



Bioremediation of PAEs-contaminated saline soil: The application of a marine bacterial strain isolated from mangrove sediment

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ABSTRACT

Phthalic acid esters (PAEs) are known as the most widely used plasticizer as well as one of the ubiquitously distributed emerging pollutants. Biodegradation and bioremediation via application of PAEs-degrading microbes is promising. In this study, a novel marine microbe, *Gordonia hongkongensis* RL-LY01, was isolated from mangrove sediment showing high di-(2-ethylhexyl) phthalate (DEHP) degradation capacity. Strain RL-LY01 could degrade a wide range of PAEs and the degradation kinetics of DEHP followed the first-order decay model. Meanwhile, good environmental adaptability, preference to alkaline conditions and good tolerance to salinity and metal ions was shown. Further, metabolic pathway of DEHP in strain RL-LY01 was proposed, with di-ethyl phthalate, phthalic acid, benzoic acid and catechol as intermediates. Additionally, one known mono-alkyl phthalate hydrolase gene (*mehpH*) was identified. Finally, the excellent performance during bioremediation of artificial DEHP-contaminated saline soil and sediment indicated strain RL-LY01 employs great application potential for the bioremediation of PAE-contaminated environments.

1. Introduction

As one of the major components of plastics, plasticizers are employed to improve the flexibility and elasticity of plastic products (Katsikantami et al., 2016). Plasticizers account for the largest proportion of plastic additives and they are consisted of a large group of synthetic organic compounds. In 2020, market volume of plasticizers worldwide amounted to nearly 10.2 million metric tons and it is forecasted that global plasticizer consumption will grow at a rate of about 3.5 % per year in the next few years (data available from: <https://www.statista.com/> and <https://www.plasticisers.org/plasticisers/>). Phthalic acid esters (PAEs), known as the most widely used plasticizers, are mainly employed in flexible PVC applications, largely for the construction, automotive and wire & cable sectors. PAEs accounted for over 55 % of world consumption of plasticizers in 2020 and it is expected to account for 50 % – 55 % of world consumption in the coming years (<https://www.ihs.com/products/plasticizers-chemical-economics-handbook.html>). Since plasticizers are so widely used, they have undergone extensive testing for possible health and environmental effects. Six kinds of PAEs have

been listed as the priority pollutants by the United States Environmental Protection Agency (EPA, 1992), the European Union (European, 1993), and China National Environmental Monitoring Center (Zhang et al., 2020a) because of their endocrine disrupting, teratogenicity, and carcinogenicity and mutagenicity properties (Duarte et al., 2021; Abdul-Ghani et al., 2012; Chang et al., 2017; Wang et al., 2019a, 2019b; Wen et al., 2022), including di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), di-*n*-octyl phthalate (DnOP), di-(2-ethyl hexyl) phthalate (DEHP), and butyl benzyl phthalate (BBP).

Over the last 40 years, great efforts have been made to understand the fate of PAEs in environment. Variety of methods are employed to eliminate PAEs from different environments (Nas et al., 2022; Bai et al., 2020; Pirsaeheb et al., 2022). Biological remediation (bioremediation) is recognized as the most efficient, safe and economic approach, including phytoremediation, fungi and bacteria mediated bioremediation, and the conjunction of these methods (Ren et al., 2018). Among these methods, bacteria mediated bioremediation is believed to be the most preferred and promising way because of their high efficiency, well environmental adaptability and low costs. Lots of PAEs-degrading bacterial strains have

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been isolated and characterized (Lu et al., 2009; Song et al., 2022; Wang et al., 2015; Zhao et al., 2018). The metabolic pathways and related molecular mechanisms have been systematically investigated. And some of these isolates have been applied to the bioremediation of PAEs-contaminated sites or samples.

Marine plastic pollution has become a global environmental issue. It was estimated that 19 to 23 million metric tons, approximately 11 %, of plastic waste generated globally in 2016, entered aquatic ecosystems (Borrelle et al., 2020). For the plastics entered the marine environment, global assessments indicated that only 1 % of these plastics remain floating in surface waters (Law, 2017). Burial in sediments is recognized as the major sink of plastic in the marine environment and it has been reported that lots of marine plastics are transported back to the coastal areas by tides, such as intertidal sediments, mangrove sediments, and coastal farmland (Li et al., 2021). Plastics enter coastal areas might be trapped and sedimented in coastal regions, and further be decomposed. During these process, marine plastics are decomposed into microplastics and the additives of plastics like plasticizers and flame retardants would be released into environments (Jambeck et al., 2015). The fate of PAEs in terrestrial ecosystems has been systematically investigated and the bioremediation of PAEs in terrestrial ecosystems has been extensively conducted while the knowledge on the fate of DEHP in marine ecosystem is still limited (Net et al., 2015). Although several PAEs-degrading marine bacterial strains have been isolated, the biodegradation and bioremediation of PAEs in coastal ecosystem is still underexplored.

In the present study, a novel PAEs-degrading marine bacterial strain RL-LY01 was isolated from mangrove sediments from the intertidal zone of Zhanjiang Bay, China. Strain RL-LY01 was identified as *Gordonia hongkongensis* by biochemical characterization, 16S rRNA gene analysis and genome-based digital DNA: DNA hybridization (dDDH) via Type Strain Genome Server (TYGS). The environmental adaptability of strain RL-LY01 was characterized through batch assays. The degradation kinetics of DEHP by strain RL-LY01 under the optimized conditions followed the first-order decay model with a correlation coefficient of 0.9957. Metabolic pathway of DEHP in strain RL-LY01 was proposed via metabolic intermediates identification and genome sequence analysis and an esterase gene involved in the hydrolysis of ester bond was identified from the genome of strain RL-LY01. Finally, strain RL-LY01 was applied for the bioremediation of DEHP-contaminated coastal saline soil to evaluate its application potential.

2. Material and methods

2.1. Chemicals and medium

The detailed information of PAEs and chemicals used in this study was presented in Table S1. PAE stock solution was prepared in methanol with a concentration of 2×10^4 mg/L and kept at 4 °C. PAE stock solution will be supplemented into the medium to obtain the desired concentration. Biological reagents and enzymes were purchased from Thermo Fisher Scientific (Table S1).

Adjusted Luria Bertani (LB) medium was used for the enrichment of PAEs-degrading bacterium while mineral salt medium was applied for the isolation of PAEs-degrading bacterium. The composition of these two media were presented as below:

Adjusted LB for enrichment: peptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 40 g/L.

MSM for isolation: KH_2PO_4 , 1.5 g/L; K_2HPO_4 , 1.5 g/L; $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 1.0 g/L; $(\text{NH}_4)_2\text{SO}_4$, 0.2 g/L; FeCl_2 , 0.05 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L; NaCl, 30 g/L and CaCl_2 , 0.001 g/L.

The solid media of LB and MSM were prepared by supplementing agar with a final concentration of 15 g/L. The pH of all media above was adjusted to 7.2 ± 0.2 with NaOH or HCl (2 M). Then, all media were sterilized by autoclaving (121 °C, 30 min).

