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Biodegradation Characteristics of Environmental Endocrine Disruptor Di-n-butyl Phthalate[1]

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Objective The biodegradation characteristics of di-n-butyl phthalate (DBP), an environmental endocrine disruptor, were studied by the method of dominant bacteria and immobilized microorganisms. **Methods** Taking DBP as the only carbon source to acclimatize the collected activated sludge, the concentration of DBP increased progressively in the process of acclimatization. Plate streaking was used to separate 1 strain of the degradation dominant bacteria after acclimatization. Better conditions to degrade DBP by the bacterium could be obtained through orthogonal experiments and the bacterium was identified. Then the acclimated activated sludge was made to immobilize the microorganism using polyvinyl alcohol as entrapment agent. The immobilized microorganism degraded DBP at different conditions. **Results** The appropriate conditions to degrade DBP by the dominant bacteria were: degradation time, 32 h; DBP concentration, 200 mg/L; rate of shaking incubator, 100 r/min; pH, 7 and temperature, 30℃. DBP could be degraded by more than 95% under such conditions. The bacteria were identified as *pseudomonas*. The proliferated immobilized microorganisms degraded DBP more effectively and more adapted to temperature and pH than the free acclimated activated sludge. **Conclusion** One strain of DBP degradation dominant bacteria was separated from the acclimatized activated sludge. It could grow with DBP as the only carbon source and energy, and degraded DBP effectively. After having been immobilized and proliferated, the dominant bacteria could keep a higher biological activity and degrade DBP more effectively than activated sludge.

Key words: Environmental endocrine disruptor; Di-n-butyl phthalate; Biodegradation; Degradation dominant bacteria; Immobilized microorganism

INTRODUCTION

Phthalic acid esters (PAEs) are one of artificially synthesized, refractory organic compounds, and they are produced with a high output and widely used mainly in chemical production such as making plastics flexible, doping, painting *etc.* As a result, they have been found in atmosphere, soil and water and have become global environmental pollutants. Because PAEs are lipophilic, they can accumulate in organisms and the concentration of PAEs in fish bodies is higher than that in water. At present, PAEs are named as environmental endocrine disruptor, which can show hormone-like function in body. The target organ is male animal genital gland. PAEs can cause testis atrophy and reduce the quantity of sperm. They also have some toxicity to embryogenesis and neural tube defect[1-3].

In this work, DBP was chosen as the research object for it existed widely in natural water and its concentration was high[4]. Then, the activated sludge was acclimatized, separated and filtrated to gain the high effective DBP degradation dominant bacteria. The appropriate degradation conditions for the bacteria were obtained through orthogonal experiments. The acclimatized activated sludge was made to immobilize the small ball using polyvinyl alcohol (PVA) as an entrapment agent and its biological characteristics of degradation on DBP were investigated.

MATERIALS AND METHODS

Experimental Materials

Activated sludge It was collected from the second sedimentation pool of Wuhan Beer Company Waste Water Treatment Station.

Inorganic salt culture solution It contained $K_2HPO_4 \cdot 3H_2O$ 1 g/L, NaCl 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.4 g/L, NH_4NO_3 0.5 g/L, $CaCl_2 \cdot 2H_2O$ 0.1 g/L, $FeCl_3$

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0.01 g/L.

Activated enrichment culture medium It consisted of pancreas peptone 10 g/L, yeast extract solution 5 g/L, NaCl 10 g/L.

Immobilized agents PVA (for industry, 1799 model, Yunnan Weinilun company), boracic acid (A. P, Beijing Hongxing Chemical Plant), and farinose activated carbon (Shanghai Activated Carbon Company) were used.

Di-n-butyl phthalate It was from Guangdong Shantou Xilong Chemical Plant and with analytical grade.

Gas chromatograph VALIANCP3800 model was from VALIAN Company, USA.

Experimental Methods

Activated sludge acclimatization The sludge was deposited for 2 days. Then, the supernatant was discarded and the impurity was filtrated out. After that the sludge was placed in a barrel to airing and acclimatized with DBP as the only carbon source at 37℃ by the method of increasing carbon source in an intermission, time-controlled and fixed-quantity way. The DBP concentration increased gradually from 50 mg/L to 500 mg/L. During the acclimatization, the soluble oxygen was controlled at 3-4 mg/L. The ratio of the inorganic salt culture solution to the activated sludge was 3:1.

Separation of DBP degradation dominant bacteria Proper agar powder was added into the inorganic salt culture solution, which was sterilized and put into a plate. Then, sterilized DBP was spread on the plate and dipped for 30 min. The acclimatized activated sludge was diluted to a certain concentration and inoculated in the plate by spread plate method. The plate was cultivated at 30℃ and different colonies were got. The pure DBP degradation dominant bacteria could be obtained by plate streaking repeatedly.

Identification of DBP degradation dominant bacteria The DBP degradation dominant bacteria with the best growth situation and degradation effect were chosen from obtained pure ones and identified according to the manual of determinative bacteriology[5].

