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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Degradation of environmental endocrine disruptor di-2-ethylhexyl phthalate by a newly discovered bacterium, *Microbacterium* sp. strain CQ0110Y

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Abstract In this study di-2-ethylhexyl phthalate (DEHP)-degradation strain CQ0110Y was isolated from activated sludge. According to the biophysical/biochemical characteristics and analysis of 16S rDNA, the strain was identified as *Microbacterium* sp. The results of this study showed the optimal pH value and optimal temperature which influenced the degradation rate in wastewater: pH 6.5–7.5, 25–35°C. Kinetics of degradation reaction had been performed at different initial concentrations and different time. Analyzed with SPSS10.0 software, the DEHP degradation can be described as the same exponential model when the initial DEHP concentration was lower than 1,350 mg/l. The kinetics equation was $\ln C = -0.4087t + A$, with the degradation half life of DEHP in wastewater (1.59 days). To the best of our knowledge, this is the first reported case of DEHP degradation by *Microbacterium* sp. strain.

Keywords Di-2-Ethylhexyl phthalate · Degradation · *Microbacterium* · Catechol-dioxygenase

Introduction

Phthalic acid esters (PAEs), a class of refractory organic compounds which are widely used in the plastic, coatings, and cosmetics industries, have received extensive attention in recent years. They are distributed in sediment, natural water, wastewater, and soils (IPCS 1992). Release of phthalates into the ecosystem or wastewater effluent occurs during the production phase and via leaching and volatilization from plastic products during their usage and after disposal (Psillakis et al. 2004). Even at very low concentrations, they are suspected of interfering with reproductive systems and behavior in humans and wildlife through disturbance of the endocrine system (Jobling et al. 1995). In addition, some of them are suspected of being teratogenic, mutagenic, and carcinogenic (Huff and Kluwe 1984). Several regulatory bodies, such as the US Environment Protection Agency, the European Union, and the China National Environmental Monitoring Center (Wang et al. 1995), classified phthalate esters as a top-priority environmental pollutant. According to our studies (Tian et al. 2004; Qiu et al. 2003), the PAE pollution in the Yangzi and Jialing rivers of China is widespread; PAEs could be found in every water sample and the concentration arises to 25 μg/l.

di-2-Ethylhexyl phthalate (DEHP) belongs to the family of PAEs. DEHP is used as a plasticizer in polyvinyl chloride (PVC) plastics to impart flexibility, strength, broad-range temperature tolerance, stability during sterilization, and optical clarity to the otherwise hard and brittle unplasticized PVC (ATSDR 2002). It is widely distributed in air, sediment, natural water, waste water, soil, and living organics (Clausen et al. 2004; Suzuki et al. 2001; Cheng et al. 2001; Hu et al. 2003; Koch et al. 2004). Exposure to high concentrations of DEHP produces a wide range of adverse effects in experimental animals, including cancer, liver damage, birth defects, and alterations of the reproductive system, especially for male animals (ATSDR 2002; Gray et al. 2003, 2004; Kavlock et al. 2002).

The metabolic breakdown of DEHP by microorganisms is considered to be one of the major routes of environmen-

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tal degradation for this widespread pollutant due to its low rate of hydrolysis and photolysis (Wolf et al. 1980). As the application of DEHP-containing PVC plastic films on agriculture dramatically increases and as large quantities of wastewater and municipal and industrial sewage sludge are deposited on agricultural land each year, DEHP is the most identified phthalate ester in agricultural soils (Kampe et al. 1988; Tao et al. 1993; Pang et al. 1995; Meng et al. 1996). In the past few years, considerable attention has been paid to the analysis of the environmental fate, general toxicity, and biological degradabililiy of DEHP (Wang et al. 1997). In recent years, a few researches have reported about DEHP's degradation by microorganisms (Quan et al. 2005; Kim et al. 2003; Zeng 1999); however, the mechanism of the biodegradation of DEHP is not very clear.

In this study, we aimed to isolate microorganisms capable of degrading DEHP, explore the mechanism of biodegradation, and characterize the environmental factors influencing the degradation process in contaminated water.

