Study on the biodegradation of di-ethyl hexyl phthalate (DEHP) by different microorganisms

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In order to study a more simple, practical, and efficient method and technology for bioremediation of environmental phthalates, di-ethyl hexyl phthalate (DEHP) was used as the research object. The influence of five kinds of microorganisms (*Aspergillus niger* (JZ1), *Penicillium* (JZ2), *Escherichia coli* (JZ3), *Bacillus subtilis* (JZ4), and yeast (JZ5)) on DEHP degradation was studied by single factor test and the best test plan, and the degradation kinetics was analyzed. According to the single factor test, the optimum conditions for DEHP degradation of JZ1, JZ2, and JZ5 were pH 5.0, 30℃, and 205 rpm, while the optimal conditions for the degradation of DEHP by JZ3 and JZ4 were pH 7.0, 35 ℃, and 205 rpm. In addition, the optimum conditions for the degradation of DEHP by JZ1 were determined by orthogonal experiment with pH 5.0, temperature 30℃, rotational speed 210 rpm, and cultural time 36h. Then, under the optimal degradation conditions, the relationship between the ability of JZ1 to degrade DEHP and the growth characteristics of JZ1 was analyzed. The degradation ability of JZ1 in logarithmic phase was the best. Within 60 hours, the degradation rate of DEHP by JZ1 was 68.75%. Finally, the degradation kinetics of these five microorganisms was analyzed. The correlation coefficient of JZ1 and JZ2 kinetic equation were more than 0.90, which indicated that the degradation curve of this strain to DEHP could well conform to the first-order kinetic equation. These results demonstrated that the five microorganisms in this study had the ability to degrade DEHP. This will provide a certain theoretical basis and technical support for the subsequent research on the separation and purification of vegetable phyllospheric microorganisms and degradation of environmental pollutants.

Keywords: Di-ethyl hexyl phthalate (DEHP); microorganism; biodegradation; degradation kinetics.

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Introduction

Di-ethyl hexyl phthalate (DEHP) is one of the most widely used phthalic acid esters (PAEs). It is a kind of environmental secretion disruptor, which is also known as environmental hormone. As a plasticizer, it is widely used in plastics, rubber, medicine, cosmetics, and other industries [1]. Due to DEHP physcically bind to the polymeric matrix, DEHP can easily leak from the polymers into the environment through manufacturing, storage, use, and disposal processes [2]. Some factors, such as solvents (oil), pH, temperature, and so on, have led to the migration of DEHP from products to the environment, even to food [3]. DEHP has caused air, water, soil, and biological pollution, and thus, it will threat the health of human beings, which has been widely evaluated by all countries over the world [4-8].The United States, Japan, Europe, and China have listed DEHP as one of the priority pollutants in water control [9, 10].

The degradation methods of environmental hormones include precipitation, adsorption, degradation (hydrolysis, thermal decomposition, photodegradation, photocatalytic degradation, biodegradation, etc.), and advanced oxidation technologies such as ozone. It has been reported that the hydrolysis and photolysis rate of phthalate esters are extremely slow, time-consuming, and poor degradation effect, due to their hydrophobicity, PAEs binding to particles in the air and organic matters in water, soil, and sediment making it hard for natural degradation processes [4]. However, some studies have suggested that almost all organic pollutants are likely to find out the microorganisms that can degrade and transform them [11-16]. Therefore, microbial degradation will be the main degradation method of DEHP. Biodegradation is an important way of environmental hormone degradation. The application of biodegradation to remove organic pollutants has the characteristics of low cost, short time, obvious effect, stability, and less by-products. In this study, five kinds of microorganisms (prokaryote and eukaryote) were selected, and the influences of these five kinds of microorganisms on DEHP degradation ability were preliminarily explored by single factor test and the best test plan, and the degradation kinetics was preliminarily analyzed.

