A novel aerobic denitrifying phosphate-accumulating bacterium efficiently removes phthalic acid ester, total nitrogen and phosphate from municipal wastewater

Lei Ren[a,b,c,1], Zhenzhao Guo[b,1], , Lele Zhang[b], Hanqiao Hu[b,c], Chengyong Li[a,b], Zhong Lin[a,b], Zhen Zhen[a,b,c], John L. Zhou[d]

[a]Shenzhen Institute of Guangdong Ocean University, Shenzhen 518108, China

[b]South China Branch of National Saline-Alkali Tolerant Rice Technology Innovation Center, Zhanjiang 524088, China

[c]College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China

[d]Centre for Green Technology, University of Technology Sydney, 15 Broadway, NSW 2007, Australia

Abstract:

Simultaneous removal of nitrogen, phosphate and emerging pollutants are critical for safe reuse of wastewater, but research in this field is limited. In the present study, a novel aerobic denitrifying phosphate-accumulating bacterial strain RL-GZ01 was found to be able to utilize phthalic acid esters (PAEs) as carbon resource for cell growth. Based on 16S rRNA gene analysis, physiological and biochemical characterization, and genome-based average nucleotide identity calculation, RL-GZ01 was identified as Rhodococcus pyridinivorans. Strain RL-GZ01 showed high DEHP degradation in alkaline conditions and good tolerance of salinity and organic solvents. The degradation of DEHP by RL-GZ01 fitted well with a modified Gompertz model (R^2 = 0.9985). Metabolic intermediates of DEHP were identified via UHPLC-MS/MS analysis and the catabolic pathway was proposed thereafter. Genes and gene clusters contributed to the utilization of DEHP were analyzed through genomic analysis. Analysis of KEGG nitrogen metabolism pathway indicated that nitrate and nitrite were further transformed into ammonium which was further used for the biosynthesis of l-glutamine and L-glutamate. Strain RL-GZ01 was further identified as a denitrifying phosphate accumulating organism which can accumulate phosphate by generating polyphosphate. Finally, strain RL-GZ01 was applied to municipal wastewater treatment for simultaneous removal of nitrogen, phosphate and DEHP. The removal percentages of DEHP (5 mg/L), TN (71.2 mg/L), NH_4^+-N (70.9 mg/L), PO_4^{3-}P (10.89 mg/L) and COD (622.4 mg/L) by strain RL-GZ01 were 89.94 %, 64.45 %, 64.94 %, 76.30 % and 63.23 % within 84 h, respectively. These demonstrated the capability of strain RL-GZ01 for the biological treatment of wastewater containing PAEs.

Keywords: Phthalic acid esters; Biodegradation; Nitrogen removal; Municipal wastewater; *Rhodococcus pyridinivorans*

1. Introduction

Plastic pollution has become a global environmental issue. It was estimated that approximately 348 million tons (Mt) of plastics were produced globally in 2017, and they were mainly used for packaging (39.7%), building and construction (19.8%), and automotive market (10.1%) (PlasticEurope 2018). Since most of these plastic products are used once, large quantities of plastics are discharged as wastes which finally enter aquatic ecosystems. It was estimated that 19 to 23 Mt, or 11%, of plastic waste generated globally in 2016 entered aquatic ecosystems which has been recognized to affect nearly every marine and freshwater ecosystem globally (Borrelle et al., 2020). When plastic wastes are released into environments, UV radiation mediated photo-oxidation makes the plastics become brittle, causing the generation of plastic debris (Wright and Kelly, 2017). Further, with the impact of wind, wave action, abrasion etc., plastic fragments will be degraded into micro-plastics (MPs) (0.1 μm - 5 mm diameter) and potentially nano-plastics (NPs) (≤0.1 μm diameter) particlesand respectively (Banerjee and Shelver, 2021). Recently, wastewater treatment plants (WWTPs) have been proposed as significant sources of MPs and NPs in freshwater and marine environments (Zou et al., 2021). Meanwhile, "Occurrence, fate, detection, and removal of microplastics in wastewater treatment" was listed as the Top 1 of research fronts in field of ecological and environmental sciences in 2020 (http://english.casisd.cn/research/rp/). Although MPs pollution in WWTPs has attracted global concerns and great efforts have been made to remove MPs from wastewater, the elimination of eco-toxic plastic additives from wastewater is still underexplored as important emerging pollutants. Plastic additives like plasticizers and flame retardants would be released from polymers when plastic debris are decomposed into MPs (Hermabessiere et al., 2017; Koelmans et al., 2014).