Materials and methods

Reagents and chemicals

DEHP, with 99.0% analytical standards, was obtained from Beijing Chemical Reagent Factory (Beijing, China). Chemicals used for dilution and extraction of DEHP were of analytical grade and were redistilled. Other chemicals used in this study were also of analytical grade. Glassware was meticulously cleaned to reduce any background contamination of phthalates. All chromic-acid-washed glassware was placed in a 300°C oven overnight. After cooling, the glassware was rinsed twice with acetone and petroleum ether and air-dried ready for use.

Isolation of microorganism resistant to DEHP

The activated sludge used for the isolation of bacteria was collected from the Tangjiaqiao sewage treatment factory of Chongqing in China that has been exposed to DEHP wastewater for more than 10 years.

The microorganisms were isolated in basal medium containing 10, 50, 100, 200, 400, 600, 800, 1,000, 1,200, 1,400, 1,600, 1,800, and 2,000 mg/l DEHP. The basal medium contained K_2HPO_4 1 g/l, NaCl 1 g/l, NH_4Cl 0.5 g/l, and $MgSO_4$ 0.4 g/l, pH 7.2, and underwent moist heat sterilization for 15 min. DEHP was used as the sole carbon source in this medium. DEHP was added to the triangular flask and the solvent dichloromethane was allowed to evaporate for 30 min before adding the basal medium. Ten grams of sludge was added into a 250-ml triangular flask with 100 ml solid medium. These flasks were incubated at 37°C in a rotary shaker (100 rpm). An aliquot of 1 ml was subcultured to fresh medium every week, the concentration of DEHP increasing from 10 to 2,000 mg/l. After 3 months, some bacteria could grow in the medium with DEHP with 2,000 mg/l; then, 10 μl of medium with bacteria was plated on nutrient agar plates to isolate and purify the bacterium capable of degrading DEHP.

Identification

The primers which were used to amplify the 16S rDNA gene were Pf: 5′-AGAGTTTGATCCTGGCTCAG-3′ and Pr: 5′-ACGGCTACCTTGTTACGACT-3′, corresponding to 8–27 and 1,495–1,514 bases of coli 16S rRNA gene, respectively. PCR amplification conditions were as follows: Each PCR mixture (25 μl) was composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.25 μM, template DNA, and 0.45 U of Taq DNA polymerase. The amplification program consisted of one cycle of 94°C for 5 min; 38 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 100 s; and finally, one cycle of 72°C for 10 min. The amplification products were subjected to gel electrophoresis in 1% agarose, followed by ethidium bromide staining. All the reagents and service of sequencing were provided by Shanghai Shenbo (Shanghai, China).

The identification of microorganisms with high DEHP biodegradation ability was made on the basis of morphological appearance and physiological characteristics (Krieg and Holt 1984; Microbiology Institute Of CAS 1978). The Vitek-AutoMicrobic System (bioMerieux, SA, Marcy-l'Etoile, France) was also used to characterize it with biochemical tests.

Detection of activity of catechol-dioxygenase

The method for detection of catechol-dioxygenase activity (Haysish 1957; Sala-Trepat and Evams 1971; Sanakis et al. 2003) was adopted. The catechol-dioxygenase activity was assayed spectrophotometrically using a Hitachi U-2000 spectrophotometer. The reaction mixture contained aliquots of the enzyme and 0.5 mM catechol (final volume, 1 ml) in 1.33 mM EDTA/50 mM phosphate (pH=7.0). The enzymatic reaction was monitored by measuring the formation of product (*cis*,*cis*-muconic acid) at 260 nm (=16.8 mM).