2.2. Isolation and identification of DEHP-degrading marine bacterial strain

Mangrove sediments were sampled from Zhanjiang Mangrove Forest Nature Reserve, China, and the detailed characteristics of collected sediments are presented in Table S2. Rocks and plant tissues were removed prior to enrichment and isolation. The schematic diagram for the enrichment and isolation of DEHP-degrading marine bacterial strain is shown in Fig. 1, and the steps as below: (a) Approximately 1 g of mangrove sediment was added into 10 mL LB liquid media with 100 mg/L of DEHP. The culture was incubated in an orbital shaker for 72 h (180 rpm, 30 °C). (b) And then, 1 mL of the culture was transfer into 9 mL of fresh LB liquid medium while the concentration of DEHP was increased to 200 mg/L. The culture was incubated in an orbital shaker for 72 h (180 rpm, 30 °C). (c) The enrichment process was repeated until the concentration of DEHP was increased to 500 mg/L. The cultures obtained from enrichment were applied for the isolation of DEHP-degrading strains. (d) An aliquot of 1 mL culture was centrifugated (6000 rpm, 5 min), washed with phosphate buffer solution (PBS, 0.1 M, pH 7.4), and re-centrifugate (6000 rpm, 5 min). The washing and centrifugation were repeated for three times to remove the constituents of LB medium efficiently and the cells were resuspended in fresh MSM liquid medium (1 mL) finally. Cells were transferred into 9 mL of fresh MSM liquid medium while DEHP was supplied as sole carbon sources with a concentration of 100 mg/L. Cultures were incubated under constituent shaking in the dark for 72 h (180 rpm, 30 °C). (e) An aliquot of 1 mL culture was used for serial dilution and the diluted culture was spread onto the MSM solid medium supplemented with 100 mg/L of DEHP as sole carbon sources. Plates were incubated under dark at 30 °C. (f) Single colony was inoculated into fresh LB medium and incubated in a rotary shaker under dark (180 rpm, 30 °C) for 24 h. (g) An aliquot of 1 mL culture was used for bacterial seeds collection by centrifugation (6000 rpm, 5 min), washing (PBS, 0.1 M, pH 7.4), and re-centrifugation (6000 rpm, 5 min). (h) The obtained bacterial seeds were resuspended in 1 mL fresh MSM medium and then inoculated into 9 mL fresh MSM medium supplied with 100 mg/L of DEHP. Cultures without inoculation were set as abiotic treatment and all cultures were incubated under dark (180 rpm, 30 °C) for 5 d. (i) The residual concentration of DEHP was determine by gas chromatography (GC). Steps (f) to (i) were repeated until DEHP-degrading strain was isolated.

The isolated DEHP-degrading marine bacterial strain was identified by physiological and biochemical characteristics analyses coupled with 16S rRNA gene analysis and complete genome sequencing. The genomic DNA of isolated strain was extracted and applied for 16S rRNA gene amplification (primers were shown in Table S3). The generated fragments were inserted into pMDTM19-T vector and sequenced by GENE-WIZ, AZENTA Life Sciences (Guangzhou, China). Vector sequences were removed via VecScreen (<https://www.ncbi.nlm.nih.gov/tools/vectscreen/>). The primary identification of isolated strain was achieved by aligning the obtained 16S rRNA gene sequence with known records via Basic Local Alignment Search Tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Further, 16S rRNA genes of related type strains were retrieved from the List of Prokaryotic names with Standing in Nomenclature (LPSN, <http://www.bacterio.net/>). MEGA 7.0 was applied to the phylogenetic analysis using Neighbor-Joining algorithm with a bootstrap value of 1000 (Kumar et al., 2016). The physiological and biochemical characteristics of isolated strain were compared with closest species according to the Bergey's Manual of Determinative Bacteriology (George et al., 2001). The cell morphology was observed with a scanning electron microscope (HITACHI, SU8010, Japan). The complete genome of isolated strain was sequenced by Biomarker Technologies Corporation (Beijing, China) with a PacBio HiFi platform and the genome sequence was applied for genome-based taxonomy. The genome-based digital DNA: DNA hybridization (dDDH) was accomplished via TYGS (https://tygs.dsmz.de/user_requests/new).

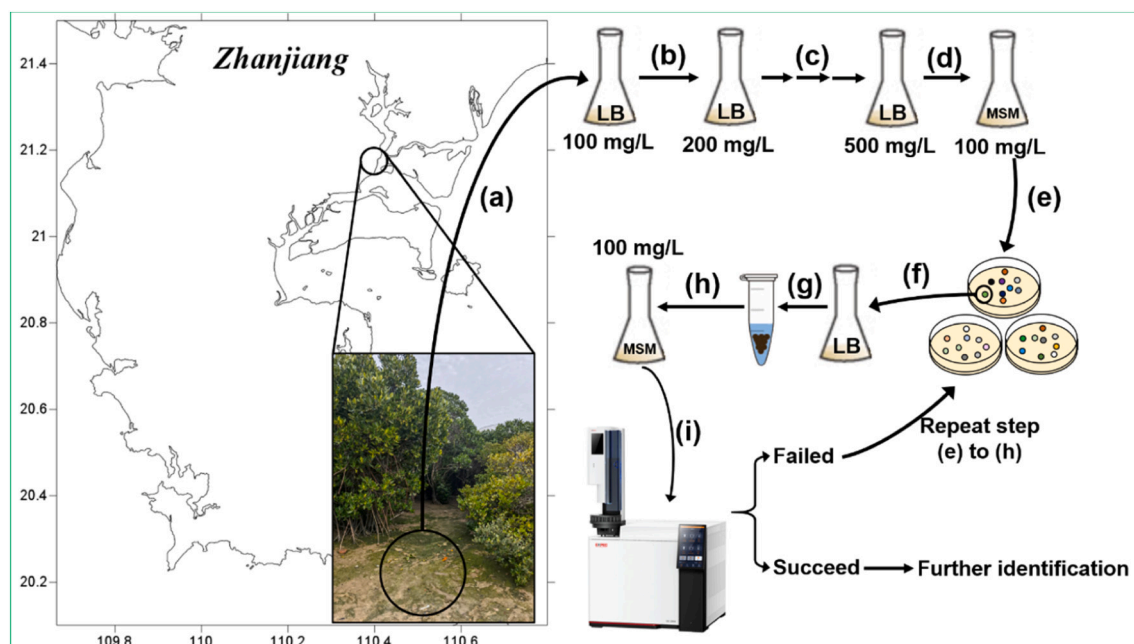


Fig. 1. The schematic diagram for the enrichment and isolation of DEHP-degrading marine bacterial strain (the concentration of DEHP is shown around the Erlenmeyer flasks).

2.3. Characterization of DEHP-degrading strain

The seeds of isolated strain for inoculum were prepared prior to batch assays. Single colony of isolated strain was inoculated into 10 mL of fresh LB medium and incubated in a rotary shaker for 24 h (180 rpm, 30 °C). Cells were harvested by centrifugation (6000 rpm, 5 min), washed by PBS (0.1 M, pH 7.4), and re-centrifugation (6000 rpm, 5 min). The washing and re-centrifugation were repeated for three times and finally resuspended in fresh MSM medium. The concentration of cell was adjusted to $OD_{600} = 0.8$, $\sim 5.0 \times 10^7$ cells/mL, and inoculum size was maintained at 1.0 % (v/v) unless stated otherwise.

The substrate range of isolated strain was determined by supplying some other plastic additives (100 mg/L of each) as sole carbon source individually, including DBP, BBP, DEP, DnOP, di-cyclohexyl phthalate (DCHP), bisphenol A (BPA), and triphenyl phosphate (TPP). The effects of different environmental factors on the degradation of DEHP (100 mg/L) by the isolated strain were determined via single-factor analysis, including initial pH (4, 5, 6, 7, 8, 9, and 10), incubation temperature (10 °C, 20 °C, 30 °C, 40 °C, and 50 °C), salinity (2.0 %, 4.0 %, 6.0 %, 8.0 %, 10.0 % and 12.0 % of NaCl, w/v), and metal ions (Mg^{2+} , Mn^{2+} , Co^{2+} , Ca^{2+} , Zn^{2+} , and Cu^{2+} , 1 mmol/L). The seeds of isolated strain were inoculated into the target medium as described above and the same cultures without inoculation were set as abiotic control. All cultures were incubated under continual shaking (180 rpm, 30 °C) in the dark. All the assays above were conducted in three replicates and the residual concentration of DEHP in each treatment was detected by GC after 72 h incubation. Finally, the degradation of DEHP under the optimized conditions was measured and the kinetics of degradation was simulated with a first-order decay model (Eq. (1)).

$$\text{The first - order decay model : } S = S_0 + A \cdot \exp.(-t/t_1) \quad (1)$$

In which S represents the substrate concentration; S_0 represents the fitted initial concentration; A is the biodegradation potential.