Materials and Methods

Preparation of microorganism suspension and culture medium

Five kinds of microorganisms including *Aspergillus niger* (JZ1), *Penicillium* (JZ2), *Escherichia coli* (JZ3), *Bacillus subtilis* (JZ4), and yeast (JZ5) were obtained from JK Microbiology Research Center (Shanghai, China) and were cultured in the corresponding medium to the later stage of logarithmic growth, centrifuged at 12,000 rpm, 20℃ for 8 minutes. The microorganisms were washed three times with 0.02 mol/L of Na_2HPO_4-NaH_2PO_4 (PBS) buffer (pH 7.0) and were prepared into microorganism suspension of pH 7.0 and OD_{600} = 0.2 with sterile water. The OD value of the medium was measured by 725N UV-Vis spectrophotometer (Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China) at 600 nm. 5 mL of microorganism suspension was inoculated into 30 mL of Basic inorganic salt medium (BSM) containing K_2HPO_4 5.8 g/L, KH_2PO_4 4.5 g/L, $(NH_4)_2SO_4$ 2.0 g/L, $MgCl_2$ 0.16 g/L, $CaCl_2$ 0.02 g/L, $Na_2MoO_4.2H_2O$ 0.0024 g/L, $FeCl_3$ 0.0018 g/L, $MnCl_2{\cdot}2H_2O$ 0.0015 g/L, at pH 7.0, and 450 mg/L of DEHP.

Determination of DEHP content by gas chromatography-mass spectrometry (GC-MS)

The cleaned samples were analyzed by using a Hewlett-Packard 5890/5971 GC-MSD (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5 trace analysis column (30 m, 0.32 mm I.D., 0.25 mm film thickness) [17, 18]. The GC oven temperature was held at 150℃ for 3 min, and programmed to increase at 20℃/min to 300℃, and finally held at 300℃ for 3 min.

The temperature of the injector was 250℃. Helium was the carrier gas at a linear flow-rate of 20.7 cm/s. Full scan electron ionization data were obtained as follows: solvent delay 5 min, electron ionization energy 70 eV, source temperature 200℃, emission current 150 μA, scan rate 4 scan/s, detector voltage 350 V. The DEHP level in the sample was taken as the average of three injections. The amounts of DEHP were calculated from a calibration curve: y = 0.0014 x + 0.0006 (r2 = 0.9911) for air samples and y = 0.0008 x + 0.0003 (r2 = 0.9848) for vegetable samples. The final contents of the vegetables were expressed as μg/g based on the amounts of the dried samples.

Study on the biodegradation conditions of DEHP

1. Single factor test

In order to determine the optimal initial pH, temperature, shaking speed, and culture time, three factors were fixed in the experiment, while one factor was changed to determine the optimal degradation condition. The residual content of DEHP was determined at 48 hours after

inoculation, and experiments were repeated three times.

(1) Temperature:
The biodegradation experiments with DEHP as the only carbon source were carried out at pH 5.0 (fungi) and pH 7.0 (bacteria), shaking speed of 200 rpm, and different culture temperatures of 15℃, 20℃, 25℃, 30℃, 35℃, 40℃, and 45℃. The content of DEHP was determined after 48 hours.

(2) pH:
The biodegradation experiments were carried out at 30℃ (fungi) and 35℃ (bacteria), 200 rpm shaking speed, and different pH values of 1.0, 3.0, 5.0, 7.0, and 9.0. The content of DEHP was determined after 48 hours.

(3) Shaking speed:
The biodegradation experiments were carried out at pH 5.0 (fungi) and pH 7.0 (bacteria), temperatures 30℃ (fungi) and 35℃ (bacteria), and different shaking speed of 55, 80, 105, 130, 155, 180, 205, 230, 255 rpm. The content of DEHP was determined after 48 hours.