Plasticizers are widely used as plastic additives which are added to plastic polymers to improve their flexibility and durability (Katsikantami et al., 2016). Since the global consumption of plasticizers in 2020 has already exceeded 10 Mt (data from: https://www.plasticisers.org/plasticisers and https://www.statista.com), the elimination of plasticizers from different environments, including WWTPs, become urgent. Phthalic acid esters (PAEs), as the most widely used plasticizers, account for 55% of global plasticizer market (data from: https://www.ihs.com/products/plasticizers-chemical-economics-handbook.html). Since PAEs are typical external plasticizer, they could be released from polymers during plastic decomposition (Ren et al., 2018). Thus, PAEs are ubiquitously detected in all the environmental compartments and have become one of the most common pollutants in municipal wastewater (Net et al.,

2015). The composition, distribution and concentration of PAEs in WWTPs have been systematically investigated (Table S1). The toxicological evaluation of PAEs on environmental and human health has been extensively conducted and many reports demonstrated that PAEs exhibited carcinogenic, developmental and reproductive toxicity (Mathieu-Denoncourt et al., 2016; Kimber and Dearman, 2010; Mu et al., 2015; Stojanoska et al., 2017). Therefore, six kinds of PAEs have been listed as the priority pollutants by the United States Environmental Protection Agency, the European Union, and China National Environmental Monitoring Center (Xu et al., 2005; EPA, 1992; European, 1993). Consequently, the removal of PAEs from municipal wastewater is critical for safe reuse of wastewater.

Different methods can be employed to eliminate PAEs from wastewater, including chemical, physical, and biological degradation (Pirsaheb et al., 2022; Bai et al., 2020; Nas et al., 2022). Among these methods, bacteria-mediated degradation has been recognized as the most ecological method for PAEs elimination. Lots of PAEs-degrading bacterial strains have been isolated and characterized, and some of these isolates have been applied for the bioremediation of PAEs-contaminated sites (Ren et al., 2018). However, the application of bacterial isolates for the elimination of PAEs from wastewater is rarely reported. Meanwhile, the removal of nitrogen and phosphorous is recognized as the emphasis in wastewater treatment field for eutrophication control. Hence, acquiring denitrifying and phosphorus-accumulating functional microorganisms capable of degrading PAEs is highly advantageous for wastewater treatment.

In the present study, a novel PAEs-degrading bacterial strain RL-GZ01, with capacity of simultaneous removal of nitrogen and phosphorus, was isolated from a municipal wastewater treatment outlet located in the intertidal zone of Zhanjiang Bay, China. Strain RL-GZ01 was identified as *Rhodococcus pyridinivorans* through the analysis of 16S rRNA gene and physiological and biochemical characteristics and the calculation of average nucleotide identity (ANI). Strain RL-GZ01 could utilize several kinds of PAEs as sole carbon source for growth and the effects of different environmental factors on the degradation of di-(2-ethylhexyl) phthalate (DEHP), one kind of PAEs as well as the most widely used plasticizer, were characterized. Further, the metabolic pathway of DEHP in strain RL-GZ01 was proposed via metabolic intermediates identification while the genes and gene clusters involved in the metabolism of DEHP and removal of nitrogen and phosphorus were identified through whole genome sequencing and analysis. Eventually, strain RL-GZ01 was applied to municipal wastewater for the simultaneous removal of DEHP, nitrogen and phosphorus to evaluate its application potential. To our

knowledge, strain RL-GZ01 is the first reported bacterial strain capable of simultaneously removing PAEs, nitrogen and phosphorus from municipal wastewater.

2. Materials and methods

2.1 Chemicals and media

PAEs, including DEHP, di-cyclohexyl phthalate (DCHP) and di-octyl phthalate (DNOP), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), di-ethyl phthalate (DEP) and di-methyl phthalate (DMP), were purchased from J&K Scientific (Beijing, China). The stock solution of PAE was prepared in menthol with a final concentration of 2×10^4 mg/L. Enzymes and biological kits were purchased from Takara (Japan) while chemical reagents were obtained from Sinopharm Chemical Reagent (Shanghai, China). The detailed information of all reagents was presented in Table S2. According to previous works (Rout et al., 2017; Ren et al., 2021; Ke et al., 2022), appropriate media for the isolation of DEHP-degrading strain and the determination of nitrogen and phosphorous removal capacity were presented in Table S3. The pH of media was adjusted to the target range with HCl (2 mol/L) or NaOH (2 mol/L). Agar with a final concentration of 15 g/L was added into the medium to obtain solid medium. Finally, all media were autoclaved under 121 °C (30 min) for sterilization.