2.4. Analysis of DEHP metabolic mechanisms

To have an insight into the metabolic mechanism of DEHP in the isolated strain, the metabolic pathway of DEHP was proposed via

metabolic intermediates identification. Catabolic intermediates of DEHP were prepared as below: (a) An aliquot of 1 mL seeds was inoculated into 9 mL of fresh MSM liquid medium and DEHP was added to a final concentration of 100 mg/L. (b) All cultures were incubated in rotary shaker for 72 h (180 rpm, 30 °C) while samples were withdrawn at a time interval of 12 h. (c) Metabolic intermediates were extracted by equal volume of ethyl acetate and the mixture was thoroughly extracted by ultrasonic extraction. The organic phase was collected while the water phase was adjusted to a final pH of 3.0. And then, the water phase (pH 3.0) was extracted again and the collected organic phases were merged. (d) The extracts were dried by nitrogen (purity above 99.99 %), redissolved in methanol, filtered with a membrane (0.22 μ m), and analyzed by ultra-high pressure liquid chromatography coupled with mass spectrum (UHPLC-MS). (e) Finally, the metabolic pathway of DEHP in the isolated strain was deduced according to the identification of metabolic intermediates. Genes and gene clusters potentially contribute to the metabolism of DEHP were analyzed by genome sequencing and analyzing.

2.5. Bioremediation of DEHP-contaminated saline soil

To determine the application potential for bioremediation of isolated strain in saline environment, the degradation percentages of DEHP in synthetic DEHP-contaminated soil and sediment were determined. Intertidal sediment, mangrove sediment and saline farmland soil were collected from Zhanjiang Bay, China. Rocks and plant tissues were removed prior to bioremediation assays. The detailed information of sediment and soil are presented in Table S2. The seeds for bioremediation were prepared as described above. In-vitro bioremediation was conducted in glass beaker and four treatments for each sample were prepared (Fig. S1): (i) 20 g soil or sediment containing 100 mg/kg of DEHP, (ii) 20 g sterilized soil or sediment containing 100 mg/kg of DEHP, (iii) 200 μ L of the seeds was added into 20 g soil or sediment containing 100 mg/kg of DEHP, and (iv) 200 μ L of the seeds was added into 20 g sterilized soil or sediment containing 100 mg/kg of DEHP. All samples were fully mixed, incubated under constant temperature (30 °C) and humidity (90 % relative humidity), and all treatments were conducted in triplicate. Soil samples were collected with specific time

interval and the residual concentration of DEHP was quantified by GC.

2.6. Analytic methods

The cell concentration was expressed as the absorbance at 600 nm (OD_{600}). Cells in culture were harvested by centrifugation (6000 rpm, 5 min) and washed by PBS buffer (0.1 M, pH 7.4). After three times centrifugation and washing, the cells were finally resuspended in PBS buffer (0.1 M, pH 7.4) and the cell density was determined with a spectrophotometer (P4PC, MAPADA, Shanghai, China). Further, the dilution plate technique was used to quantify the colony-forming units (CFUs) of the cell suspension.

DEHP in MSM liquid medium was extracted by equal volume of ethyl acetate and the mixture was thoroughly extracted by ultrasonic extraction for 10 min (40 kHz). Organic phase was filtered by a membrane (0.22 μ m) and then applied to GC analysis. The recovery rates of DEHP (1 mg/L, 10 mg/L, and 100 mg/L) from MSM liquid medium were all above 99.0 %. DEHP in sediment or soil was extracted by ethyl acetate three times. Extracts were merged, filtered (0.22 μ m), evaporated and redissolved in 2 mL of methanol. And then, solid phase extraction was performed with the extracts using a dSPE extraction tube (SBEQ-CA8649-glass, Anpel, Shanghai, China) following the protocol. The recovery rates of DEHP from sediment and soil (1 mg/kg, 10 mg/kg, and 100 mg/kg) were all above 95.0 % (average = 97.6 %, min = 95.8 %, and max = 99.2 %).

DMP, DBP, BBP, DEP, DnOP, DCHP, and DEHP were quantified by a GC (GC-2010 pro, SHIMADZU, Japan) equipped with a WondaCap 5 column (GL Sciences Inc., Japan, 30 m \times 0.25 mm \times 0.25 μ m) and an electron capture detector (ECD). The detection parameters were as follows: (a) the initial oven temperature was maintained at 100 $^{\circ}$ C for 5 min, increased to 280 $^{\circ}$ C with a rate of 20 $^{\circ}$ C/min, and finally maintained at 280 $^{\circ}$ C for 15 min; (b) the temperatures of inlet and detector were maintained at 300 $^{\circ}$ C and 280 $^{\circ}$ C, respectively. An aliquot of 1 μ L sample was injected and nitrogen (purity >99.999 %) served as the carrier gas with a flow rate of 2 mL per minute. GC data was acquired and analyzed via software LabSolutions (version 5.90, Shimadzu, Japan). A high-performance liquid chromatograph (Agilent 1260), equipped with an Eclipse Plus C18 (4.6 \times 250 mm \times 5 μ m) column and a variable wavelength detector (VWD), was used for the quantification of BPA and TPP. For the detection and quantification of BPA, methanol (65.0 %) and water (35 %) were used as the mobile phase with a flow rate of 1.0 mL/min, the column temperature was maintained at 28 $^{\circ}$ C, the injection volume was 2 μ L and the wavelength for detection was 254 nm. For the detection and quantification of TPP, methanol (80.0 %) and water (20.0 %) were used as the mobile phase with a flow rate of 1.0 mL/min, the column temperature was maintained at 28 $^{\circ}$ C, the injection volume was 2 μ L and the wavelength for detection was 250 nm. External standard method was applied for the establishment of standard curves for the selected substrates (Table S4). All detections were performed in triplicate.

The degradation percentage of target substrate was calculated with Eq. (2) as below:

$$\text{Degradation percentage (\%)} = (C_{ck} - C_f) / C_{ck} \times 100 \quad (2)$$

where C_{ck} represents the final concentration of specific substrate in control treatment while C_f means the final concentration of this substrate in the target treatment.

The metabolic intermediates of DEHP were identified by Bio-novogene Co. Ltd. (Suzhou, China) with an ultra-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS). The ultra-high pressure liquid chromatography (UHPLC) separation was performed with a Thermo Ultimate 3000 UHPLC system equipped with an ACQUITY UPLC HSS T3 column (150 \times 2.1 mm, 1.8 μ m, Waters). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH 9.75) (A) and acetonitrile (B). The

analysis was carried with elution gradient as follows: 0–0.5 min, 95 % B; 0.5–7.0 min, 95 % \sim 65 % B; 7.0–8.0 min, 65 % \sim 40 % B; 8.0–9.0 min, 40 % B; 9.0–9.1 min, 40 % \sim 95 % B; 9.1–12.0 min, 95 % B. The column temperature was maintained at 25 $^{\circ}$ C. The auto-sampler temperature was 6 $^{\circ}$ C, and the injection volume was 2 μ L. The ESI-MSn assays were conducted with a Thermo Q Exactive Focus mass spectrometer while the spray voltage for positive and negative modes were set as 3.8 kV and –2.5 kV, respectively. The capillary temperature was 325 $^{\circ}$ C while the sheath gas and auxiliary gas were set at 30 and 10 arbitrary units, respectively. The mass range (m/z) of 100–500 was scanned with a mass resolution of 70, 000. Data dependent acquisition (DDA) MS/MS experiments were accomplished with HCD scan and the normalized collision energy was set as 30 eV. MS raw data (.wiff) files were converted to the mzXML format by ProteoWizard, and processed by R package XCMS. The process includes peak deconvolution, alignment and integration. Minfrac and cut off are set as 0.5 and 0.3 respectively. The identification of specific compounds depending on MS/MS spectra was done with selected MS/MS database (Table S5). The processing, visualization and analysis of mass spectrometry based molecular profile data was executed on MZmine 2 (version 2.53) (Pluskal et al., 2010).