Biodegradation of DEHP by CQ0110Y

DEHP was used as the sole carbon source in basal medium with 2,000 mg/l DEHP. Two hundred microliters of bacterial suspension (OD330=0.6) was added into a 250-ml triangular flask with 100 ml basal medium. These triangular flasks were incubated at 30°C in a rotary shaker

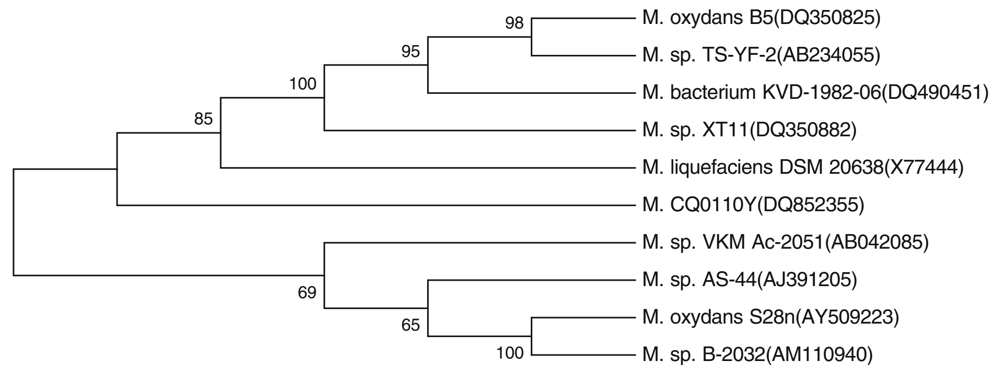

Fig. 1 The phylogeny tree of CQ0110Y

(100 rpm) and kept from the light to avoid photodegradation of DEHP. By investigating the degradation rate under different pH (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) and temperature (10, 15, 20, 25, 30, 35, 40, 45, and 50°C) conditions, the optimum degradation conditions were determined.

Analytical methods

The liquid phase and bacterial cell were separated by centrifugation at 8,000×*g*. Phthalate in the supernatant was extracted with dichloromethane. DEHP contents were analyzed by gas chromatography (SC2000, Chuanyi, Chongqing, China) with a stainless steel column (3 mm× 2 m, OV101 silicon packed). The flame ionization detector was used. The temperatures of the column, injection port, and flame ionization detector were 250, 260, and 280°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 20 ml/min, and the hydrogen and airflow rates were 45 and 230 ml/min, respectively. The biodegradation products of DEHP were analyzed by GC/MS [GC6890/5973MSD, Agilent, Palo Alto, CA, USA, with an HP-5 MS column (Agilent, length 30 m, i.d. 0.25 mm, film thickness 0.25 μm)], in reference to Wu et al. (2005). The conditions were (1) 50°C, 8°C/min, 180°C (2 min), 8°C/min, 250°C (10 min); (2) the scan style was 230°C, 150°C (tetrapolar); (3) the injection port temperature was 270°C, interface temperature was 280°C, pressure was 50.5 kPa, and flow rate was 1.0 ml/min; and (4) sample injection volume was 1 μl. The products were determined by the score matching to the standard library.

Results

Isolation and identification of the DEHP-degrading bacterium

The bacterium capable of utilizing DEHP as the sole source of carbon and energy was isolated from activated sludge. The initial concentration of DEHP was 10 mg/l. After 3 months, one strain that could survive in the concentration of 2,000 mg/l was studied in the following experiments. We designated it as CQ0110Y. The colony showed round morphology, low prominency, smooth and glossy surface, and yellowish coloring. The cell was aerobic and gram-positive.

The complete 16s rDNA sequence of CQ0110Y was determined. The sequence was compared with published 16s rDNA sequences by blasting in GenBank; the results showed that the similarity to *Microbacterium* sp. was 99.5%. The accession number of CQ0110Y in GenBank is DQ852355. Combined with the analysis of morphology, physio-biochemical character, and genetic specificity, CQ0110Y was identified as a *Microbacterium* sp. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al. 2004). The phylogeny tree is shown in Fig. 1.

