Journal of Hazardous Materials 168 (2009) 938–943

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Biodegradation of dimethyl phthalate, diethyl phthalate and di-*n*-butyl phthalate by *Rhodococcus* sp. L4 isolated from activated sludge

Yi Lu, Fei Tang, Ying Wang, Jinhui Zhao, Xin Zeng, Qifang Luo, Lin Wang*

Institute of Environmental Medicine, MOE Key Laboratory of Environmental and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

ARTICLE INFO

Article history:
Received 8 September 2008
Received in revised form 18 February 2009
Accepted 23 February 2009
Available online 6 March 2009

Keywords:
Biodegradation
Dimethyl phthalate
Diethyl phthalate
Di-*n*-butyl phthalate
Wastewater
Rhodococcus ruber

ABSTRACT

In this study, an aerobic bacterial strain capable of utilizing dimethyl phthalate (DMP), diethyl phthalate (DEP) and di-*n*-butyl phthalate (DBP) as sole carbon source and energy was isolated from activated sludge collected from a dyeing plant. According to its morphology, physiochemical characteristics and 16S rDNA sequence, the strain was identified as *Rhodococcus ruber*. The biodegradation batch tests of DMP, DEP and DBP by the *Rhodococcus* sp. L4 showed the optimal pH value, temperature and substrate concentration: pH 7.0–8.0, 30–37 °C and PAEs concentration ≤450 mg/L. Kinetics of degradation have also been performed at different initial concentrations. The results show that the degradation can be described with exponential model. The half-life of degradation was about 1.30 days when the concentration of PAEs mixture was lower than 300 mg/L. PAEs contaminated water samples (300 mg/L) with non-emulsification and completed emulsification were prepared to investigate the effect on PAEs degradation rate. Little difference between the above two sample preparations was observed in terms of ultimate degradation rate. *Rhodococcus* sp. L4 can also grow on phenol, sodium benzoate or naphthalene solution as sole carbon source and energy which suggests its ability in resisting environmental toxicants. This work provides some new evidence for the possibility of applying *Rhodococcus* for contaminated water remediation in the area of industry.

1. Introduction

Phthalic acid esters (PAEs) which are widely used in plastic, coatings and cosmetics industries are a class of refractory organic compounds. Some of PAEs have received more and more attention in recent years since they are considered as estrogenic disrupting compounds (EDCs) and may have reproductive development toxicity [1–4]. Animal studies have proved PAEs can cause repetitive abortions [5,6] and male sterility [7]. In addition, some PAEs are suspected to be mutagens and carcinogens [8–10]. The United States Environmental Protection Agency and China National Environmental Monitoring Center have classified most of the PAEs, such as diethyl phthalate (DEP), benzyl butyl phthalate (BBP), di-*n*-butyl phthalate (DBP) and di-(2-ethyl hexyl) phthalate (DEHP) as priority pollutants [11,12].

There are more than 60 kinds of PAEs produced and consumed for diverse purposes, mainly as plasticizers. In particular, soft polyvinyl chloride (PVC) has been manufactured with more than 60% by weight of plasticizers [13]. As it bound covalently to the plastic resin, PAEs are able to migrate into the environment during use or disposal. Because of their low water solubility and high octanol-water partition coefficients, they tend to accumulate in the soil or sediment and in the biota living in the PAEs containing waters [14]. Presently, pollution of PAEs is ubiquitous in variable water environment including river water, ground water, drinking water, open ocean and lake [15–18], which may do harm to aquatic organisms and human health through food chain transmission and bioamplification.

Unfortunately, due to its chemical structure, PAEs can not be removed well by natural processes such as hydrolyzation and photodecomposition. However, metabolic breakdown of PAEs by microorganisms is considered as one of the major routes of environmental degradation for this widespread pollutant [19,20]. Many bacterial strains with the ability of degrading PAEs and their isomers have been isolated from different sources such as activated sludge, mangrove sediment and wastewater [21,22].

In the present study, an aerobic bacterial strain was isolated from PAEs acclimated activated sludge of a dyeing plant. This strain was recognized to have the ability to utilize DMP, DEP and DBP individually and all together. Besides, some physiochemical characteristics, the environmental factors and the kinetics of biodegradation were also investigated.

* Corresponding author at: Department of Environmental Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan, 430030, People's Republic of China. Tel.: +86 27 83691962; fax: +86 27 83692333.
E-mail address: lwangtj@yahoo.com.cn (L. Wang).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.02.126

2. Material and methods

2.1. Chemicals

DMP, DEP and DBP (A.P.) were purchased from Beijing Chemical Reagent Factory, China. Dichloromethane (DCM, C.P.) and acetone (A.P.) were obtained from Shanghai Chemical Reagent Factory, China. Other chemicals used in this study were all of analytical grade and commercially available.