2.7. Accession numbers

Strain RL-LY01 is available from Guangdong Microbial Culture Collection Center (GDMCC) with accession number of GDMCC No.1.3280. The genome sequence of strain RL-LY01 was deposited in GenBank under the accession number of CP121270.

3. Results and discussion

3.1. Isolation and identification of DEHP-degrading bacterial strain

After several weeks of enrichment and domestication, one bacterial strain with orange colony was isolated from mangrove sediments which was capable of utilizing DEHP as sole carbon source for growth and named as RL-LY01. Strain RL-LY01 could degrade \sim 98.5 % of DEHP (100 mg/L) in 24 h with a significant increase of biomass (Fig. 3A). The colony of strain RL-LY01 was orange and round shaped (Fig. 2A), and the cells were rod shape without flagellum (Fig. 2B). The partial sequence of 16S rRNA gene with a length of 1474 bp was amplified from the genome of strain RL-LY01. The results of the BLAST search suggested that strain RL-LY01 belongs to genus *Gordonia*, and then the phylogenetic tree was constructed (Fig. 2C). The results indicated that strain RL-LY01 was clustered with *Gordonia hongkongensis* HKU50^T (LC072670). The physiological and biochemical characteristics of strain RL-LY01 were presented in Table 1. The dDDH values between the genome of strain RL-LY01 and the genome of closest genomes were shown in Table 2, and the results indicated that strain RL-LY01 belongs to *Gordonia hongkongensis*. Finally, strain RL-LY01 was identified as *Gordonia hongkongensis* according to the physiological and biochemical characteristics, 16S rRNA gene analysis and genome-based taxonomy.

Genus *Gordonia* is a phylogenetically and catabolically diverse group that has been isolated from various environments for its versatile ability to degrade a wide range of natural and synthetic organic compounds. Lots of xenobiotics-degrading *Gordonia* spp. have been isolated from various environments and several PAEs-degrading *Gordonia* spp. strains have been reported (Table S6). These strains were mainly isolated from terrestrial ecosystem while the reports of PAEs-degrading *Gordonia* spp. from marine ecosystem are limited. *Gordonia* sp. MTCC4818 is the first reported PAEs-degrading *Gordonia* strain which could completely degrade 1000 mg/L of BBP within 4 days (Chatterjee and Dutta, 2003). *Gordonia alkanivorans* YC-RL2 is known as an alkali tolerant DEHP-degrading strain which could efficiently degrade DEHP (100 mg/L) under a wide range of pH (6.0–11.0, degradation percentages above 80.0 %) (Nahurira et al., 2017). *Gordonia terrae* RL-JC02 was isolated from red soil with long-term usage of plastic mulch which showed great

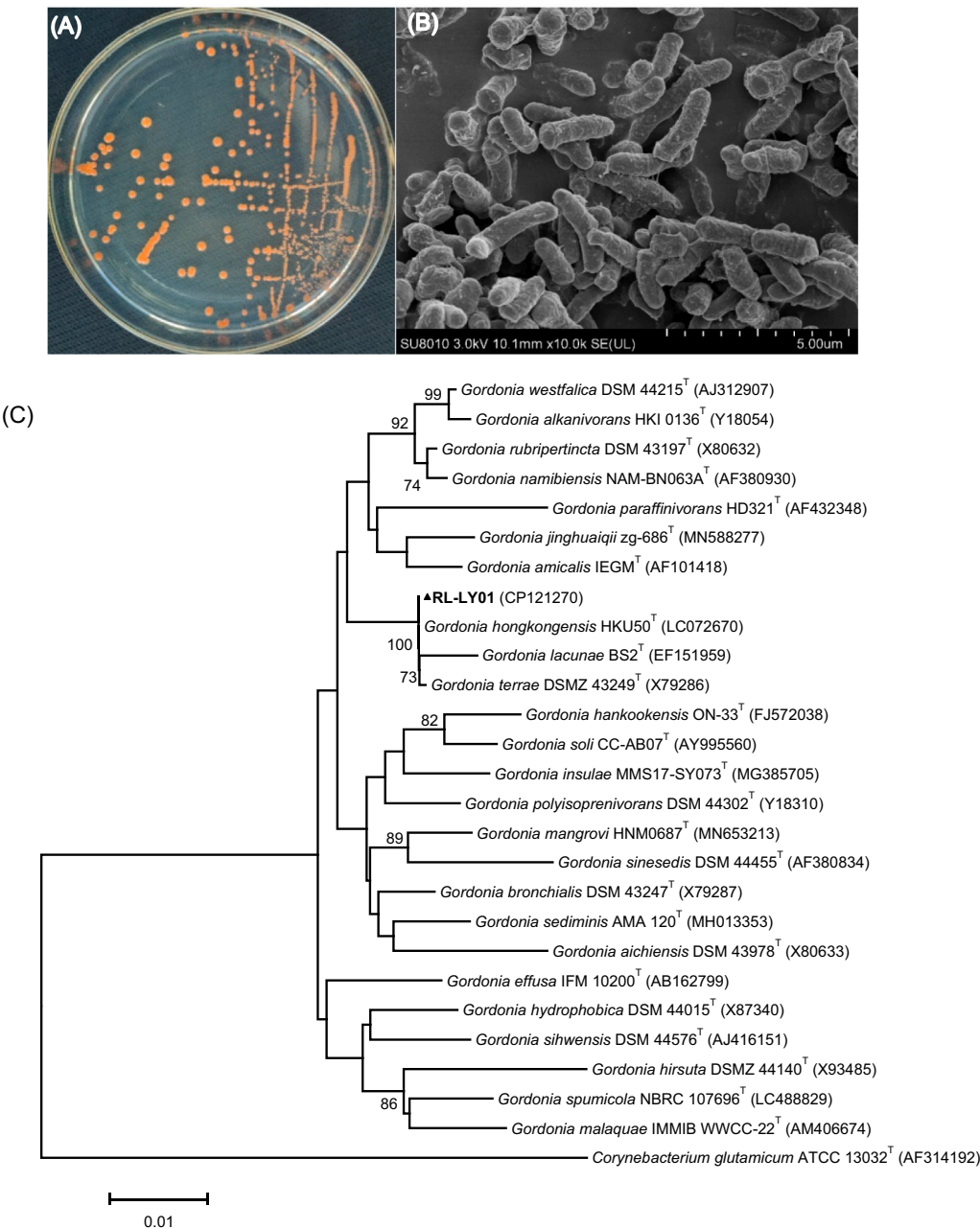


Fig. 2. Identification of strain RL-LY01 (A: colony morphology, B: cell morphology, C: phylogenetic tree of 16S rRNA gene).

Table 1
The physiological and biochemical characteristics of strain RL-LY01.

Characteristics	Description	Characteristics	Description
Gram staining	Positive	Urease test	Positive
Indole test	Negative	Hydrogen sulfide production	Positive
Methyl Red test	Negative	Antibiotic resistance (Ampicillin)	Negative
V-P test	Negative	Antibiotic resistance (Kanamycin)	Negative
Gelatin hydrolysis	Negative	Antibiotic resistance (Tetracycline)	Negative

application potential for the bioremediation of DEHP contaminated soil (Zhang et al., 2020a). However, the reports of marine PAEs-degrading *Gordonia* spp. strains are limited as well as the application of *Gordonia*

strains for the bioremediation of PAEs-contaminated marine samples. Thus, the isolation of marine PAEs-degrading *Gordonia* strains and the application of these isolates with marine samples are necessary and significant.