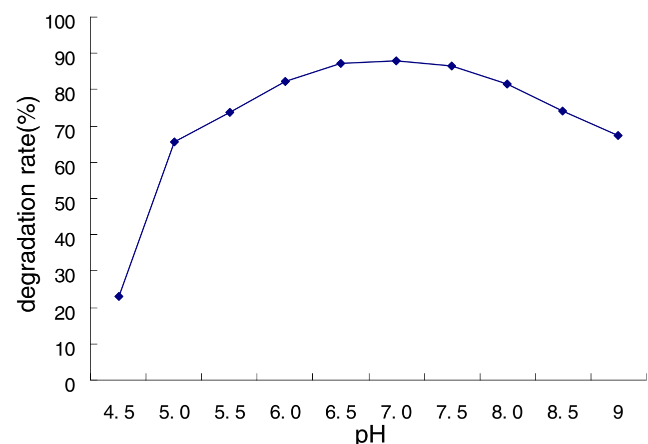

Fig. 2 Effects of pH on DEHP degradation rate. The rate of DEHP degradation increased quickly when the pH value of the soil was increased from 4.5 to 5.0. A high rate was achieved for CQ0110Y when the pH value was 7.0. When the pH value was >7.5, the degradation efficiency decreased. The optimal pH values for CQ0110Y to degrade DEHP were from 6.5 to 7.5

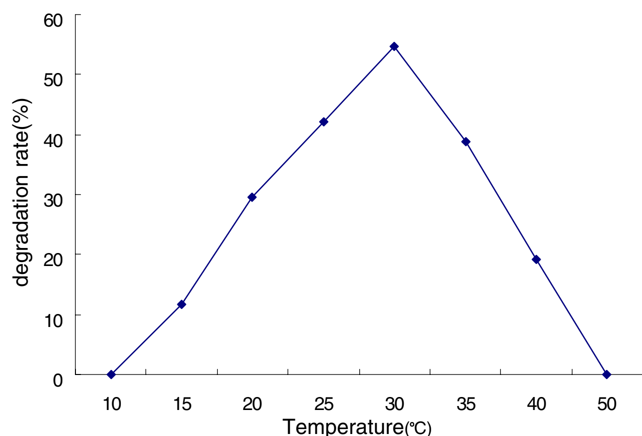

Fig. 3 Effects of temperature on DEHP degradation rate. The degradation rate increased with the increase of temperature between 10 and 30°C. A high rate was achieved for CQ0110Y when the temperature was sustained between 25 and 35°C. Higher temperature resulted in the lowering of the degradation rate. The result indicated that the optimum temperature for CQ0110Y to degrade DEHP was 25–35°C, at which temperature the degradation-related enzyme reached the highest activity

Environmental factors influencing the degradation process in wastewater

The relationship between the degradation rate constants and pH for CQ0110Y is shown in Fig. 2. The rate of DEHP degradation increased quickly when the pH value of the soil was increased from 4.5 to 5.0. A high rate was achieved for CQ0110Y when the pH value was 7.0. When pH value was >7.5, the degradation efficiency decreased. The optimal pH value for CQ0110Y to degrade DEHP was from 6.5 to 7.5.

Bacterium growth is sensitive to environmental temperature. In order to determine the optimal temperature for CQ0110Y to degrade DEHP, different temperature conditions (10, 15, 20, 25, 30, 35, 40, 45, and 50°C) were assessed. As is shown in Fig. 3, the degradation rate increased with the increase of temperature between 10 and 30°C. A high rate was achieved for CQ0110Y when the temperature was sustained between 25 and 35°C.

Characteristics of DEHP degradation kinetics in wastewater

In order to investigate the effect of initial DEHP concentration on degradation activity, the degradation kinetics character in wastewater was studied. Different initial DEHP concentrations (100, 500, 850, 1,350, 1,600, and 2,000 mg/l) were assessed. As shown in Fig. 4, the time course of degradation rate was recorded.

Detection of activity of catechol-dioxygenase

As shown in Table 1, the activity of catechol 1,2-dioxygenase other than catechol 2,3-dioxygenase could be detected in the crude enzyme of CQ0110Y strain. In addition, the higher activity of catechol 1,2-dioxygenase was detected only after DEHP inducing.