2.2 Isolation and identification of DEHP-degrading strain

Sediment samples were collected from a municipal wastewater treatment outlet located in the intertidal zone of Zhanjiang Bay, China. The characteristics of sediment were presented in Table S4. Approximately 5 g of sediment sample was added into 50 mL mineral salt medium (MSM) while DEHP was supplemented as sole carbon source with a final concentration of 100 mg/L. Culture was incubated in a constant shaker under 30°C (180 rpm) for 5 d. Then, 1 mL of culture was transferred into 9 mL fresh MSM and the concentration DEHP was increased to 200 mg/L. The incubation and inoculation were repeated until the concentration of DEHP reached 500 mg/L.An aliquot of 1 mL culture was applied to centrifugation (6000 rpm, 5 min) and the cell pellets were washed with phosphate buffer solution (PBS, 0.2 M, pH 7.2). The centrifugation and washing were repeated for three times and the cells were resuspended in 1 mL of fresh MSM liquid medium. Then, the cell suspension was spread on solid MSM medium supplied with 500 mg/L of DEHP and 0.01 g/L of Tween 80. All plates were incubated at 30°C and checked every day. Well grown colonies with hydrolytic halos were selected for the confirmation of DEHP-degrading capacity. Briefly, (a) single colony of target strain was inoculated into fresh Luria-

Bertani (LB) liquid medium and incubated in a constant shaker (180 rpm) at 30°C for 24 h, (b) an aliquot of 1 mL culture was centrifugated (6000 rpm, 5 min) to collect the cell pellets, (c) cell pellets were washed by PBS (0.2 M, pH 7.2) and re-centrifugated (6000 rpm, 5 min) to harvest the cell pellets again, (d) the centrifugation and washing were repeated for three times, (e) the cell pellets were finally resuspended in 1 mL of fresh MSM liquid medium and inoculated into 9 mL of MSM liquid medium supplied with 100 mg/L of DEHP as sole carbon source, (f) same culture (10 mL of MSM, 100 mg/L of DEHP) was set as abiotic control treatment, (g) all cultures were incubated in a constant shaker (180 rpm) at 30°C while the residual concentration of DEHP was quantified with a gas chromatograph (GC) after 5 days' incubation. Steps (a) to (g) were repeated until stable DEHP-degrading strain was isolated. Meanwhile, the nitrogen and phosphorous removal capacity of isolated DEHP-degrading strains was verified with nitrification medium (NM, 100 mg/L of NH_4^+-N), denitrification medium (DM, 100 mg/L of NO_3^--N or NO_2^--N) or phosphate uptake medium (PUM, 20 mg/L of PO_4^{3-}-P), separately. Finally, isolated strains capable of simultaneously eliminating of DEHP, nitrogen and phosphorous were selected for further investigation.

2.3 Identification of isolated strain

The identification of isolated strains was accomplished via the analysis of 16S rRNA gene and physiological and biochemical characteristics, and the calculation of average nucleotide identity (ANI). Firstly, the 16S rRNA gene of target strain was amplified from the genome via polymerase chain reaction (PCR) with universal primers of 27F and 1492R. The PCR products were purified and inserted into the p-MD19T vector. The positive inserts were sequenced by GENEWIZ (Guangzhou, China). The obtained sequences were searched against reported sequences via the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and then the 16S rRNA genes of related type strains were obtained from the List of Prokaryotic names with Standing in Nomenclature (LPSN, http://www.bacterio.net/) for the construction of phylogenetic tree (Parte, 2018). Secondly, the physiological and biochemical characteristics of isolated strain were further determined as referenced in Bergey's Manual of Determinative Bacteriology (George et al., 2001). Thirdly, the complete genome of isolated strain was sequenced by Biomarker Technologies Corporation (Beijing, China) and the genome sequence was applied for genome-based identification of target strain via average nucleotide identity (ANI) analysis. The ANI analysis was accomplished with ANI calculator provided by Kostas lab (http://enve-omics.ce.gatech.edu/ani/index).

2.4 Characterization of isolated strain

Prior to experimental assays, the seeds for inoculation were prepared in LB medium. Single colony of target strain was inoculated in 10 mL of fresh LB medium and incubated in a constant shaker (180 rpm, 30°C) for 24 h. An aliquot of 2 mL culture was applied to centrifugation (6000 rpm, 5 min) and the cell pellets were washed by PBS buffer (0.2 M, pH 7.2). The centrifugation and washing were repeated for three times. The cells were resuspended in fresh MSM medium and the OD_{600} value of the suspension was adjusted to 0.8 (~5.6×10^7 cell/mL). The obtained cell suspension was used as inoculants and the inoculation ratio was maintained at 1.0% (*v*/*v*) unless stated otherwise.