3.2. Characterization of *Gordonia hongkongensis* RL-LY01

The performance of xenobiotics-degrading bacteria is significantly affected by the environmental adaptability of strains. Simultaneously, the environmental adaptability might decide the application potential of these isolates. Thus, the effects of pH, temperature, salinity, and metal ions on DEHP degradation by strain RL-LY01 were evaluated and the metabolic capability of some other plastic additives was also determined. As shown in Fig. 3A, DEHP could be rapidly removed by strain RL-LY01 with a significant increasing of biomass. Approximately 98.5 %

Table 2

Genome information of closet strains and dDDH values between the genome strain RL-LY01 and the closet genomes.

Strains	Accession numbers ^a	Genome length (bp)	G + C percent (%)	dDDH against strain RL-LY01 (%) ^b	Diff. G + C Percent (%)
Strain RL-LY01	PRJNA951700	5, 175, 544	67.95	/	/
<i>Gordonia hongkongensis</i> JCM 31934	PRJNA824702	5, 409, 529	67.82	79.7	0.13
<i>Gordonia lacunae</i> BS2	PRJNA386254	5, 756, 385	68.08	35.5	0.14
<i>Gordonia terrae</i> NRRL B-16283	PRJNA472951	5, 708, 950	67.8	34.9	0.14
<i>Gordonia terrae</i> ATCC 25594	PRJNA330541	5, 701, 501	67.81	34.9	0.14
<i>Gordonia terrae</i> NBRC 100016	PRJDA73365	5, 669, 149	67.84	34.8	0.11
<i>Gordonia jinghuaiqii</i> zg-686 T	PRJNA647679	5, 184, 113	67.56	24.8	0.39
<i>Gordonia westfalica</i> DSM 44215	PRJNA303710	6, 405, 003	66.9	24.4	1.05
<i>Gordonia alkanivorans</i> NBRC 16433	PRJNA66367	5, 071, 550	67.41	24.1	0.53
<i>Gordonia rubripertincta</i> NBRC 101908	PRJDA71851	5, 204, 225	67.38	23.9	0.56
<i>Gordonia amicalis</i> DSM 44461	PRJNA622446	4, 917, 585	67.43	23.8	0.52
<i>Gordonia amicalis</i> NBRC 100051	PRJDB393	4, 915, 471	67.42	23.8	0.52
<i>Gordonia namibiensis</i> NBRC 108229	PRJDB143	4, 936, 023	67.59	23.8	0.35
<i>Gordonia bronchialis</i> NCTC 10667	PRJEB6403	5, 326, 567	67.03	22.1	0.92
<i>Gordonia hydrophobica</i> NBRC 16057	PRJDB466	4, 579, 443	67.52	20.2	0.43

^a The accession numbers of BioProject were provided in this study;^b The dDDH value presented in the table is d4 value and the reason can be found in <https://tygs.dsmz.de/faqs>.

of DEHP (100 mg/L) was degraded with the inoculation of strain RL-LY01 within 72 h while DEHP concentration was maintained at a high level in the abiotic treatment (*P* value shown in Table S7). Actually, the degradation of DEHP by strain RL-LY01 was mainly achieved in 24 h with 95.6 % of DEHP was degraded and no lag phase was observed during this process. The cell growth showed similar trends with DEHP degradation as OD₆₀₀ increased rapidly in 24 h without lag phase and the growth entered stationary phase after 30 h with a maximum OD₆₀₀ of ~1.37. The substrate profile assay (Fig. 3B) indicated that strain RL-LY01 could utilize a wide range of PAEs for growth, including short side-chain PAEs (DEP and DBP), long side-chain PAEs (DnOP and DEHP), complex side-chain PAEs (BBP), and circular side-chain (DCHP), but failed to degrade BPA and TPP. Further, strain RL-LY01 showed good tolerance to different initial pH and the degradation percentages of DEHP under different initial pH were 51.61 % (pH 4.0), 72.43 % (pH 5.0), 89.99 % (pH 6.0), 97.00 % (pH 7.0), 98.18 % (pH 8.0), 95.68 % (pH 9.0), and 80.91 % (pH 10.0). Therefore, the optimal initial pH for the degradation of DEHP by strain RL-LY01 should be pH 8.0. Specifically, strain RL-LY01 could efficiently degrade DEHP under a wide range of temperature and the inhibiting effect of the relatively high temperature (50 °C) is limited since the degradation percentage reached 71.58 %. Even under the relatively low temperature (10 °C), approximately 45.16 % of DEHP (103.9 mg/L) could be removed. The optimal incubation temperature should be 30 °C for the highest degradation percentage (98.30 %) of DEHP under 30 °C. As expected, strain RL-LY01 showed good tolerance to high salinities and the degradation percentages of DEHP were all above 75.0 % when salinities ranged from 2.0 % to 8.0 %. When the salinity was increased to 10.0 %, the degradation percentage of DEHP was decreased to 51.26 % while the degradation was completely inhibited for the salinity of 12.0 %. Additionally, most of the selected metal ions did not exhibit inhibiting effects to the degradation of DEHP by strain RL-LY01. For the treatment with Zn²⁺, the degradation of DEHP by strain RL-LY01 was slightly inhibited and the degradation percentage of DEHP was decreased to 60.15 % while the addition of Cu²⁺ almost completely inhibited the degradation of DEHP by strain RL-LY01. Finally, the degradation kinetics of DEHP by strain RL-LY01 was simulated with the first-order decay model under the optimized conditions (pH 8.0 and 30 °C) and the results demonstrated that the degradation process fitted with the model with a correlation coefficient of 0.9957. The degradation kinetics analysis demonstrated that DEHP could be rapidly degraded by strain RL-LY01 with a half-life of 5.14 h and degradation constant (*k*) of 0.13469.

Strain RL-LY01 was expected to harbor good environmental adaptability, especially for the temperature and salinity, since the samples for isolation were collected from intertidal mangrove sediment located in subtropical region. The substrate profile assay indicated that strain RL-

LY01 employed robust metabolic capability to different kinds of PAEs while the incapability to utilize BPA and TPP for growth is potentially due to the specificity of enzyme-mediated degradation process. Although it has been reported that the degradation of long or complex side-chain PAEs is more difficult than the short side-chain PAEs (Ren et al., 2018; Zhang et al., 2020a), the degradation percentages of all the selected PAEs (including typical short, long, complex and circular PAEs) by strain RL-LY01 were above 95.0 % after 72 h incubation. These results demonstrated that strain RL-LY01 is a robust PAEs degrader. Strain RL-LY01 could efficiently degrade DEHP with a wide range of initial pH and showed preference to alkaline conditions, which is consisted with known reports (Nahurira et al., 2017; Yang et al., 2018; Kamaraj et al., 2022; Wang et al., 2012). In line with most microbes isolated from saline environments (Ren et al., 2021; Yang et al., 2018), strain RL-LY01 showed good tolerance to high salinities. Additionally, the excellent tolerance of alkaline and saline conditions suggested that strain RL-LY01 should be favored for the bioremediation of PAEs-contaminated saline-alkali environments. Metal ions in mangrove ecosystems have been widely reported which might explain the good tolerance of metal ions of strain RL-LY01 (Zhou et al., 2022; Wang et al., 2020; Jiang et al., 2020). To simulate the degradation process of pollutants, kinetics analysis has been widely used which might provide important information to evaluate the degradation efficiencies of different isolates (Ahuaactzin-Pérez et al., 2016; Jin et al., 2012; Zhang et al., 2020a; Zhang et al., 2020b). The first-order decay model was extensively applied to simulate the enzyme catalytic degradation process.