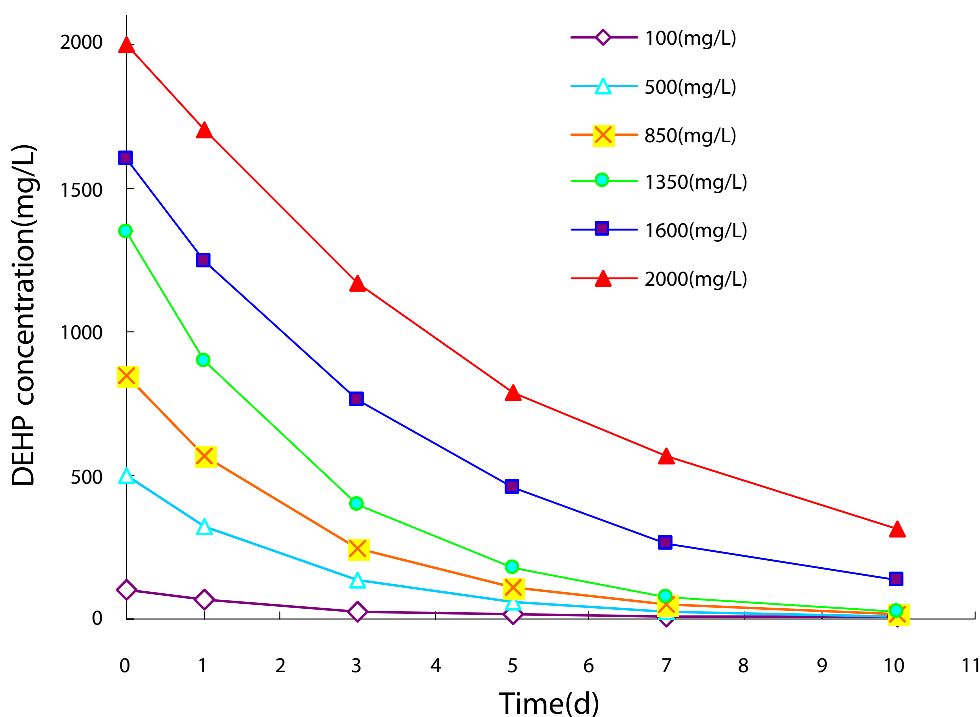

Fig. 4 DEHP-degradation kinetics character in wastewater by CQ0110Y. The time course of degradation rate was recorded in different initial DEHP concentrations (100, 500, 850, 1,350, 1,600, and 2,000 mg/l). When DEHP initial concentration is <1,350 mg/l, DEHP biodegradation reaction fit with the first-order kinetics. The half life of DEHP in soil was 1.59 days

Table 1 Activities of catechol-dioxygenase in CQ0110Y

Catechol 1,2-dioxygenase (U/mg)		Catechol 2,3-dioxygenase (U/mg)	
After DEHP inducing	Before DEHP inducing	After DEHP inducing	Before DEHP inducing
7.536±0.182*	0.153±0.005*	0.089± 0.037**	0.094± 0.002**

$^*p<0.01$; $^{**}p>0.05$

Degradation products of DEHP by CQ0110Y

In this study, the degradation products of DEHP by CQ0110Y were determined at the half life time ($t_{1/2}$) to clearly understand the degradation pathway. The determined products are shown in Table 2 when the degradation process was at 1.5 days, the half life time of DEHP by CQ0110Y.

Discussion

We isolated a DEHP-degrading bacterium utilizing DEHP as sole carbon and energy sources from activated sludge, which is designated as CQ0110Y. According to the analysis of genetic specificity, morphology, and physio-biochemical character, the strain CQ0110Y belongs to *Microbacterium* sp. To the best of our knowledge, this is the first reported case of DEHP degradation by *Microbacterium* sp. strains. Metabolic breakdown of PAEs by a microorganism is considered to be one of the major routes of environmental degradation. It is well known that PAEs with longer alkyl chains (i.e., di-octyl phthalate, DEHP) are more difficult to biodegrade than those with shorter alkyl chains (i.e., diethyl phthalate, di-butyl phthalate), and it is also confirmed that a correlation exists between increasing length of the ester side-chain and decreasing biodegradability (O'Grady et al. 1985).