The substrate profile assay of isolated strain was conducted to determine its catabolic capacity of different PAEs. BBP was selected as representative of complex side-chain PAE, DCHP was selected as representative of cyclic side-chain PAE, DNOP and DEHP were selected as representatives of long side-chain PAEs, and DBP and DEP were selected as representatives of short side-chain PAEs. The assays were performed in 50 mL Erlenmeyer's flasks containing 10 mL of MSM. One kind of PAE was individually added into the medium with a final concentration of 100 mg/L and the seeds of target strain were inoculated as described above. Same medium (MSM, 100 mg/L of PAE) was set as abiotic control and all the cultures were incubated in a constant shaker (180 rpm, 30°C). Environmental factors affecting DEHP degradation in isolated strain were characterized via single-factor assays, including the initial pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0), temperature (10°C, 20°C, 30°C, 40°C, and 50°C), salinities (4.0%, 6.0%, 8.0%, and 10.0%, *w*/*v*), and organic solvents (*p*-xylene, toluene, ethyl acetate, and biphenyl, 500 mg/L of each). Same cultures without inoculation were set as abiotic control. Thereafter, the degradation of DEHP was determined under the optimized condition and the kinetics of DEHP degradation by isolated strain was simulated with a first-order decay model (Eq. (1)) and a modified Gompertz model (Eq. (2)) (Zhang et al., 2020).

The first−order decay model: $S = S_0 + A\cdot\exp(-t/t_1)$ (1)

The modified Gompertz model: $S = S_0 - A\cdot\exp\left\{-\exp\left[\frac{Vm\cdot e}{A}\cdot(L-t)+1\right]\right\}$ (2)

where *S* represents the substrate concentration; S_0 represents the initial substrate concentration; *A* is the biodegradation potential; *Vm* means the maximum biodegradation rate; and *L* represents the lag phase.

All cultures above were incubated under constant shaking (180 rpm, 30°C). Samples were collected at a time interval of 6 h and the residual DEHP concentration was determined by gas chromatography (GC, maker? Model?). All assays were conducted in triplicate.

2.5 Metabolites and metabolic pathways of DEHP

The metabolic intermediates of DEHP in isolated strain were identified via ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS, maker, model) analysis and then the metabolic pathway of DEHP was deduced. Seeds were inoculated into fresh MSM liquid medium supplemented with 100 mg/L of DEHP as sole carbon source while samples were collected every 4 h. For the collected cultures, equal volume of *n*-hexane was added and then mixed with a vortex for 30 s. After that, the mixture was fully extracted by ultrasound with a working frequency of 45 kHz (10 min) on ice-water bath. The organic phase was collected while the residual aqueous phase was adjusted to pH 3.0 with HCl (2.0 M) and applied to extraction again. The extracts were merged, evaporated, redissolved in methanol and subsequently applied for metabolites identification. Finally, the metabolic pathways of DEHP in the isolated strain were proposed according to the identification of metabolites.

2.6 Identification of enzymes in the removal of DEHP, nitrogen and phosphorus

To have an insight into the molecular mechanisms of DEHP degradation, nitrogen and phosphorus removal in the isolated strain, whole genome sequencing and analysis were conducted. Seeds of target strain were inoculated in fresh LB liquid medium and incubated in a constant shaker (180 rpm, 30 °C) for 24 h. Cells were harvested by centrifugation (6000 rpm, 5 min) and washed by PBS buffer (0.2 M, pH 7.2). The washing and centrifugation were repeated for three times and the obtained cell pellets were applied to genome extraction with a Bacterial Genomic DNA Extraction kit (Takara, Japan). DNA integrity, quality and concentration were determined by agarose gel electrophoresis, a Qubit fluorometer (Thermo, USA) and a NanoDrop 1000 spectrophotometer (Thermo, USA), respectively. The complete genome sequencing of strain RL-GZ01 was accomplished via Oxford Nanopore Technology (ONT) coupled with highly accurate Illumina sequencing (Biomarker Technologies Corporation, Beijing, China). The assembled genome and plasmid sequences were submitted to Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nih.gov/genome/annotation_prok/) for automatic gene prediction and in-depth annotation. In addition, the predicted coding sequences (CDSs) were searched against the Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups Database (eggNOG, http://eggnog.embl.de) and the Kyoto Encyclopedia of Genes and Genomes database (KEGG,

http://www.genome.jp/kegg/) to analyze the gene functions and metabolic pathways, respectively. The circular representation of genome and plasmids was generated by circos (version 0.69) (Krzywinski et al., 2009). Genes and gene clusters potentially involved in the degradation of DEHP and removal of nitrogen and phosphorus were manually checked and the related molecular mechanisms were summarized.