2.2. Preparation of medium and PAEs wastewater

The inorganic salt solution used for acclimation of the activated sludge contained NaCl 1.0 g/L, NH_4NO_3 0.5 g/L, $FeCl_3$ 0.01 g/L, $K_2HPO_4{\cdot}3H_2O$ 1.0 g/L, $MgSO_4{\cdot}7H_2O$ 0.4 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L.

The Luria-Bertani (LB) broth for bacteria enrichment consisted of yeast extract (YE) 5 g/L, NaCl 10 g/L, peptone 10 g/L, pH 7.2. Nutrient agar plate was made as LB broth but add powdered agar (1.5%). The mineral salt medium (MSM) used for the degradation tests contained 1.0 g/L NaCl, 1 g/L $K_2HPO_4{\cdot}3H_2O$, 0.5 g/L NH_4Cl and 0.4 g/L $MgSO_4{\cdot}7H_2O$. Media were sterilized at 121 °C for 20 min.

PAEs synthetic wastewater was made by adding DMP, DEP and DBP equally to 100 ml of MSM solution. DMP, DEP and DBP were filter sterilized through 0.22 μm pore size filters.

2.3. Acclimation and screening of dominant bacteria for PAEs degradation

Activated sludge was collected at a dyeing plant of Wuhan, an industrial city located in central region of China. Eight litres were mixed with 24 L of the inorganic salt solution in an aerated basin. The acclimation process was conducted at room temperature. The DMP, DEP and DBP were equally added as sole carbon and energy source. The total concentration of PAEs was increased gradually from 30 to 600 mg/L. After 8 weeks of acclimation, the activated sludge was used to inoculate nutrient agar plates under aseptic conditions. The plates were incubated at 37 °C and different colonies grew after 36 h. The pure clones were obtained by plate streaking repeatedly. Then, each isolated bacterial strain was put into wastewater in order to screen dominant bacterium. All the flasks were put in swing bed with speed of 140 rpm under 37 °C. After 1 week degradation, the dominant PAEs-degrading bacterium could be confirmed in term of turbidity and degradation rate. After this screening test, we obtained a high-performance strain named strain L4.

2.4. 16S rDNA sequence analysis of strain L4

The strain L4 was enriched overnight in LB medium, and its genomic DNA was extracted by Takara MiniBEST Bacterial Genomic DNA extract kit (Takara, Japan). 16S rRNA gene of the strain was amplified from the genomic DNA by PCR. The eubacterial primers F27 and R1492 used for amplifying the full length of 16S rRNA gene fragments were described elsewhere [23]. Takara Biotechnology (DaLian) co., Ltd. provided all the related reagents and service of DNA sequencing.

The 16S rDNA sequence was submitted to GenBank (accession number is EU552925). The similarity of the nucleotide sequence was determined by BLAST search in NCBI (National Center for Biotechnology information Databases).

2.5. Orthogonal test

Orthogonal experiment using L_{16} $(4)^3$ table including three factors (temperature: A; pH value: B; substrate concentration: C) and four levers were designed to explore the optimal PAEs degrading conditions of strain L4 (Table 1). The degrading experiments were carried out in 250 ml Erlenmeyer flasks, pretreated with chromic acid to prevent PAEs contamination and filled with 100 ml of MSM. DMP, DEP, and DBP dissolved in acetone were equally added to MSM. The Erlenmeyer flasks were water-bathed at 65 °C in a swing bed with speed of 200 rpm during 30 min to evaporate the acetone. Each flask was inoculated with 10^8 degrading cells/ml bacteria, which were collected from a 24 h LB culture and washed twice with phosphate buffer saline (PBS, pH 7.4). The flasks were kept rocking in air-bath rocking bed with speed of 140 rpm during 5 days. Blank control was set up as above but without cells addition. All the tests were carried out under sterilized conditions. Each experimental group designed in Table 1 was conducted in duplicate.

Table 1
Factors and levels for orthogonal test.

Variable	Level			
	1	2	3	4
(A) Temperature (°C)	12	23	30	37
(B) pH value	5	6	7	8
(C) Initial concentration (mg/L)[a]	50	100	150	200

[a] The initial concentrations of single chemical DMP, DEP and DBP were 50, 100, 150 and 200 mg/L. So the concentrations of total PAEs in each level were 150, 300, 450 and 600 mg/L.