Biodegradation test of DBP degradation dominant bacteria A vergae of strain was placed in 50 mL of sterilized enrichment culture medium and cultivated with vibration for 12 h. Then the medium was centrifuged and the thallus was collected and washed three times with buffer (0.02 mol/L Na_2HPO_4- NaH_2PO_4, pH 7) and suspended in the buffer and made into bacteria suspending solution. One mL of the solution was added to the inorganic salt culture solution containing DBP and degradation tests were undertaken in different conditions.

Preparation of immobilized ball The immobilized ball was cultured for 3 days[6-7].

Degradation of immobilized microorganisms on DBP In order to make the degradation tests of immobilized microorganisms and activated sludge more comparable, an “equal biomass” was adopted in the tests, it means that, the amount of activated sludge entrapped in the immobilized ball was equal to the amount of sludge in free activated sludge degradation tests. The immobilized ball, acclimatized activated sludge and water sample (mixed by DBP and tap water according to the test) were placed in a conical flask (volume 250 mL) in some proportion. Then they were placed in a shaking incubator and degradation tests were progressed at different shaking rate of incubator, temperature, pH and initial concentration.

DBP analytical methods Sample processing: water sample was extracted with dichloromethane (20 mL, 10 min) three times. Three aliquots of dichloromethane were combined and dried by anhydrous Na_2SO_4, and then concentrated into 5 mL. DBP concentrations of all samples in this work were determined by gas chromatography (VALIANCP model 3800, VALIAN company, USA) analysis. The conditions were as follows: OV-101 capillary column; carrier gas, high pure nitrogen gas (5 mL/min); injector temperature, 260℃; column temperature and programmed temperature from 100℃ to 230℃ at 20℃/min and 230℃ for 8 min; FID detector, temperature 300℃; injection volume, 1 μL.

RESULTS AND DISCUSSION

Suitable Conditions for DBP Degradation Dominant Bacteria

Temperature, pH, oxygen amount, initial DBP concentration and degradation time taken as major factors and each factor containing four levels are shown in Table 1. An equal amount of the bacteria was added to each water sample and DBP degradation tests were done according to the orthogonal table $L_{16}(4^5)$. Table 2 gives the results in number 5 and number 14 tests. Their degradation rate was 93.4% and 95.5%, respectively. According to the range analysis, the sequence of 5 factors was degradation time, DBP concentration, rate of incubator, pH and temperature. The better conditions of the dominant bacteria on DBP degradation were as follows: degradation time, 32 h; DBP concentration, 200 mg/L; rate of incubator, 100 r/min; pH, 7 and temperature, 30℃.

The dominant bacteria were identified according

to the ordinary classification method. Table 3 shows the shape and characteristics of the bacteria, which illustrated that the dominant bacteria belonged to *pseudomonas.*

Degradation Characteristics of Immobilized Microorganisms on DBP

Effects of DBP initial concentration on degradation The DBP initial concentrations of water samples were 100, 200, 300, 400, and 500 mg/L. The tests were done by shaking incubator (30 ℃, 100 r/min). The retention time was 24 h. Table 4 gives the results.

Table 4 shows that the suitable concentration of the free activated sludge to degrade DBP was below 300 mg/L. However, the concentration of immobilized microorganisms reached up to 500 mg/L. This fact suggested that the immobilized microorganisms had much more tolerance to DBP loading than activated sludge.

Influence of different pH The pH of water samples was 5.0, 6.0, 7.0, 8.0, and 9.0, which were regulated with 10% NaOH and HCl. The initial DBP concentration was 100 mg/L. Other conditions were identical to the activated sludge acclimatization. The degradation rate could be seen in Table 5.

As shown in Table 5, both the activated sludge and the immobilized microorganisms had a higher degradation rate when pH was 6-8. The DBP degradation rate of the activated sludge group dropped obviously when pH was below 6 or above 8, but such rate of the immobilized microorganisms vibrated slightly. This fact showed that the immobilized microorganisms had a better adaptability to pH of the water samples.

Influence of temperature on degradation The initial DBP concentration of the water samples was 100 mg/L. pH was 7.0. Shaking rate of incubator was 100 r/min. The degradation rates at 20℃, 25℃, 30℃, 35℃, and 40℃ are given in Table 6.

Table 6 shows that the immobilized microorganisms had a higher adaptability to the change of temperature than activated sludge. But the degradation rate of immobilized microorganisms dropped apparently when the temperature reached up to 40℃, showing that the degradation temperature of immobilized microorganisms on DBP should not be too high.

Effects of different shaking rate incubator of on degradation The shaking rates of incubator were 0, 50, 100, and 150 r/min. The initial DBP concentration was 100 mg/L. Other conditions were the same as the activated sludge acclimatization. The results of the degradation tests are shown in Table 7.