The DEHP biodegradation data we collected fit well with the exponential model, $C=b_0*e^{(b1*t)}$. A first-order kinetics model, $\ln C=-kt+A$, could be constructed by logarithmic transformation, where C is the initial concentration (mg/l), k is the biodegradation rate constant, t is the time period, A is the constant, and $t_{1/2}=\ln 2/k$ is the half life. Table 3 shows the different DEHP degradation kinetics equations at different DEHP initial concentrations. The results showed that when DEHP initial concentration <1,350 mg/l, DEHP biodegradation reaction fit with the first-order kinetics. Analyzed by SPSS10.0, the DEHP degradation kinetics equation in wastewater by CQ0110Y was $\ln C=-0.4087t+A$, and the half life of DEHP was 1.59 days. When the initial concentration of DEHP is higher than 1,600 mg/l, the half life will increase due to the inhibition by the higher concentration of DEHP.

Table 2 The degradation products of DEHP detected at the half life time ($t_{1/2}$)

Products	Match score
Monoethylhexylphthalate	87
Phthalic acid	96
Benzenecarboxvlic acid	99
Orthohydroxybenzoic acid	78
Pyrocatechin	98
Muconic acid	96
Pyruvic acid	89

Table 3 DEHP degradation kinetics equation in different initial concentrations

Initial concentration (mg/l)	Kinetics equation	Half life (day)
100	$\ln C=-0.3911t+4.6052$	1.63
500	$\ln C=-0.4183t+6.2146$	1.57
850	$\ln C=-0.4119t+6.7452$	1.58
1,350	$\ln C=-0.4097t+7.2079$	1.59
1,600	$\ln C=-0.2552t+7.3778$	2.06
2,000	$\ln C=-0.1871t+7.6009$	2.36

Catechol is the intermediate product of aromatic hydrocarbon compounds, and the cleaving benzene ring is the common pathway of aromatic hydrocarbon metabolism. Ring opening reaction could be catalyzed by catechol 1,2-dioxygenase and catechol 2,3-dioxygenase (Strachan et al. 1998). Because the activity of catechol 1,2-dioxygenase is higher than that of catechol 2,3-dioxygenase in the crude enzyme of the CQ0110Y strain, catechol was suspected to be an intermediate product of DEHP and cleaving the benzene ring was catalyzed by catechol 1,2-dioxygenase. The later detection of degradation products confirmed the occurrence of catechol during the degradation process. For the degradation pathway of phthalates, it is generally supposed that hydrolysis of the ester side-chain of the di-alkyl phthalate through mono-alkyl phthalate occurs, leaving phthalic acid and alkyl alcohols available for further conversion. According to the degradation products (the supposed degradation pathway of DEHP by strain CQ0110Y is shown in Fig. 5), DEHP was hydrolyzed to monoethylhexylphthalate and phthalic acid by the action of esterase, then benzenecarboxvlic acid would form with the decarboxylation of phthalic acid; through the hydroxylation at the consecutive position of benzenecarboxvlic acid, orthohydroxybenzoic acid would be produced, and then to pyrocatechin and muconic acid; after the tricarboxylic acid cycle, the terminal degradation products were CO_2 and H_2O. Quan et al. (2005) proposed the pathways for DEHP