2.6. Kinetics studies

The kinetics of PAEs mixture degradation in water at different initial concentrations were investigated on the strain L4 during 6 days. The initial concentrations of the PAEs synthetic wastewater (pH 7.0) were 150 mg/L, 300 mg/L, 450 mg/L and 600 mg/L by equally adding 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L of DMP, DEP and DBP, respectively. The flask in each concentration was removed from the rocking bed (37 °C, 140 rpm) everyday and the degrading activity was stopped by addition of concentrated sulfuric acid. In addition, PAEs contaminated water samples (300 mg/L) with non-emulsification and completed emulsification were prepared to investigate the effect on PAEs degradation rate. Non-emulsification solution was made by adding DMP, DEP and DBP directly into 100 ml of MSM, while the completed emulsification one was prepared as described in 2.5.

2.7. Analytical methods

The total volume of each flask was liquid–liquid extracted by DCM three times. The total dosage of DCM was 50 mL. The extracts were merged and volatilized to dry with rotary evaporator (Heidolph, Germany) at 60 °C. The dried residue was redissolved with 20 mL DCM. Concentration of PAEs was determined by Agilent 6890N gas chromatogram (GC) equipped with FID detector and HP-5 capillary column (30 m × 0.32 mm × 0.25 μm). The conditions were as follows: FID detector, 280 °C; carrier gas, high pure nitrogen gas (1 ml/min); injector temperature, 250 °C; column temperature, programmed temperature increasing which started at 70 °C with 2 min and was ramped up to 130 °C at 20 °C/min and then to 270 °C at 30 °C/min before being held for 5 min; injection volume, 1 μl.

2.8. Utilization of other aromatic compounds by strain L4

Frequent aromatic compounds including phenol, 2, 4-dinitrophenol, p-nitrophenol, sodium benzoate and naphthalene were used to explore the utilization spectrum of strain L4. The concentration of each compound in MSM was 4 mmol/L. The bacteria were inoculated and cultured in swing bed with speed of 120 rpm under 37 °C. The biomass of strain L4 was recorded

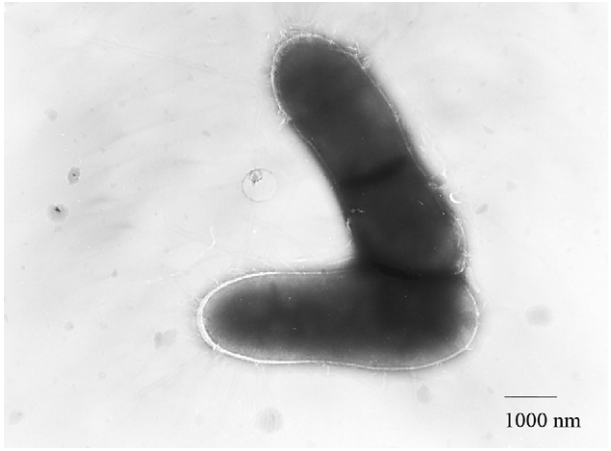

Fig. 1. Transmission electron micrograph of strain L4 (10000×, negative staining).

consecutively through monitoring OD600. In order to avoid adsorbance interference with chemicals, the same solutions without adding bacteria were prepared and uniformly operated as blank.

3. Results and discussion

3.1. Identification and characterization of the bacterial strain L4

Viable bacteria were isolated from activated sludge of a dyeing plant after 8 weeks PAEs acclimation in an aerated basin. One pure bacterial strain named strain L4 was obtained as dominant PAEs-degrading strain through screening test. The strain with size of 1.1–1.9 μm × 4.0–5.4 μm was gram-positive, non-flagellated and short rod shape. The morphology of strain L4 was shown in Fig. 1. The colonies of this strain with diameter of 0.2–0.5 mm on LB agar cultured in 18–24 h showed ivory, opaque and round morphology. The color would turn to salmon pink if culture time was extended to 48 h. The strain L4 can grow well in LB broth with temperature between 30–37 °C.

The physiochemical properties of strain L4 were shown in Table 2. The strain L4 can be identified as *Rhodococcus ruber* through its morphology, physiochemical characteristics and 16S rDNA sequence.

Table 2
Physiochemical properties of the *Rhodococcus* sp. L4.

Characteristics	Strain L4
Aerobic growth	+
Oxidase	+
Catalase	+
D-Glucose	+
D-Lactose	–
Saccharose	w
Maltose	–
D-Galactose	–
D-Cellobiose	–
α-Hydroxybutyric acid	+
L-Lactic acid	+
3-Methyl-glucose	–
Acetic acid	+
Adenosine	–
α-Ketoglutaric Acid	+
Uridine	–
D-Ala	–
L-Ala	–
L-Alanyl-Gly	–
L-Serine	–
Hemisuccinat	+
Control	–

Positive (+); negative (–); weakly positive (w).