(4) Cultural time:
The biodegradation experiments were carried out at pH 5.0 (fungi) and pH 7.0 (bacteria), temperatures 30℃ (fungi) and 35℃ (bacteria), shaking speed of 200 rpm, and different cultural times of 12h, 24h, 36h, 48h, and 60h.The content of DEHP was determined at different time point.

2. Multiple factors test (*Aspergillus niger* (JZ1))
(1) Orthogonal test:
Under the premise of single factor experiment, four degradation factors including temperature, pH, shaker speed, and culture time were optimized by orthogonal test. The factor level (4 factors and 3 levels) (L9 (3^4)) was set by SPSS (IBM Company, Armonk, New York, USA) as shown in Table 1. The residual amount of DEHP was measured. The degradation rate of DEHP was taken as the index to determine the optimal degradation conditions.

Table 1. Orthogonal design table (*Aspergillus niger* (JZ1)).

Levels	Factors			
	pH	Temperature (℃)	Speed (rpm)	Cultural time (h)
1	4	25	180	36
2	5	28	200	48
3	6	30	210	60

(2) Optimization of degradation conditions of DEHP:
Under the optimal degradation conditions, the microorganism suspension was cultured in inorganic salt medium (DEHP concentration 450 mg/L) for 60 h. The samples were taken every 6 h from the 0 h. Three parallel samples were taken each time to determine the residual amount of DEHP. The degradation curve of DEHP by JZ1 under the best degradation conditions was drawn with time as abscissa and DEHP concentration as ordinate.

(3) Growth of microorganism under optimal degradation conditions:
Under the optimal degradation conditions, the microorganism suspension was cultured in inorganic salt medium (DEHP concentration 450 mg/L) for 60 h. The samples were taken every 6 h with 0 h as blank control. Three parallel samples were taken each time to determine the OD_{600} values. The growth curve of JZ1 under the optimal degradation conditions was plotted with time as abscissa and OD_{600} as ordinate.

Statistical analysis
Data were presented as means together with standard deviations (SDs). In addition, SPSS analysis of variance was also used to optimize the multiple degradation conditions.

Results and discussion

The effect of pH on the degradation of DEHP
As shown in Figure 1, the optimal degradation pH of two kinds of fungi was 5.0, that of yeast was 5.0, and that of two kinds of bacteria was 7.0. For the tested mold and yeast, when the pH value

was between 3.0-7.0, the degradation rate of DEHP was high. For the tested bacteria, when the pH value was higher than 7.0, the degradation rate of DEHP was high. The results indicated that fungi could degrade DEHP better in acid condition, while bacteria could degrade DEHP better in neutral to alkaline condition. The results also showed that the DEHP degradation ability of microorganisms was related to their optimum growth pH. Some studies have reported that, when the microorganisms were in the optimum growth pH, their physiological functions were in the best state, which might be more conducive to promoting the degradation of DEHP [19].

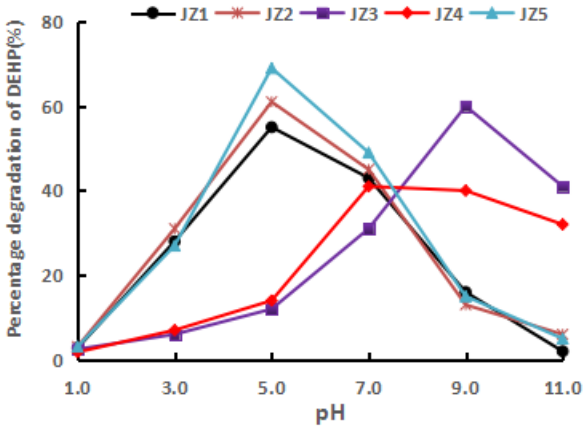

Figure 1. Effect of pH on degradation of DEHP.