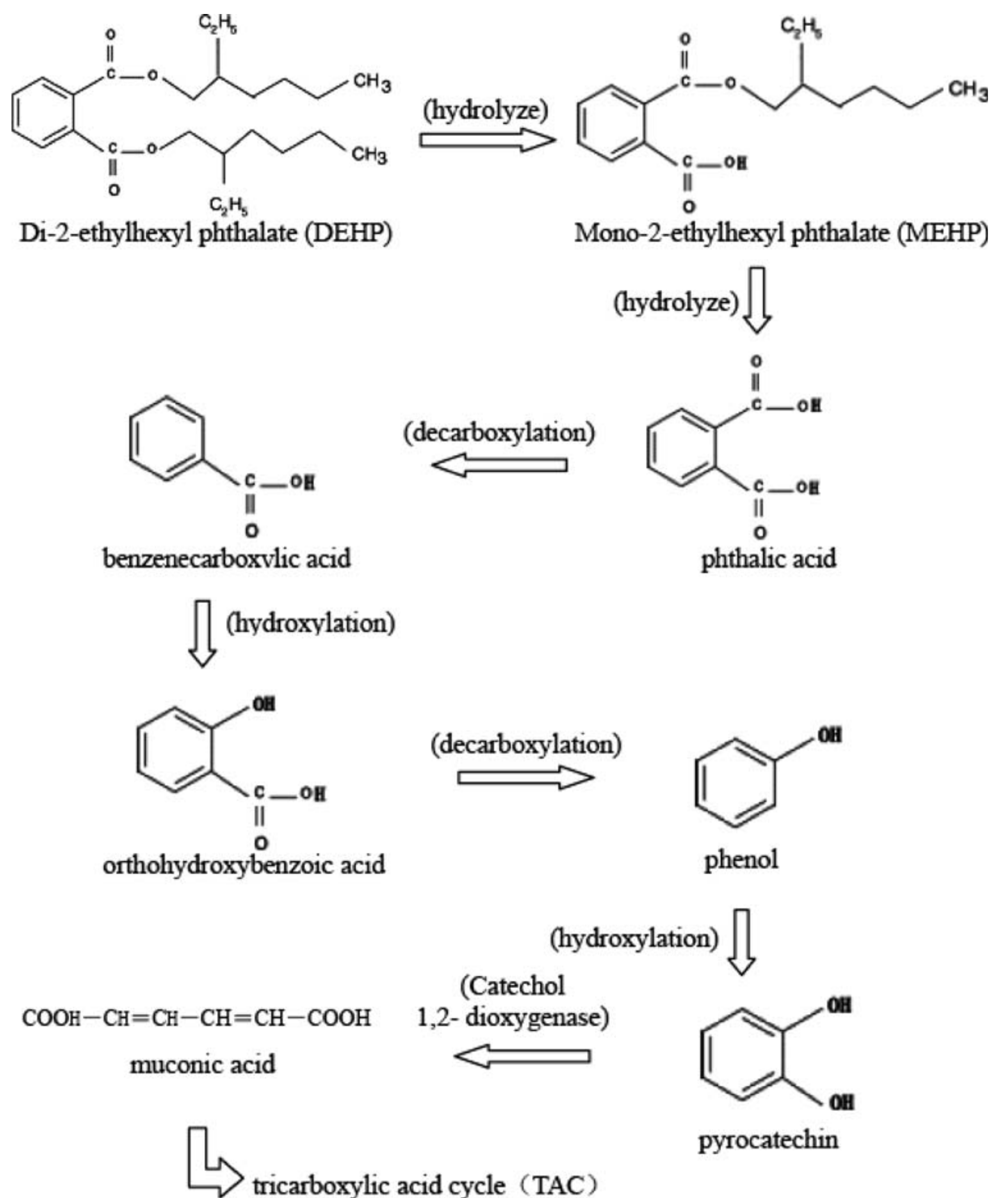

Fig. 5 The supposed degradation pathway of DEHP by strain CQ0110Y. By detecting the degradation products of DEHP at half life time, we supposed the degradation pathway of DEHP by strain CQ0110Y: DEHP was hydrolyzed to monoethylhexylphthalate and phthalic acid by the action of esterase, then benzenecarboxvlic acid would form with the decarboxylation of phthalic acid; through the hydroxylation at consecutive position of benzenecarboxvlic acid, orthohydroxybenzoic acid would be produced, and then to pyrocatechin and muconic acid; after the tricarboxylic acid cycle, the terminal degradation products were CO_2 and H_2O

degradation by strain number 66 as follows: one ester bond of DEHP was hydrolyzed by the strain to form mono-alkylphthalate and alcohol, and then the mono-alkylphthalate was hydrolyzed to protocatechuic acid and alcohol. Although the process from DEHP to phthalic acid is the same, the hydroxylation and decarboxylation is different. The activities of catechol-dioxygenase in CQ0110Y also confirm the ring cleavage beginning with the break at the consecutive position; Table 1 shows the activity of catechol 1,2-dioxygenase higher than that of catechol 2,3-dioxygenase; with the oxidization by catechol 1,2-dioxygenase, the muconic acid is produced.

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