Table 3
Results of orthogonal test of *Rhodococcus* sp. L4 on DMP, DEP and DBP biodegradation.

No.	A	B	C	Degradation rate (%)[a]			
				DMP	DEP	DBP	Total[c]
1	12	5	50	27.0	16.4	16.5	20.0
2	12	6	100	26.0	28.9	12.9	22.6
3	12	7	150	23.4	21.5	11.3	18.7
4	12	8	200	23.8	22.4	1.7	16.0
5	23	5	100	17.7	14.3	1.9	11.3
6	23	6	50	37.3	32.8	57.0	42.4
7	23	7	200	19.8	21.6	0.0	13.8
8	23	8	150	38.8	30.0	16.1	28.3
9	30	5	150	71.2	77.5	42.3	63.7
10	30	6	200	31.6	44.2	48.7	41.5
11	30	7	50	100.0	100.0	83.2	94.4
12	30	8	100	40.6	40.3	53.7	44.9
13	37	5	200	41.7	64.3	28.6	44.9
14	37	6	150	90.3	97.7	87.4	91.8
15	37	7	100	100.0	100.0	97.2	99.1
16	37	8	50	100.0	100.0	88.1	96.0

	A	B	C
DMP			
K_1	25.1	39.4	66.1
K_2	28.4	46.3	46.1
K_3	60.9	60.8	55.9
K_4	83.0	50.8	29.2
R[b]	57.9	21.4	36.9
DEP			
K_1	22.3	43.1	62.3
K_2	24.7	50.9	45.9
K_3	65.5	60.8	56.7
K_4	90.5	48.2	38.1
R[b]	68.2	17.7	24.2
DBP			
K_1	10.6	22.3	61.2
K_2	18.8	51.5	41.4
K_3	57.0	47.9	39.3
K_4	75.3	39.9	19.8
R[b]	64.7	29.2	41.4
Total			
K_1	19.3	35.0	63.2
K_2	24.0	50.0	44.5
K_3	61.1	56.5	50.6
K_4	83.0	46.3	29.1
R[b]	63.6	21.5	34.2

[a] Degradation rate (%) = $(C_{initial} - C_{final})/C_{initial}$.
[b] R value refers to the result of extreme analysis; $R = \max\{K_1, K_2, K_3, K_4\} - \min\{K_1, K_2, K_3, K_4\}$.
[c] Total biodegradation rate refers to the change of amount of total PAEs.

3.2. Optimal conditions for PAEs degradation

Orthogonal test was used to explore the optimal degrading conditions. In the orthogonal test, K_i ($i = 1, 2, 3 \ldots$) was defined as mean value of sum of degradation rate of every level and the optimal level of variable can be confirmed by comparing the value of K_i. R value was used to estimate the effect of variables, and $R = \max\{K_1, K_2, \ldots K_i\} - \min\{K_1, K_2, \ldots K_i\}$. High R value of variable means this variable has strong effect on the results. The results and data analysis of orthogonal test were listed in Table 3. From orthogonal test, regardless of single chemical or their mixture, the influence of the variables to the degradation rate decreased in the order: $A > C > B$. Temperature was found to be the most important factor in degrading process, which was prior to substrate concentration and pH value. For degradation, 37 °C, neutral pH and concentration of wastewater lower than 450 mg/L would be the optimal condition. However, in the degrading experiment, this bacterium can survive in all the above conditions and this adaptability must be a good quality for biodegradation. The relationship between factorial level

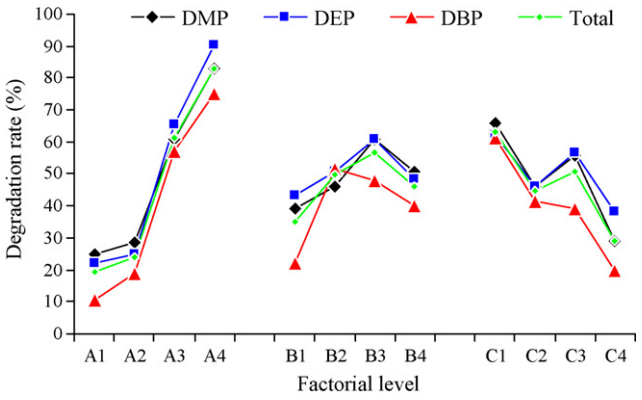

Fig. 2. The relationship between factorial level and the degrading rates of *Rhodococcus* sp. L4 in orthogonal experiment. Temperature: 12 °C (A1), 23 °C (A2), 30 °C (A3), 37 °C (A4); initial pH value: 5.0 (B1), 6.0 (B2), 7.0 (B3), 8.0 (B4); initial concentration of each PAE: 50 mg/L (C1), 100 mg/L (C2), 150 mg/L (C3), 200 mg/L (C4).