The effect of temperature on the degradation of DEHP

Figure 2 showed that the optimum temperature for DEHP degradation was 30℃ by five kinds of microorganisms. When the temperature was 25-35℃, the five microorganisms showed the ability to degrade DEHP with a high degradation rate. When the temperature was above 40℃, the degradation rate of DEHP by five kinds of microorganisms were obviously decreased. The results showed that the DEHP degradation ability of microorganism was also related to the optimum growth temperature. When the microorganism was at the optimum growth temperature, its split generation was the shortest or the growth rate was the highest, which was more conducive to promoting the degradation of DEHP [19].

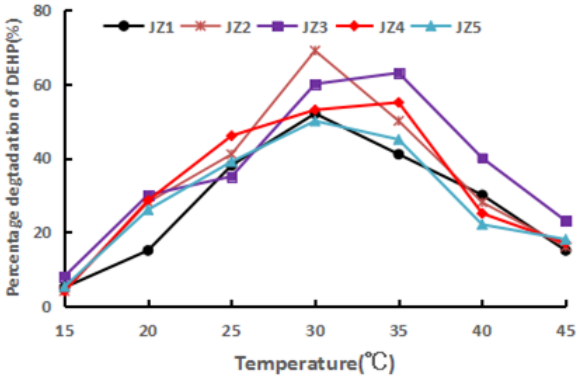

Figure 2. Effect of temperature on degradation of DEHP.

The effect of shaking speed on the degradation of DEHP

Figure 3 showed that the degradation rate of DEHP by the five kinds microorganisms was low at low speed, while the degradation rate increased with the increase of speed. When the rotating speed reached 205 rpm, the degradation rates of five kinds microorganisms reached the highest values. The rotating speed can increase the oxygen content in the reaction solution, which promotes the growth and reproduction of microorganisms, and improves the degradation rate of DEHP. With the further acceleration of rotating speed, the degradation rates of DEHP by three kinds microorganisms (JZ3, JZ4, JZ5) were decreased quickly, while the other two (JZ1, JZ2) were decreased slowly. The results may be related to the relationship between microorganism and oxygen because JZ3, JZ4, and JZ5 are facultative anaerobes while JZ1 and JZ2 are specific anaerobes. For facultative anaerobes in aerobic bacteria, they can grow under aerobic conditions or anaerobic conditions. The faster the rotation speed was, the higher the oxygen content input quantity and frequency was, and the faster the growth speed of microorganisms was. Thus, in the absence of replenishment, the microorganisms entered the apoptosis stage quickly, which ultimately affected the degradation ability [19].

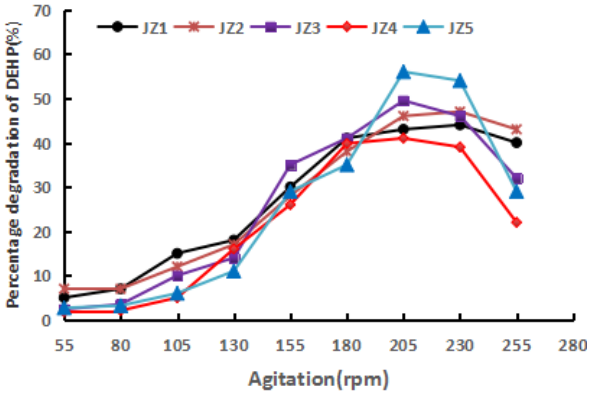

Figure 3. Effect of agitation on degradation of DEHP.

The effect of culture time on the degradation of DEHP

Figure 4 demonstrated that the degradation rate of DEHP by five kinds of microorganisms increased with the time going on during 12-48 h. During 48-60 h, the degradation rates of JZ1, JZ2, and JZ5 were still rising except for JZ3 and JZ4. These results may be related to the nature of the microorganism because JZ3 and JZ4 are prokaryotes, while the other three are eukaryotes. It may also be related to the depletion of nutrients, excessive metabolites, or the metabolites that were not conducive to the degradation of DEHP.