2.7 Simultaneous removal of nitrogen and phthalic acid esters from municipal wastewater

Wastewater was collected from Xiashan Municipal Wastewater Plant (Zhanjiang, China) and the detailed characteristics of wastewater was presented in Table S4. DEHP was added into 10 mL wastewater in a 100 mL flask to generate a final concentration of 5 mg/L. The seeds of isolated strain were prepared and inoculated as described above, and the same wastewater (containing 5 mg/L of DEHP) without inoculation was selected as control treatment. All these flasks were incubated in a constant shaker (180 rpm, 30 °C) and the samples were collected at a time interval of 4 h. The residual concentration of DEHP, NH_4^+-N, NO_3^--N, NO_2^--N, total nitrogen (TN), PO_4^{3-}-P, and chemical oxygen demand (COD) were determined.

2.8 Analytic methods

Cell growth of isolated strain was assessed by detecting the OD_{600} values with a spectrophotometer (P4PC, MAPADA, Shanghai, China) while the cell density was quantified by the dilution plate technique and presented as colony-forming units (CFUs). Depending on the standard methods, the concentration of COD, NH_4^+-N, NO_3^--N, NO_2^--N, TN and TP were measured by the potassium dichromate method, Nessler's reagent spectrophotometry, the ultraviolet spectrophotometric method, α-naphthylamine spectrophotometry, and antimony molybdenum blue spectrophotometry, respectively (APHA, 2017).

The nitrogen and phosphorus removal percentage and removal rate were calculated with Eq. (3) and Eq. (4) as below:

$$\text{Removal percentage (\%)} = (S_0 - S_t)/S_0 \times 100\% \quad (3)$$

$$\text{Removal rate (mg/L/h)} = (S_0 - S_t)/t \quad (4)$$

where S_0 and S_t are the concentrations, at the beginning and time t, of TN, NH_4^+-N, NO_3^--N, NO_2^--N or PO_4^{3-}-P, respectively.

The quantification of PAEs was performed with a GC system (GC-2010 pro, SHIMADZU, Japan) equipped with a WondaCap 5 column (GL Sciences Inc., Japan, 30 m × 0.25 mm × 0.25 μm) and electron capture detector (ECD) according to (Zhang et al., 2020). The recovery rates of PAEs were all above

97.0% (average = 98.3%, max. = 99.3%, min. = 97.4%). The standard curves of selected PAEs were established and presented in Table S5. The identification of DEHP metabolites was accomplished with a UHPLC-MS/MS platform in Bionovogene Co. Ltd. (Suzhou, China) as described by Ren et al. (2021). The degradation percentage of pollutants were calculated with Eq. (3):

2.9 Accession numbers

Strain RL-GZ01 has been deposited in Guangdong Microbial Culture Collection Center (GDMCC) under accession number 62401. The 16S rRNA gene of strain RL-GZ01 has been deposited in GenBank under accession number MK787328. Genome information for the chromosome and plasmids of strain RL-GZ01 were deposited in GenBank under the accession numbers CP013297, CP013298 and CP013299, respectively.

3.Rusult and discussion

3.1 Isolation and identification of DEHP-degrading bacterial strain RL-GZ01

One bacterial strain (RL-GZ01) capable of utilizing DEHP as sole carbon source for growth was isolated from sediment samples. Strain RL-GZ01 could completely degrade 100 mg/L of DEHP in 24 h with a significant increase of OD_{600} value (Fig. 1A). However, the cell growth was relatively lagging behind the degradation of DEHP. Meanwhile, the nitrogen removal performance of strain RL-GZ01 was presented in Fig. 1. When $(NH_4)_2SO_4$ was supplied as the sole nitrogen source, no obvious lag phase of cell growth was observed and 95.6% of NH_4^+-N was reduced with a maximum removal rate of 2.85 mg/L/h between 24 h and 36h (Fig. 1B). With a short time (12 h) lag phase, NO_3^--N and NO_2^--N were rapidly removed between 24 h and 48 h and the removal percentages were 93.8% and 94.4% within 72 h, respectively (Fig. 1C and 1D). Phosphate could be rapidly removed by strain RL-GZ01 with 91.5% removal (84 h) and a maximum removal rate of 0.43 mg/L/h (between 48 h and 60 h). The colony morphology of strain RL-GZ01 on the LB solid plate was round, orange-colored, sheeny, opaque, prominent, smooth on the surface and with regular edges (Fig. 2A). The SEM image showed that strain RL-GZ01 was rod-shaped without flagellum (Fig. 2B). The 16S rRNA gene sequence of strain RL-GZ01 was amplified and sequenced with a length of 1361 bp. The phylogenetic analysis of 16S rRNA gene indicated that strain RL-GZ01 has the closest relationship with the species of *Rhodococcus pyridinovorans* (Fig. 2C). The physiological and biochemical characteristics of strain RL-GZ01 were presented in Table 1. The ANI value between the genome of strain RL-GZ01 and the reference genome