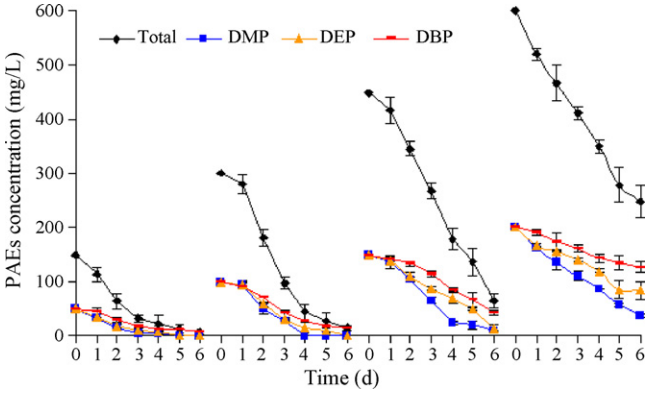

Fig. 3. Degradation of PAEs synthetic wastewater by *Rhodococcus* sp. L4. The initial concentrations of each compound were 50, 100, 150 and 200 mg/L. The degradation time was 6 days.

and the degrading rates in orthogonal experiment was revealed in Fig. 2. From Fig. 2, it can also be found that the biodegradation rate of DBP in five days was lower than DEP or DMP. In other words, the degradation rate may decrease with increasing alkyl chain length, since the branched chain of DBP is longer than DEP and DMP. Therefore, the effect of stereospecific blockade of DBP is stronger than others. Similar reports can be found in previous studies [24,25].

Control experiments were carried out on PAEs synthetic wastewater with no bacteria. The results showed no obvious degradation which suggests that abiotic losses can be neglected in this study. The recovery rates of PAEs after sample pretreatment were between 95–105%.

3.3. Kinetics of PAEs degradation

The PAEs degradation in water by *Rhodococcus* sp. L4 was fit well with exponential model. 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. The degradation half life ($t_{1/2}$) of PAEs is $\ln 2/k$. Table 4 showed the PAEs degradation kinetics equation with different initial concentration. From the results, it can be found that the half-life of degradation was about 1.30 days when the concentration of total PAEs lower than 300 mg/L. The half life would be extended while the initial concentration increased.

These kinetics equations are different from those of the Gompertz model referred by Jiaxi Li to be used to describe *R. ruber* Sa in transformation of dimethyl phthalate (DMP) and its isomers, dimethyl isophthalate (DMI) and dimethyl terephthalate (DMT) [26]. Gompertz model is usually applied to describe sigmoidal curves. That is to say, lag phases must exist during degradation process. For the study of Li et al., DMI and DMT were easily degraded by *R. ruber* Sa compared to DMP. The transformation of DMP started after DMI and DMT depleted (Inhibitor disappeared). So the lag time cannot be ignored when describing DMP degradation. Actually, apparent lag time was observed in DMP transformation by *R. ruber* Sa. The competitive inhibition between the compounds and the amount of dominant bacteria should be considered. However, the exponential model is more suitable to fit the degradation process with vigorous reaction at the beginning. In our research, the tendency of degrading curve of PAEs by strain L4 appeared in the first degrading day and persisted to the last day (Fig. 3). The competitive inhibition among DMP, DEP and DBP seemed not strong. No lag time was observed in the degradation of the three compounds.

Table 4
PAEs degradation kinetics equation under different initial concentrations.

Total initial concentration (mg/L)	Kinetics equation	Half life (days)	Correlation coefficient
150	$\ln C = -0.5336t + 5.1496$	1.30	0.9951
300	$\ln C = -0.5420t + 6.0371$	1.28	0.9853
450	$\ln C = -0.3113t + 6.3403$	2.23	0.9618
600	$\ln C = -0.1497t + 6.4226$	4.63	0.9944

The kinetics of degradation of PAEs wastewater with non-emulsification and completed emulsification at an initial concentration of 300 mg/L were compared. Data were shown in Fig. 4. In non-emulsification group, during the degrading process, the strain L4 can conglutinate and coat the oil droplets gradually to form cytorrhyctes with different sizes (the diameter was from 0.2 mm to 1.5 mm) (Fig. 5), which can be easily observed by naked eyes. These orange droplets were picked out and the amount of bacteria was counted subsequently. The result showed that the amount of bacteria of cytorrhyctes with different size can reach to 10^3–10^6 cfu. The cytorrhyctes would disappear along with the degrading process, from which it could be concluded that the formation of cytorrhyctes may be a critical step for strain L4 in catabolism of PAEs oil droplets. *Rhodococcus* adhere to oil droplets may be due to their hydrophobic cell surface containing mycolic acids [27]. Regardless of total or single PAE, little difference between the above two sample preparations was observed in terms of ultimate biodegradation rate after 5