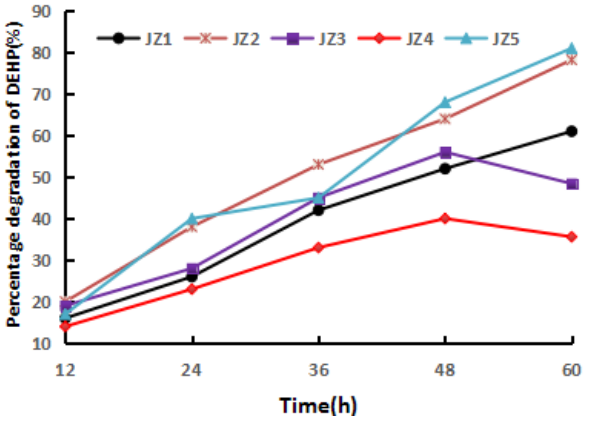

Figure 4. Effect of time on degradation of DEHP.

Determination of the best degradation conditions of DEHP by *Aspergillus niger* (JZ1)

Based on the single factor experiments, the degradation conditions of JZ1 including pH, temperature, rotation speed, and cultural time were optimized by orthogonal test. Table 2 and Table 3 showed that, with the degradation rate of DEHP as the evaluation index, the order of the factors affecting the degradation of DEHP by JZ1 from large to small was as follows: cultural time (D), temperature (B), pH (A), and rotation speed (C). Cultural time is the main factor, and the first level is the best; the second is temperature and the best level is the third level; the third is pH and the best level is the second level; the last is rotation speed, and the third level is the best. From this, we can get the best scheme of the ability of JZ1 to degrade DEHP including pH 5.0, temperature 30°C, rotational speed 210 rpm, and cultural time 36 h, which is $A_2B_3C_3D_1$.

Table 2. Orthogonal design result.

No.	Factors				Index	
	A	B	C	D	DEHP Content (mg/L)	Rate of Degradation (%)
1	1	1	1	1	297.1	25.7
2	1	2	2	2	242.5	39.4
3	1	3	3	3	258.9	35.3
4	2	1	2	3	286	28.5
5	2	2	3	1	125.5	68.6
6	2	3	1	2	198.7	50.3
7	3	1	3	2	285.5	28.6
8	3	2	1	3	313.4	21.7
9	3	3	2	1	195.2	51.2

Note: A: pH; B: temperature; C: rotation speed; D: cultural time.

Determination of the degradation effect of *Aspergillus niger* (JZ1) on DEHP under the optimal degradation conditions

It can be seen from Figure 5 that, with the increase of culture time, the content of DEHP in the culture medium decreased slowly at first, and then decreased rapidly during 12-30 h. This may be related to the fact that JZ1 is in logarithmic phase and stable phase in this period of time, and the cell vitality is strong, so it has strong

Table 3. Analysis of the results of orthogonal experiment.

Index	K Value、Range (R)	Factor A	Factor B	Factor C	Factor D
Degradation rate	K1	100.4	82.9	97.7	145.5
	K2	147.5	129.7	119.1	118.3
	K3	101.5	136.8	135.0	85.5
	k1	33.5	27.6	32.6	48.5
	K2	49.2	43.2	39.7	39.4
	K3	33.8	45.6	45.0	28.5
	Range (R)	17.3	18	12.4	20

Note: Factor A: pH; Factor B: temperature; Factor C: rotation speed; Factor D: cultural time.