3.3. Metabolic mechanisms of DEHP in strain RL-LY01

To have a further insight into the metabolic mechanisms of DEHP in strain RL-LY01, the metabolic pathway of DEHP was deduced by metabolites identification while genes potentially contributed to the metabolism of DEHP were obtained by gene cloning. Firstly, the metabolic pathway (Fig. 4A) of DEHP in strain RL-LY01 was deduced via metabolic intermediates identification (Fig. 4B and Fig. S2). Apart from the parent compound DEHP, five metabolic intermediates of DEHP were identified by UHPLC-MS, including DBP, DEP, phthalic acid (PA), benzoic acid (BA), and catechol. Therefore, the metabolic pathway of DEHP in strain RL-LY01 was inferred as: (a) DEHP was initially transformed into DBP and DEP step-by-step via β -oxidation; (b) DEP was transformed into PA via de-esterification; (c) PA was decarboxylated into BA which was further transformed into catechol; (d) catechol was finally utilized for cell growth through the catechol branch of β -ketoadipate pathway.

The genome of strain RL-LY01 was sequenced to have an insight into the molecular mechanisms of PAEs metabolism. One circular chromosome was finally assembled with a length of 5, 175, 544 bp and G + C

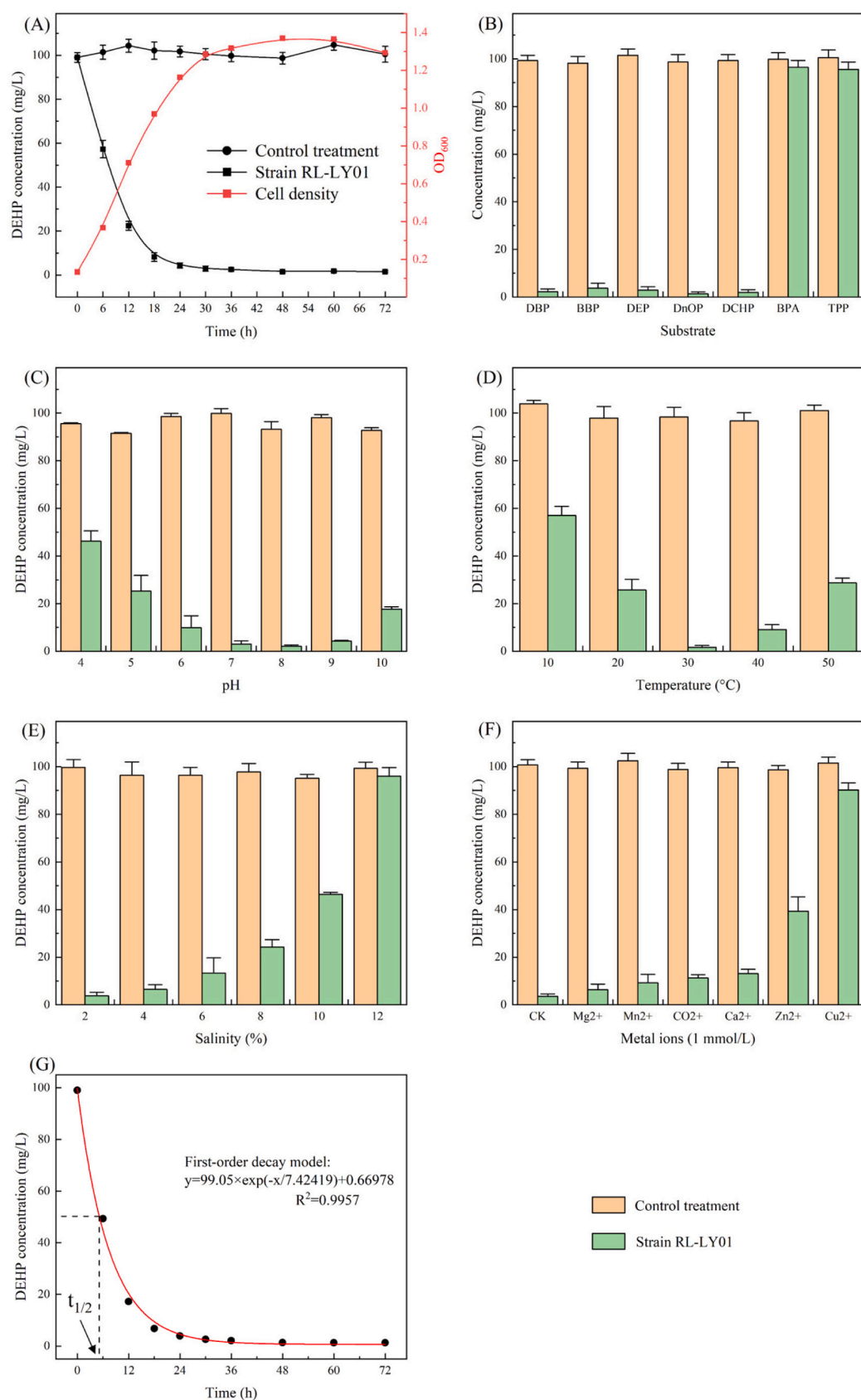


Fig. 3. Characterization of strain RL-LY01 (A: degradation of DEHP and cell growth, B: substrate range, C: effects of pH, D: effects of temperature, E: effects of salinity, F: effects of metal ions, G: the fitted curve for DEHP degradation with the first-order decay model).

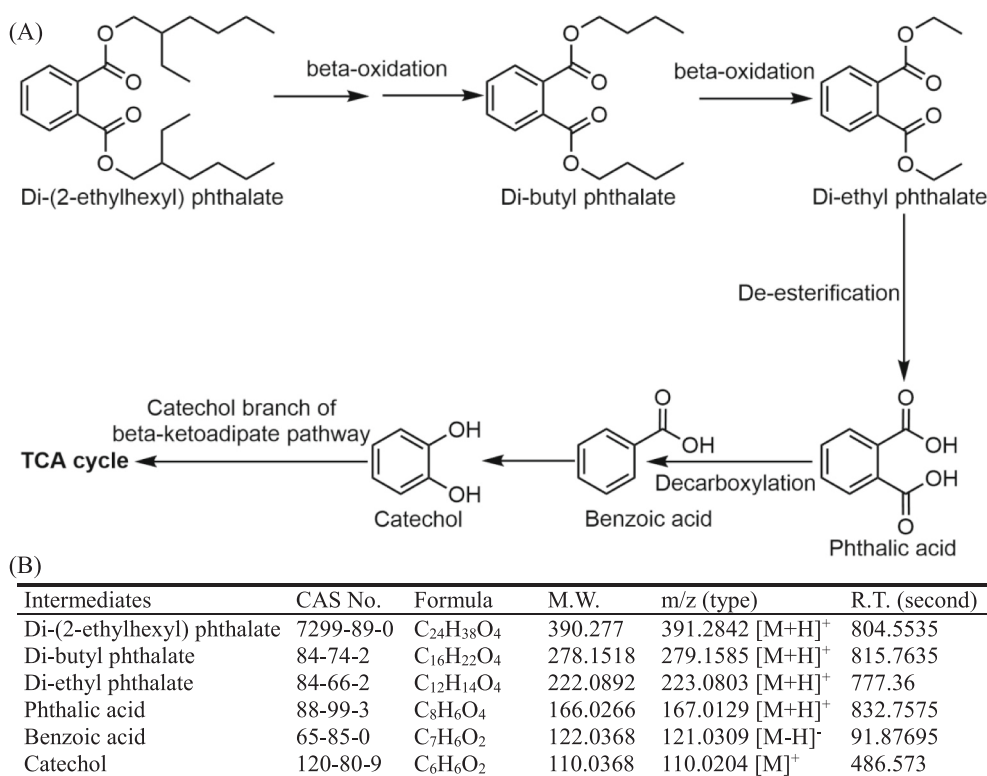


Fig. 4. Metabolic pathway of DEHP in strain RL-LY01 (A) and the identified metabolic intermediates of DEHP in strain RL-LY01 (B). (M.W.: molecular weight, R.T.: retention time).

content of 67.95 %. The circular map of genome was shown in Fig. 5A and 4615 genes were predicted, including 4615 of protein coding genes, 9 of rRNA genes and 48 of tRNA genes. The assembled genome sequence was submitted to Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nih.gov/genome/annotation_prok/) and Rapid Annotation using Subsystem Technology server (<https://rast.nmpdr.org/>) for automatic gene prediction and in-depth annotation. Genes and gene clusters potentially involved in the metabolism of DEHP were shown in Fig. 5B which are in accord with the proposed metabolic pathway of DEHP. Briefly, one hydrolase gene (*mehpH*, P9A14.12890), encoded the esterase involved in the transformation of mono-alkyl phthalates to PA, was identified in the genome of strain RL-LY01. Benzoate was transformed into catechol by BenABC while the utilization of catechol was achieved via CatABCED. Nevertheless, the gene involved in the transformation of PAEs to mono-alkyl phthalates was still unknown.