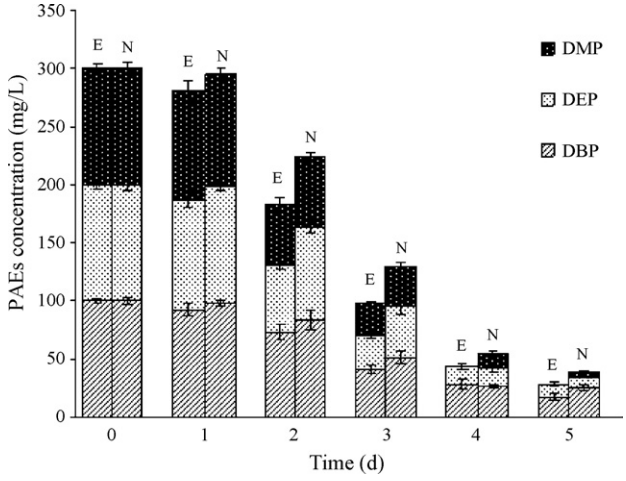

Fig. 4. Degradation of DMP, DEP and DBP mixture in water by *Rhodococcus* sp. L4 with two different sample preparation: Emulsification (E) and non-Emulsification (N). The initial concentration of each PAE was 100 mg /L.

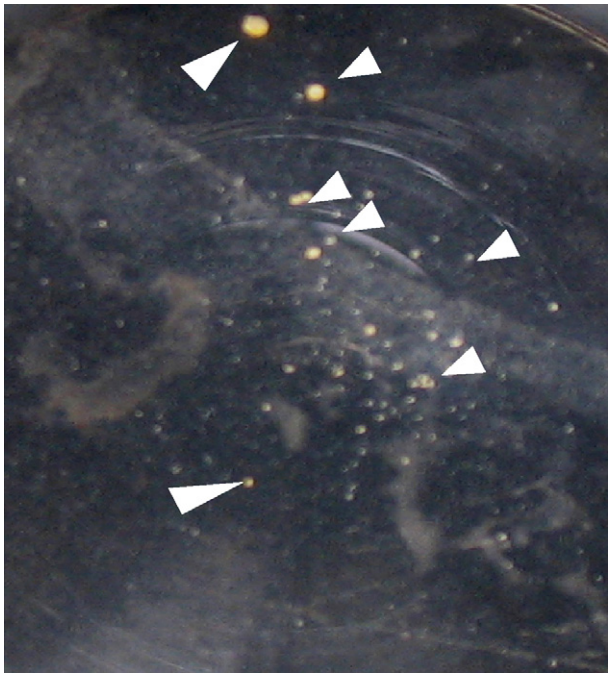

Fig. 5. Cytorrhyctes formed (△) during degradation process. Formation of coating bodies may extend the contact area between bacteria and its objective oil droplets. This could be a critical process for improving degrading efficacy in non-emulsification group. The picture was taken from the top of the Erlenmeyer flask.

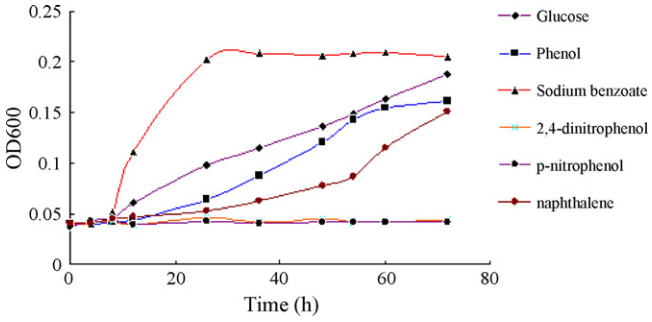

Fig. 6. The biomass of strain L4 grew in glucose, phenol, sodium benzoate, naphthalene, p-nitrophenol and 2, 4-dinitrophenol as the sole carbon source and energy.

days. For non-emulsification group, it may be conferred that conglutination or production of biosurfactants [28] may offset the effect of emulsification.

3.4. Utilization of other aromatic compounds

In order to investigate the utilization spectrum of aromatic compounds by strain L4 in water, some organic compounds including phenol, 2, 4-dinitrophenol, *p*-nitrophenol, sodium benzoate and naphthalene were tested as sole carbon source and energy. The biomass of L4 was monitored continuously with OD600. The result showed the strain L4 can grow well in MSM containing phenol, sodium benzoate or naphthalene but cannot grow by utilizing 2,4-dinitrophenol or p-nitrophenol (Fig. 6).