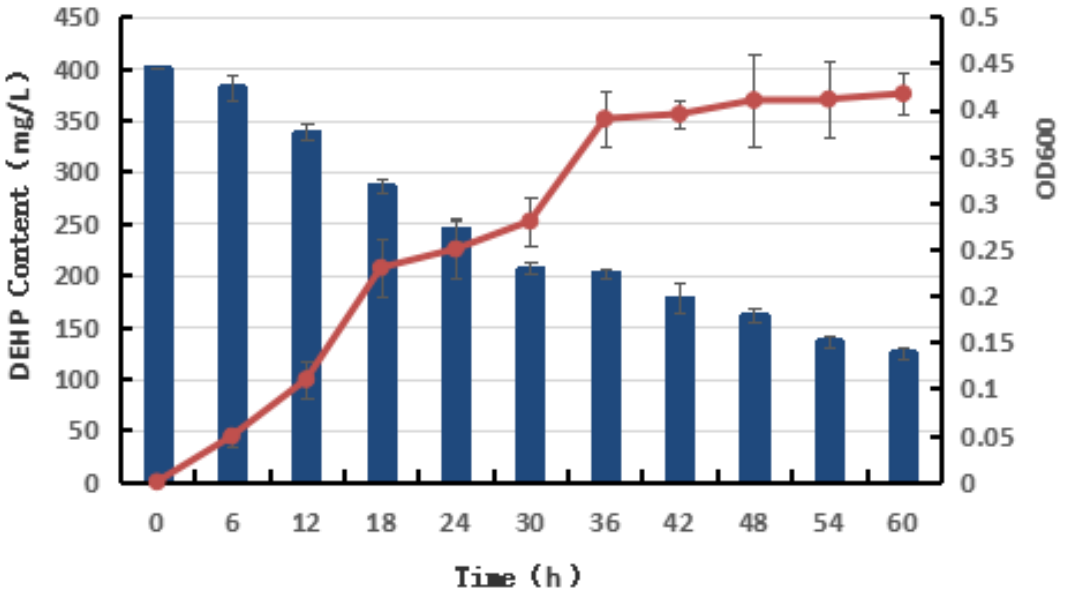

Figure 5. The Biodegradation effect of DEHP under optimum condition and growth of the isolates.

degradation ability to DEHP. When the culture time reached 60 h, the degradation rate of DEHP reached 68.75%. The growth curve of JZ1 was similar to that of filamentous microorganism. In the first 12 hours, the OD_{600} value of the culture medium did not change much, which may be related to the delay period of JZ1 just inoculated into the fresh medium. From 12 h to 36 h, OD_{600} value increased rapidly with the passage of time, because this stage has entered the logarithmic growth phase of JZ1, the number of cells is in the vigorous period, the number of cells is gradually increased, and the concentration of microorganism solution is also increasing. At the same time, the degradation rate of DEHP also increased. From 36 h to 60 h, the OD_{600} value was stable with the increase of time, which might be due to the depletion of nutrients in the culture medium and the accumulation of harmful metabolites, which led to the gradual decrease of the reproduction rate of JZ1. At the same time, the ability of JZ1 to degrade DEHP was also gradually weakened.

Kinetic analysis of DEHP degradation by five kinds of microorganisms

If the degradation reaction of five microorganisms to DEHP follows the first-order

Table 4. The kinetic equation of DEHP degradation.

Microorganisms	Kinetic equation	Half-life (hour)	R^2
JZ1	lnA=6.2146-0.0348t	19.87	0.9055
JZ2	lnA=6.2005-0.0285t	24.33	0.9102
JZ3	lnA=6.2046-0.0313t	22.17	0.8641
JZ4	lnA=6.1944-0.0187t	37.02	0.8052
JZ5	lnA=6.2086-0.0166t	41.79	0.7828

reaction kinetic formula, it will be: $lnA = lnA_0 - KT$, where A_0 is the concentration of reactant at the beginning of reaction, A is the concentration of reactant at the time of T, K is the rate constant, the negative first power of time unit, and T is the degradation time (unit: hour). Half-life formula will be: T1 / 2 = LN2 / k = 0.693/k. According to the degradation curve of DEHP, the kinetic equation in Table 4 can be obtained by using the formula. The correlation coefficient of JZ1 and JZ2 kinetic equation were more than 0.90, which indicates that the degradation curve of this strain to DEHP can well conform to the first-order kinetic equation.

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