Lots of studies have demonstrated that microbes preferred to reduce the side-chain length of PAEs prior to de-esterification, such as *Mycobacterium phocaicum* RL-HY01, *Gordonia* sp. Lff, *Pseudomonas* sp. YJB6, and consortium LF (Ren et al., 2021; Feng et al., 2021; Li et al., 2018; Wang et al., 2019a). The advantages of this process have been extensively discussed and can be summarized as bellow: (i) the reduction of side-chain length could extinguish the steric-hindrance effects of long or complex side-chains, (ii) the β -oxidation could make full utilization of side-chains for cell growth, and (iii) the reduction of side-chain length might alleviate the potential toxic effects of sides and their metabolites. Importantly, the recognition of side-chain toxicity was underrepresented while some of these side-chains have been proved to be toxic to environmental livings and be more recalcitrant for biodegradation, such as 2-ethylhexanol (side-chain of DEHP) (Horn et al., 2004; Nalli et al., 2006). Thus, strain RL-LY01 should be recognized as an environment friendly bacterial strain for the bioremediation of PAEs-contaminated environments. The metabolic pathway of PA, the main metabolic intermediates of PAEs, has been systematically investigated which could be transformed into several kinds of certain intermediates like

protocatechuic acid, benzoate, catechol, and gentisate, and further utilized for cell growth via ring-cleavage. In strain RL-LY01, PA was utilized for cell growth via a typical catechol branch of the β -ketoadipate pathway. Additionally, the gene cloning and sequencing suggested that the *mehpH* gene was included in the genome of strain RL-LY01 and the encoded protein showed 100 % identity with known *MehpH* (BAE78500, *Gordonia* sp. P8219) (Nishioka et al., 2006). However, the gene involved in the transformation of di-alkyl phthalates to mono-alkyl phthalates is still unknown and therefore, further investigation is needed.

3.4. Bioremediation of DEHP in different saline soil

As expected, strain RL-LY01 showed good performance in the bioremediation of artificial DEHP-contaminated soil (Fig. 6). In the treatment without inoculation of strain RL-LY01 (both non-sterilized and sterilized soil), DEHP concentration maintaining at a high level was observed except for the non-sterilized mangrove sediment (*P* value shown in Table S8). These results suggested the natural degradation of DEHP was very slow. For the non-sterilized mangrove sediment, approximately 22.2 % of DEHP (100 mg/kg) was degraded within 7 d which indicated that the indigenous microbes in mangrove sediment could contribute to the elimination of DEHP. With the inoculation of strain RL-LY01, the degradation percentages of DEHP in the non-sterilized intertidal sediment, mangrove sediment, saline farmland soil and normal farmland soil were 84.9 %, 96.3 %, 92.2 % and 79.9 % while the degradation percentages of DEHP in the sterilized treatments were 84.0 %, 93.8 %, 82.7 % and 75.2 %, respectively. Although the degradation of DEHP in the same sample of non-sterilized and sterilized treatments showed same tendency, the degradation percentage of DEHP in the non-sterilized treatment is always higher than the sterilized treatment. These results indicated that the indigenous microbes might promote the degradation of DEHP by strain RL-LY01. Further, the degradation percentages of DEHP in the mangrove sediment and saline

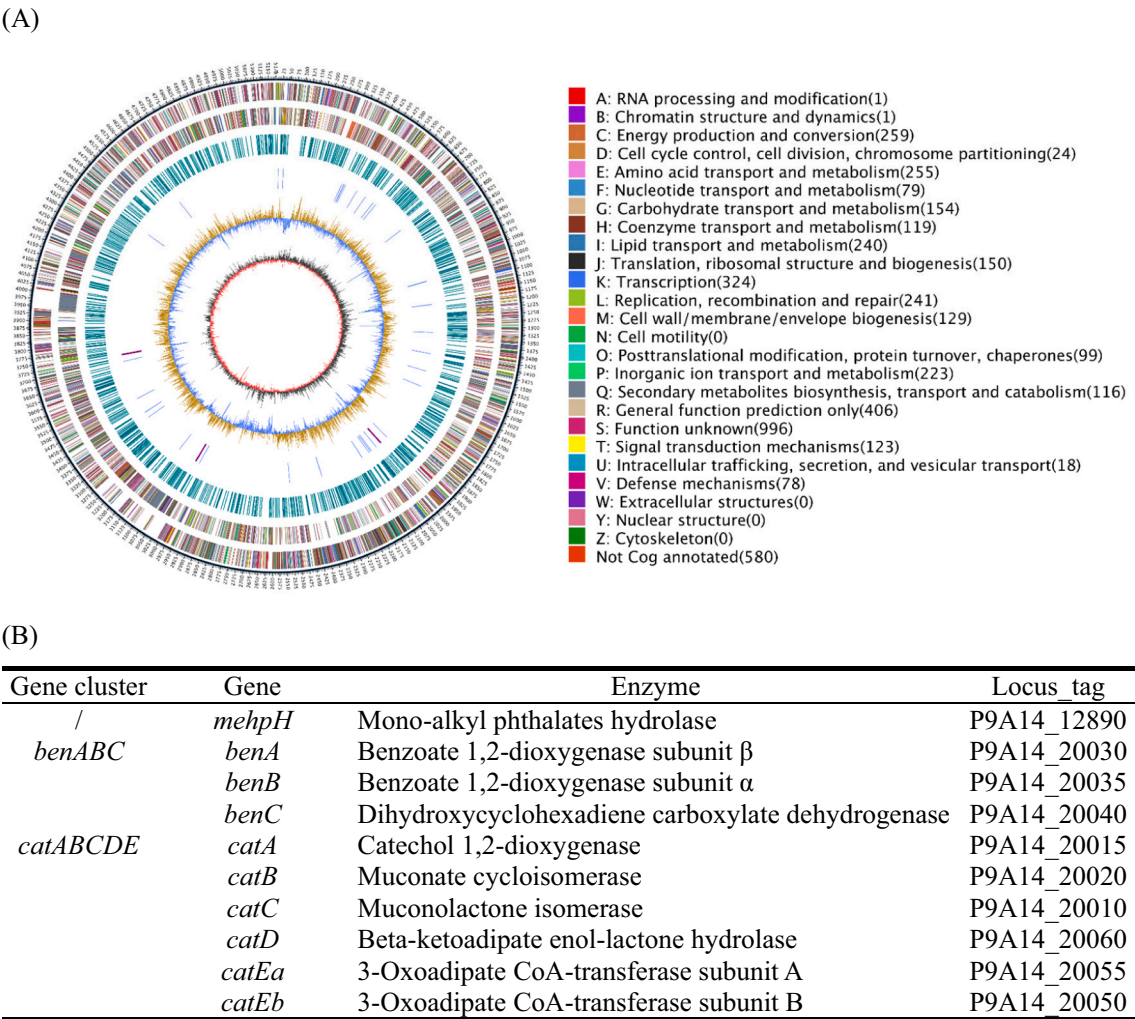


Fig. 5. The circular map of genome (A) and enzymes involved in the metabolism of DEHP (B).

farmland soil are higher than the degradation percentages of DEHP in intertidal sediment. Interestingly, the overall degradation percentages of DEHP in the saline samples are always higher than the non-saline samples (normal farmland soil).

