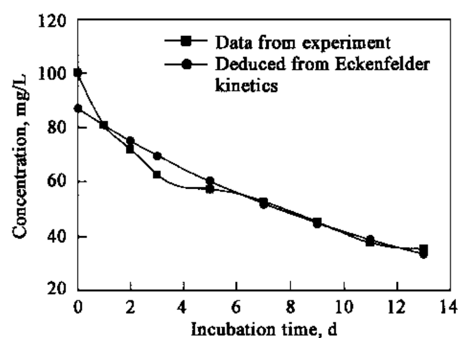

Fig. 6 2, 6-DTBP degradation by strain F-3-4 under optimal growth conditions

2.5 Effect of initial concentration of 2,6-DTBP

Fig. 7 shows that both strain growth and 2, 6-DTBP removal amounts increased with increasing initial concentration of the substrate till 200 mg/L. However, the strain growth was severely inhibited above the initial substrate concentration of 200 mg/L, so was the substrate removal amount. Results indicated that 200 mg/L was the optimal point where the largest amounts of strain growth and 2, 6-DTBP removal were achieved, i.e., OD_{net} = 0.974 and 2,6-DTBP removal amount = 94.3 mg/L (corresponding to an removal efficiency of 47.8%). This implies that the bio-refractory compound, 2, 6-DTBP, can be biodegraded if suitable bacterium strains are selected.

In comparison with other phenol-degrading bacteria, strain F-3-4 exhibited a relatively slower substrate degradation rate due to the higher toxicity of 2,6-DTBP (Zhang, 1999; Walker, 1995). Above 200 mg/L of 2,6-DTBP, the strain would be significantly inhibited due to the substrate overload. However, the concentration of 2,6-DTBP in the wastewater was determined to be only about 4.0 mg/L. Therefore, it is reasonably expected that all 2,6-DTBP would be effectively removed by strain F-3-4 from the wastewater. Considering that other toxic pollutants present in the wastewater (Zhang, 1999) may inhibit the strain F-3-4, strain immobilization technique is probably suitable to reduce the environmental toxicity so that an effective degradation of 2,6-DTBP can be

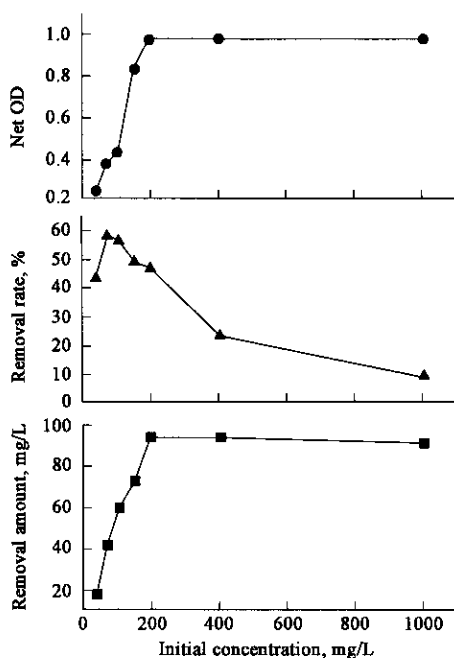

Fig. 7 Effect of initial concentration of 2,6-DTBP on the degradation ability of strain F-3-4

achieved (Balfanz, 1991; Wu, 1992; Anselmo, 1992; Menke, 1992; O'Reilly, 1989; Hu, 1994).

In addition, it is found that the strain F-3-4 could not degrade carbohydrates, such as glucose, D-fructose, D-xylose, and D-seminose. This characteristic may enhance the removal efficiency of 2,6-DTBP by strain F-3-4 because the competition from different substrates is reduced.

3 Conclusions

This work isolated a high efficiency bacterium strain, named F-3-4 and identified as *Alcaligenes* sp., for the degradation of 2,6-DTBP. The optimal conditions for both strain growth and substrate removal were determined to be: temperature 37℃, pH 7.0, inoculum size 0.10%, and shaker rotary speed 250 r/min. Under the optimal conditions, the removal efficiency of 2,6-DTBP reached 62.4% at an initial substrate concentration of 100 mg/L. Above 200 mg/L of initial substrate concentration, the strain exhibited severe inhibition due to the toxicity of 2,6-DTBP.

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