Rhodococcus pyridinivorans (NC_023150) is 98.68% (Fig. S1). Finally, strain RL-GZ01 was identified as *Rhodococcus pyridinivorans* according to 16S rRNA gene analysis, physiological and biochemical characterization and ANI analysis. The genus *Rhodococcus* is a phylogenetically and catabolically diverse group that are frequent components of microbial communities in diverse natural environments, including polar and alpine regions (Kim et al., 2018). Numerous strains of *Rhodococcus* spp. were isolated for their versatile ability to degrade a wide variety of natural and synthetic organic compounds (Kim et al., 2018). Species *Rhodococcus pyridinivorans* is known as pyridine-degrading bacterium and several xenobiotics degrading strains of *Rhodococcus pyridinivorans* have been reported (Table 2). Among these strains, *Rhodococcus pyridinivorans* XB and *Rhodococcus pyridinovorans* DNHP-S2 were identified as PAEs-degrading isolates (Wang et al., 2022; Zhao et al., 2018). Although *Rhodococcus pyridinovorans* has been recognized as robust xenobiotics degrader, its ability to simultaneously remove emerging pollutants and N/P nutrients is rarely reported as well as its application in wastewater treatment.

3.2 Characterization of strain RL-GZ01

PAEs consist of dozens of compounds and are distinguished by the side chains. The substrate profile assay indicated that strain RL-GZ01 could completely degrade all the selected PAEs within 24 h (Fig. 3A), including typical short side-chain PAEs (DEP and DBP), long side-chain PAEs (DNOP and DEHP), complex side-chain PAEs (BBP), and circular side-chain (DCHP). The results also suggested that the steric effect of side chains might affect the degradation efficiency of PAEs since the complete degradation of DEP is faster than the others and the process was accomplished in 12 h. Strain RL-GZ01 could degrade DEHP under a wide range of pH, although the results indicated that strain RL-GZ01 preferred the alkaline environments (Fig. 3B). As shown in Fig. 3B, 100 mg/L of DEHP could be completely degraded within 18 h under pH 8.0 which was therefore the optimal pH. The degradation of DEHP was significantly inhibited under pH 4.0 and 5.0 while 100 mg/L of DEHP could be completely degraded from pH 9.0 to 10.0 within 72 h. The effects of incubation temperature on the degradation of DEHP by strain RL-GZ01 were presented in Fig. 3C and the results suggested that too low (10°C) or too high (50°C) temperature was unfavorable for DEHP degradation. The optimal incubation temperature is 30°C since 100 mg/L of DEHP could be completely degraded within 24 h under 30°C while it took 60 h and 72 h for 20°C and 40°C, respectively. Further, strain RL-GZ01 showed good tolerance of environmental salinity (Fig. 3D). When the salinity was increased to 4.0%, no significant inhibiting effects was observed and 100 mg/L of DEHP was completely degraded by strain RL-GZ01 within 36 h. Although the degradation of DEHP was

slowdown under salinities 6.0% and 8.0%, the complete degradation of 100 mg/L of DEHP was still accomplished within 72 h. This is probably because strain RL-GZ01 was isolated from intertidal sediments of wwhere bacteria are acclimatized to saline environment. The abilities of strain RL-GZ01 to degrade DEHP under the stress of toxic organic solvents were determined (Fig. 3E). Although the selected organic solvents could inhibit the degradation of DEHP to some content, 100 mg/L of DEHP could be completely degraded for all treatments within 72 h. Specifically, limited effects of ethyl acetate and toluene were observed since 100 mg/L of DEHP was completely removed within 48 h while it took a longer time for the treatments of *p*-xylene (60 h) and biphenyl (72 h). Finally, the degradation of DEHP under pH 8.0 and 30°C was fitted with the modified Gompertz model and the first-order decay model. The results revealed that the degradation of DEHP by strain RL-GZ01 followed the modified Gompertz model with a higher correlation coefficient (R^2 = 0.9985) than the first-order decay model (R^2 = 0.8689). The best-fitted curve for DEHP degradation by strain RL-GZ01 using the modified Gompertz model was presented in Fig. 3F. The fitted curves and related parameters for the first-order decay model were shown in Fig. S2. The kinetics analysis demonstrated that strain RL-GZ01 could degrade DEHP with a maximum biodegradation rate of 28.21 mg/L/h and a lag phase of 5.34 h.