Table 7 shows that both the activated sludge and the immobilized microorganisms had a higher DBP degradation rate under vibration and no significant change was shown in the degradation rate when the shaking rate of incubator was above 100 r/min. This fact illustrates that vibration could speed up transporting and prompt entering and dissolving of oxygen. If simulation equipment were used, a higher degradation rate would be gained as long as enough dissolving oxygen was provided and vibration was given properly.

CONCLUSION

DBP degradation dominant bacteria separated from the acclimatized activated sludge could grow with DBP as the only carbon source and energy. Through orthogonal experiments, the appropriate degradation conditions of the bacteria were: degradation time, 32 h; DBP concentration, 200 mg/L; shaking rate of incubator, 100 r/min; pH, 7 and temperature, 30℃. The DBP degradation rate could reach up to 95% under the above conditions. The bacteria were identified as *pseudomonas.*

DBP degradation bacteria could keep a higher biological activity after being immobilized and cultivated, thus degrading DBP effectively. The immobilized microorganisms are more adaptable to temperature, pH, and DBP load than the activated sludge.

TABLE 1

Design of Orthogonal Test

Level	Factor				
	Shaking Rate of Incubator (r/min) A	DBP Concentration (mg/L) B	pH C	Temperature (℃) D	Degradation Time (h) E
1	0	100	6.0	20	8
2	50	200	6.5	25	16
3	100	300	7.0	30	24
4	150	400	7.5	35	32

TABLE 2

Results of Orthogonal Test

Test Number	Factor					Degraded Rate (%)
	A	B	C	D	E	
1	1	1	1	1	1	28.5
2	1	2	2	2	2	60.1
3	1	3	3	3	3	77.8
4	1	4	4	4	4	70.3
5	2	1	2	3	4	93.4
6	2	2	1	4	3	83.2
7	2	3	4	1	2	70.4
8	2	4	3	2	1	35.1
9	3	1	3	4	2	71.5
10	3	2	4	3	1	51.3
11	3	3	1	2	4	92.8
12	3	4	2	1	3	69.4
13	4	1	4	2	3	85.5
14	4	2	3	1	4	95.5
15	4	3	2	4	1	40.4
16	4	4	1	3	2	53.6
Ⅰj	236.7	278.9	258.1	263.8	155.3	
Ⅱj	282.1	290.1	263.3	273.5	255.6	
Ⅲj	285.0	281.4	279.9	276.1	315.9	G=1078.8
Ⅳj	275.0	228.4	277.5	265.4	352.0	
Rj	12.1	15.4	5.5	3.1	49.2	

TABLE 3

Identified Characteristics of Degradation Dominant Bacteria

Identification Items	Results
Colony Characteristics	Buff, Wet, Enation, Smooth, and Glossy, Border Neat
Gram Staining	Negative
Individual Shape	Staff
Size	0.8×2um
Mobility	Positive
Glucose Fermentation	Positive
Glucose Oxidation	Negative
Contact Enzyme Test	Positive
Oxidase Test	Positive
Pseudomonas Medium Test	Grow Well

TABLE 4

Effects of DBP Initial Concentration on Degradation

Initial Concentration (mg/L)	Activated Sludge		Immobilized Microorganisms	
	Concentration (24 h, mg/L)	Degraded Rate (%)	Concentration (24 h, mg/L)	Degraded Rate (%)
100	25.2	74.8	18.8	81.2
200	35.2	82.4	29.2	85.4
300	67.3	77.6	62.1	79.3
400	171.4	57.1	115.2	71.2
500	251.8	47.6	191.5	61.7

TABLE 5

Effects of pH on Degradation

pH	Activated Sludge		Immobilized Microorganisms	
	Concentration (24 h, mg/L)	Degraded Rate (%)	Concentration (24 h, mg/L)	Degraded Rate (%)
5.0	38.0	62.0	28.8	71.2
6.0	26.2	73.8	22.3	77.7
7.0	25.2	74.8	18.8	81.2
8.0	25.5	74.5	23.8	76.2
9.0	37.0	63.0	27.2	72.8

TABLE 6

Degradation Results of DBP at Different Temperature

T (℃)	Activated Sludge		Immobilized Microorganisms	
	Concentration (24 h, mg/L)	Degraded Rate (%)	Concentration (24 h, mg/L)	Degraded Rate (%)
20	33.4	66.6	25.7	74.3
25	23.2	76.8	20.1	79.9
30	25.2	74.8	18.8	81.2
35	24.5	75.5	21.7	78.3
40	44.2	55.8	32.7	67.3

TABLE 7

Degradation Results of DBP at Different Incubator Rate

Incubator Rate(r/min)	Activated Sludge		Immobilized Microorganisms	
	Concentration (24 h, mg/L)	Degraded Rate (%)	Concentration (24 h, mg/L)	Degraded Rate (%)
0	44.7	55.3	34.8	65.2
50	31.8	68.2	27.7	72.3
100	25.2	74.8	18.8	81.2
150	24.9	75.1	18.1	81.9

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