The genus *Rhodococcus* is a very diverse group of bacteria that possesses the ability to degrade a large number of organic compounds, including some of the most difficult compounds with regard to recalcitrance and toxicity such as endosulfan, quinoline, 2,4,6-trichloro-phenol, and so on [29–31]. These studies described the industrial value of *Rhodococcus*. Li et al. [32] have reported that *R. ruber* CQ0301 after DBP–inducing can express catechol 1, 2–dioxygenase significantly higher than the one before DBP–inducing. To our knowledge, catechol is the metabolic intermediate product of aromatic hydrocarbon compounds and cleaving benzene ring is the common pathway of aromatic hydrocarbon metabolism.

Rhodococcus is a bacterial genus distributing in variable environments, including sediments, soil, sludge or waste water. In other words, the genus has ability to stand with variable environmental pollutants. This is just why we can isolate *Rhodococcus* strain to utilize different substrates from different environments. In order to use this genus to remediate polluted environment more effectively and rationally, the mechanism of catabolism should be further investigated in future on the level of molecular biology.

4. Conclusions

A strain able to degrade PAEs, isolated from activated sludge of a dyeing plant was identified as *R. ruber* through analysis of morphology, physiochemical properties and 16S rDNA sequence. This strain can survive by using DMP, DEP and DBP mixture as carbon source and energy. The article showed the optimal pH value, temperature and substrate's concentration that influenced the degradation rate in water. Kinetics of degradation can be described with exponential model. Little difference was observed in terms of ultimate degradation rate between PAEs contaminated water samples with non-emulsification and samples with completed emulsification. Besides DMP, DEP and DBP, *Rhodococcus* sp. L4 can also metabolize phenol, sodium benzoate and naphthalene. This broad-spectrum utilization suggested *Rhodococcus* sp. L4 be used as a potential candidate for remedying PAEs containing wastes.

Acknowledgements

This study was supported by grants from the National Science Council, Republic of China (No. 30400346 and No. 30771776). The authors wish to express their appreciation to Dr. D. Zheng, who has given technical assistance in GC analysis.

References

[1] S. Oishi, K. Hiraga, Testicular atrophy induced by phthalic acid esters, effect on testosterone and zinc concentrations, Toxicology 15 (1980) 197–202.
[2] S. Jobling, T. Reynolds, R. White, M.G. Parker, J.P. Sumpter, A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic, Environ. Health Perspect. 103 (1995) 582–587.
[3] M. Ema, E. Miyawaki, K. Kawashima, Further evaluation of developmental toxicity of di-*n*-butyl phthalate following administration during late pregnancy in rats, Toxicol. Lett. 98 (1998) 87–93.
[4] S.D. Gangolli, Testicular effects of phthalate esters, Environ. Health Perspect. 45 (1982) 77–84.
[5] L.E. Gray, J. Ostby, J. Furr, M. Price, D.N.R. Veeramachaneni, L. Parks, Perinatal exposure to the phthalates DEHP, BBP and DINP, but not DEP, DMP or DOTP, alters sexual differentiation of the male rat, Toxicol. Sci. 58 (2000) 350–365.
[6] A.M. Saillenfait, J.P. Sabate, F. Gallissot, Developmental toxic effects of diisobutyl phthalate, the methyl-branched analogue of di-*n*-butyl phthalate, administered by gavage to rats, Toxicol. Lett. 165 (2006) 39–46.
[7] G. Latini, A. Del Vecchio, M. Massaro, A. Verrotti, C. De Felice, Phthalate exposure and male infertility, Toxicology 226 (2006) 90–98.
[8] J.E. Huff, W.M. Kluwe, Phthalate esters carcinogenicity in F344/N rats and B6C3 F mice, Prog. Clin. Biol. Res. 141 (1984) 137–154.
[9] A.R. Singh, W.H. Lawrence, J. Autian, Mutagenic and antifertility sensitivities of mice to di-2-ethylhexyl phthalate (DEHP) and di-methoxyethyl phthalate (DMEP), Toxicol. Appl. Pharm. 29 (1974) 35–36.
[10] A.M. Jha, A.C. Singh, M. Bharti, Germ cell mutagenicity of phthalic acid in mice, Mutat. Res. 422 (1998) 207–212.
[11] US. EPA, Code of federal regulation, 40 CFR Part 136, 1992.
[12] J.L. Wang, P. Liu, Y. Qian, Microbial degradation of di-*n*-butyl phthalate, Chemosphere 31 (1995) 4051–4056.
[13] M.J. Bauer, R. Herrmann, Estimation of the environmental contamination by phthalic acid esters leaching from household wastes, Sci. Total Environ. 208 (1997) 49–57.
[14] J.L. Wang, X. Zhao, W.Z. Wu, Biodegradation of phthalic acid esters (PAEs) in soil bioaugmented with acclimated activated sludge, Process Biochem. 39 (2004) 1837–1841.