It is widely accepted that the isolation of novel xenobiotics degrading bacteria is significant for the bioremediation of polluted sites and wastewater treatment. In addition, the application potential of isolated strains is mainly depended on their degrading efficiency and environmental adaptability. The capability of degrading different PAEs is highly important for PAEs-degrading isolates since a range of PAEs co-exist in the real environment. Although strain RL-GZ01 could efficiently degrade different types of PAEs, the results also suggested that the side-chains in PAEs could hinder the degradation efficiency which is consist with known studies (Wu et al., 2010; Zhang et al., 2020), and the steric effect of long or complex side chain might be the reason (Ren et al., 2018). For the most reported PAE-degrading strains, a preference for neutral or alkaline environments was shown, such as *Gordonia alkanivorans* strain YC-RL2, *Arthrobacter* sp. ZH2 and *Rhodococcus ruber* YC-YT1 (Wang et al., 2012; Nahurira et al., 2017; Yang et al., 2018). Strain RL-GZ01 showed good tolerance to high salinity and could efficiently degrade DEHP with a salinity ranging from 2.0% to 8.0%. And the degradation of DEHP was completely inhibited until the salinity was increased to 10.0%. Several salt tolerant PAEs-degrading been reported and they were mainly isolated from salinity environments, such as marine plastic debris, intertidal and mangrove sediments (Ren et al., 2021; Yang et al., 2018). The characteristics of salt tolerance is unique and critical

for the bioremediation of saline wastewater and sediments. The tolerance of solvents has also been recognized as important characteristics microbial-based remediation, especially for the elimination of hydrophobic compounds (Wang et al., 2021; Navacharoen and Vangnai, 2011). The solvent tolerant mechanisms of strain RL-GZ01 might be illustrated in our future works. Kinetics analysis has been extensively used to simulate the degrading process and evaluate the degradation efficiencies of different isolate (Zhang et al., 2020; Cao et al., 2019; Hasan et al., 2012; Jin et al., 2012; Lin and Yuan, 2005; Zeng et al., 2004). For the most studies of degradation kinetics, the modified Gompertz model and the first-order decay model were widely used while the modified Gompertz model was found to be more applicable to the degradation with lag phase (Ren et al., 2021; Cao et al., 2019; Zhang et al., 2020; Zhang et al., 2020). In the present study, the degradation of DEHP by strain RL-GZ01 was better fitted with the modified Gompertz model than by the first order kinetics model.

3.3 Metabolic pathway of DEHP in strain RL-GZ01

The metabolic pathway of DEHP in strain RL-GZ01 was deduced via intermediates identification and presented in Fig. 4 and the detailed information of metabolic intermediates was listed in Table S6. Apart from the parent compound DEHP, DBP (retention time (RT), 9.873 min; m/z, 279.1591, $[M+H]^+$), DEP (RT, 7.962 min; m/z, 223.1416, $[M+H]^+$), phthalic acid (RT, 5.328 min; m/z, 165.0544 $[M-H]^-$), benzoate (RT, 6.375 min; 121.1213, $[M-H]^-$), and catechol (RT, 4.116 min; 109.1157, $[M-H]^-$) were identified as the metabolic intermediates of DEHP. All these compounds were undetectable after 72 h incubation, which suggested that they were completely utilized by strain RL-GZ01. According to the identification of metabolic intermediates, we found that strain RL-GZ01 could transform the long side-chain PAE (DEHP) into short side-chain PAE (DEP) prior to ester bond hydrolyzation. The reduction of side-chain in DEHP was always accomplished by β-oxidation while the transformation of DEP to PA was achieved through step-by-step de-esterification. Further, PA was transformed into catechol via benzoate while catechol was exploited for cell growth via the catechol branch of the β-ketoadipate pathway.