Although it is widely recognized that the degradation of pollutants in diverse environments is mainly mediated by microbes, these processes are affected by the properties of the habitat, such as environmental pH, oxidant, indigenous microbes, and sunlight irradiation. In this study, strain RL-LY01 could rapidly degrade DEHP in different environmental samples as that in the test media. Compared with intertidal zone, the mangrove ecosystem represents the coastal ecosystem with rich nutrition and high microbial diversity and abundance. The intertidal sediment in this study is a typical sandy sample in which the lack of indigenous microbe and nutrition might be major limitations for the elimination of DEHP by strain RL-LY01. Meanwhile, the higher degradation percentages of DEHP in saline soil and sediment demonstrated that strain RL-LY01 is favored to bioremediation in the saline conditions. In all, the performance of strain RL-LY01 in sediment and soil samples indicated that strain RL-LY01 could be a robust candidate for the bioremediation of PAEs-contaminated soil and sediment, especially the saline soil and sediment.

4. Conclusion

Biodegradation and bioremediation of PAEs by microbes have been extensively investigated while application of isolated strains with saline

environmental samples remains underexplored. In the present study, biodegradation of DEHP by *Gordonia hongkongensis* RL-LY01 was investigated by examining the characteristics of strain RL-LY01, degrading kinetics of DEHP, and the metabolic pathway of DEHP. Strain RL-LY01 could utilize a wide range of PAEs for cell growth, including short, long, complex and circular side-chain PAEs. Strain RL-LY01 showed preference to alkaline conditions and good tolerance to salinity and metal ions, and the good adaptability of strain RL-LY01 indicated its great application potential. The kinetics analysis of DEHP degradation by strain RL-LY01 under the optimized conditions indicated that the process was well fitted with the first-order decay model ($R^2 = 0.9985$). The metabolic intermediates of DEHP by strain RL-LY01 were identified by UHPLC-MS and the metabolic pathway was deduced thereafter. DBP and DEP were detected during the degradation of DEHP by strain RL-LY01 which demonstrated that the side-chain length of DEHP were reduced by strain RL-LY01 via β -oxidation. DEP was further hydrolyzed into PA while PA was decarboxylated into BA. BA was transformed into catechol and further utilized for cell growth via the catechol branch of the β -ketoadipate pathway. Further, genes and gene clusters potentially involved in the metabolism of DEHP were identified by genome sequencing and analyzing, and the results were in line with the proposed metabolic pathway of DEHP. In addition, a reported mono-alkyl phthalate hydrolase gene (*mehpH*) was identified in the genome of strain RL-LY01. Finally, the bioremediation of artificial DEHP-contaminated soil and sediment by strain RL-LY01 was conducted and strain RL-LY01 showed high DEHP degradation in the selected samples.

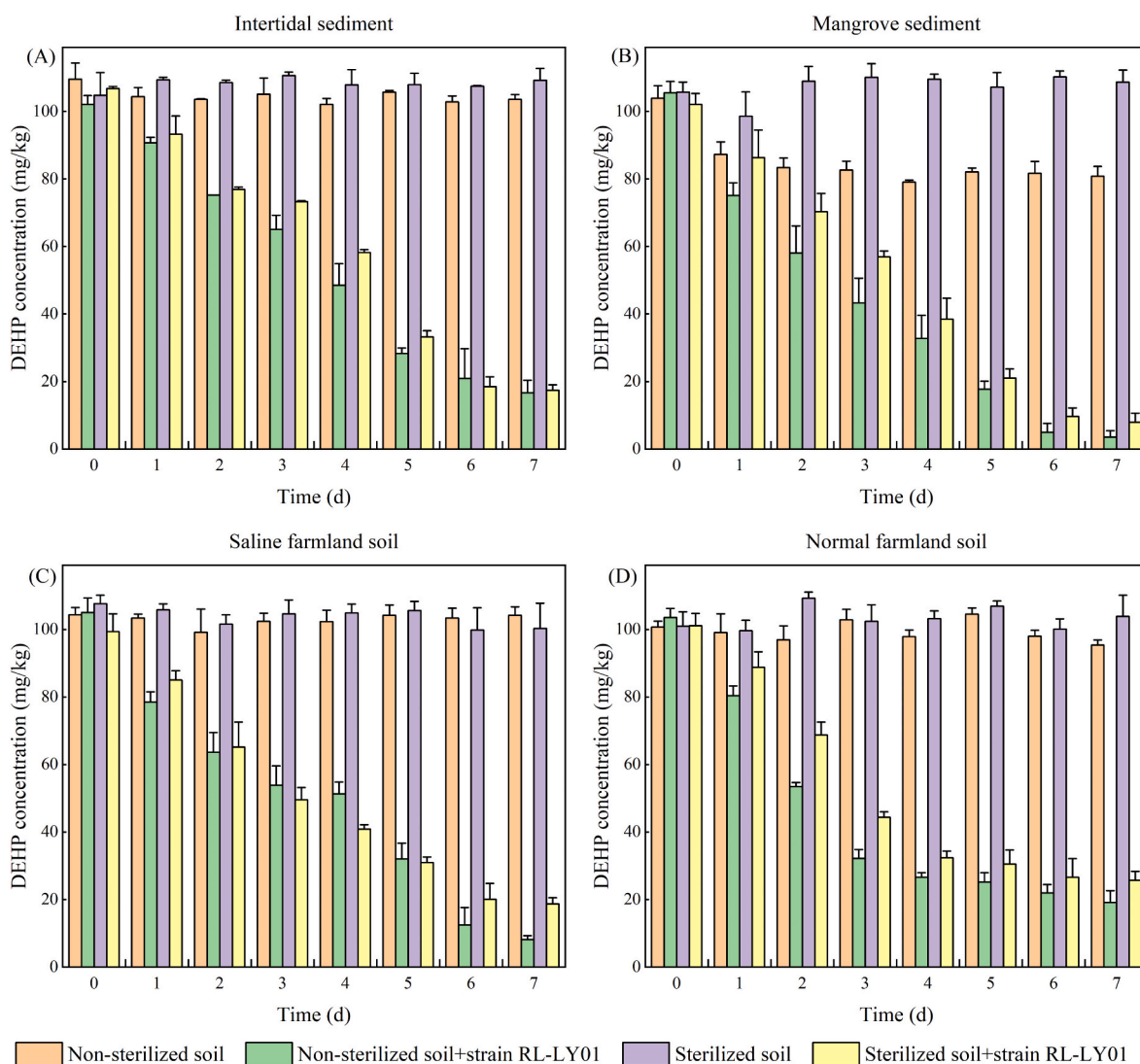


Fig. 6. Bioremediation of artificial DEHP-contaminated soil. (A: intertidal sediment, B: mangrove sediment, C: saline farmland soil, D: normal farmland soil)

Specifically, (i) the maximum degradation percentage of DEHP (100 mg/kg, 96.3 %) by strain RL-LY01 was observed in the mangrove sediment, (ii) indigenous microbes might promote the degradation of DEHP by strain RL-LY01 since the degradation percentages of DEHP in non-sterilized samples were always higher than the sterilized samples, (iii) strain RL-LY01 showed preference to the saline environments since the degradation percentages of DEHP by strain RL-LY01 in saline environmental samples were higher than the degradation percentages of DEHP in normal farmland soil. These results suggested that strain RL-LY01 exhibited robust application potential for the bioremediation of PAEs-contaminated soil and sediment, especially the saline soil and sediment.

CRediT authorship contribution statement

Hanqiao Hu and Yang Jia designed research. Lei Ren and Liyun Weng contributed to experimental work. Danni Chen, John L. Zhou and Yang Jia finished the data analysis. Lei Ren and Hanqiao Hu wrote the manuscript. Lei Ren, John L. Zhou and Yang Jia revised the manuscript. All authors read and approved the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2023.115071>.

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