[15] F.L. Mayer, D.L. Stalling, J.L. Johnson, Phthalate esters as environmental contaminants, Nature 238 (1972) 411–413.
[16] M. Vitali, M. Guidotti, G. Macilenti, C. Cremisini, Phthalate esters in freshwaters as markers of contamination sources-a site study in Italy, Environ. Int. 23 (1997) 337–347.
[17] C.A. Staples, T.F. Parkerton, D.R. Peterson, A risk assessment of selected phthalate esters in North American and Western European surface waters, Chemosphere 40 (2000) 885–891.
[18] I.C. Beck, R. Bruhn, J. Gandrass, Analysis of estrogenic activity in coastal surface waters of the Baltic Sea using the yeast estrogen screen, Chemosphere 63 (2006) 1870–1878.
[19] N.L. Wolf, W.C. Steen, L.A. Burns, Phthalate ester hydrolysis: linear free energy relationship, Chemosphere 9 (1980) 939–942.
[20] C.A. Stales, D.R. Peterson, T.F. Parkerton, W.J. Adams, The environmental fate of phthalate esters: a literature review, Chemosphere 35 (1997) 667–749.
[21] R. Kleerebezem, J. Mortier, L.W. Hulshoff Pol, G. Lettinga, Anaerobic pretreatment of petrochemical effluents: terephthalic acid wastewater, Wat. Sci. Technol. 36 (1997) 237–248.
[22] D.W. Liang, T. Zhang, H.H. Fang, J. He, Phthalates biodegradation in the environment, Appl. Microbiol. Biotechnol. 80 (2008) 183–198.
[23] V. Kisand, R. Cuadros, J. Wikner, Phylogeny of culturable estuarine bacteria catabolizing riverine organic matter in the Northern Baltic Sea, Appl. Environ. Microbol. 68 (2002) 379–388.
[24] J.L. Wang, X. Zhao, W.Z. Wu, Biodegradation of phthalic acid esters (PAEs) in soil bioaugmented with acclimated activated sludge, Process Biochem. 39 (2006) 1837–1841.
[25] B.V. Chang, C.M. Yang, C.H. Cheng, S.Y. Yuan, Biodegradation of phthalate esters by two bacteria strains, Chemophere 55 (2004) 533–538.
[26] J.X. Li, J.D. Gu, L. Pan, Transformation of dimethyl phthalate, dimethyl isophthalate and dimethyl terephthalate by *Rhodococcus ruber* Sa and modeling the processes using the modified Gompertz model, Int. Biodeter. Biodegr. 55 (2005) 223–232.
[27] L.G. Whyte, S.J. Slagman, F. Pietrantonio, L. Bourbonniere, S.F. Koval, J.R. Lawrence, W.E. Inniss, C.W. Greer, Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15, Appl. Microbiol. Biot. 65 (1999) 2961–2968.
[28] J. Philp, M. Kuyukina, I. Ivshina, S. Dunbar, N. Christofi, S. Lang, V. Wray, Alkanotrophic *Rhodococcus ruber* as a biosurfactant producer, Appl. Microbiol. Biot. 59 (2002) 318–324.
[29] K. Verma, N. Agrawal, M. Farooq, R.B. Misra, R.K. Hans, Endosulfan degradation by a *Rhodococcus* strain isolated from earthworm gut, Ecotox. Environ. Safe. 64 (2006) 377–381.
[30] S.N. Zhu, D.Q. Liu, L. Fan, J.R. Ni, Degradation of quinoline by *Rhodococcus* sp. QL2 isolated from activated sludge, J. Hazard. Mater. 160 (2008) 289–294.
[31] M.Y. Pamukoglu, F. Kargi, Biodegradation kinetics of 2,4,6-trichlorophenol by *Rhodococcus rhodochrous* in batch culture, Enzyme Microb. Tech. 43 (2008) 43–47.
[32] J. Li, J.A. Chen, Q. Zhao, X. Li, W.Q. Shu, Bioremediation of environmental endocrine disruptor di-*n*-butyl phthalate ester by *Rhodococcus ruber*, Chemosphere 65 (2006) 1627–1633.