The metabolic pathways of PAEs have been systematically investigated and the process can be divided into two main steps: (i) transformation of PAEs into PA, and (ii) utilization of PA (Ren et al., 2018). As to the transformation of PAEs into PA, some strains were capable of hydrolyzing the ester bonds directly and generating PA thereafter, such as *Mycobacterium* sp. YC-RL4, *Gordonia terrae* RL-JC02, and *Halomonas* sp. ATBC28 (Wright et al., 2020; Ren et al., 2016; Zhang et al., 2020). Some other strains could reduce the side-chain's length prior to ester bond hydrolyzation, for example, DBP to DEP

(β-oxidation), DEP to ethyl methyl phthalate (EMP) (trans-esterification), and DEP to DMP (demethylation) (Amir et al., 2005). Although most of these strains are capable of transforming PAEs into PA, some of these are incapable of utilizing PA for growth, such as *Gordonia* sp. JDC-2 and *Camelimonas* sp. M11 (Chen et al., 2015; Wu et al., 2010). PA is the main metabolic intermediates of PAEs degradation, which could be further utilized through ring-cleavage. The catabolic pathway of PA has been extensively investigated while PA might be transformed into some certain intermediates like PCA, benzoate, catechol, and gentisate. And then, these intermediates could be utilized for cell growth via specific metabolic pathway. Particularly, the reduction of side-chain length should be significant for PAEs degradation even though the related studies were still limited. Two reasons for this viewpoint: (i) the reduction of side-chain length could extinguish the steric-hinerance effects of long or complex side-chains and make full utilization of side-chains for growth at the same time; (ii) the recognition of side-chain toxicity was underrepresented while some of these side-chains have been proved to exhibit acute toxicity to environmental organisms and be more recalcitrant for biodegradation, such as 2-ethylhexanol (side-chain of DEHP) (Nalli et al., 2006; Horn et al., 2004). Thus, strain RL-GZ01 should be recognized as an environment friendly bacterial strain for bioremediation and wastewater treatment.

3.4 Molecular mechanisms of DEHP metabolism, nitrogen and phosphorus removal

The genome of strain RL-GZ01 comprises a circular chromosome (4,948,512 bp, G+C content = 68.04%), one linear plasmid (pGZ01, 236,455 bp, G+C content = 64.98%) and two circular plasmids (pGZ02, 100,921 bp, G+C content=63.92%; pGZ03, 87,329 bp, G+C content= 65.48%). In total, 5,081 genes have been predicted in the chromosome and plasmids of strain RL-GZ01. Among these genes, 4,988 of protein coding genes (CDSs) and 93 of RNA genes (12 rRNA genes, 55 tRNA genes, and 26 ncRNA genes) were predicted. Functional categorization of CDSs were performed with eggNOG database and the results shown that 4,235 (84.9%) of the predicted CDSs were assigned to the eggNOG function classification (Fig. S3). The top three classifications with known function were transcription (357, 8.33%), energy production and conversion (284, 6.62%), and replication, recombination and repair (269, 6.27%). The alignment of predicted CDSs with KEGG database revealed that 1,973 of genes were involved in 112 pathways. The KEGG nitrogen metabolism pathway analysis demonstrated that ammonium was removed by assimilation while nitrate and nitrite were removed by ammonification and assimilation (Fig. 4).

The genes and gene clusters involved in the metabolism of DEHP, nitrogen and phosphorus are

listed in Table 3. The extracellular nitrate and nitrite could be transferred into the cells with the assistance of nitrate and nitrite transporter (NarT) while ammonium was transferred by ammonium transporter (Amt). Nitrate was transformed into nitrite by respiratory nitrite reductase (NarGHIJ) while nitrite was further transformed into ammonium through nitrite reductase (NirBD). Ammonium was used for the biosynthesis of L-glutamine and L-glutamate via glutamine synthetase and glutamate synthase, respectively. In addition, *cat* gene cluster, contributed to the catechol branch of β-ketoadipate pathway, was identified according to KEGG annotation. As to the hydrolyzation of ester bonds of PAEs, a mono-alkyl phthalates hydrolase gene, *mehpH*, was identified through local BLAST while the gene involved in the transformation of PAEs to mono-alkyl phthalates (MAPs) was still unknown. For the removal of phosphorus, genes involved in the sensing and accumulating of environmental inorganic phosphate were identified via genome annotation and local BLAST analysis. The Pho regulon integrates the sensing of environmental inorganic phosphate (Pi) availability with coregulation of gene expression, mediating an adaptive response to Pi concentration (Allenby et al., 2005; Monds et al., 2006). Phosphate transport system regulatory protein (PhoU) could regulate the transport of extracellular phosphorus into cells while the transportation was mediated by a low-affinity inorganic phosphate transporter (LAT). Two poly phosphate kinase (PPK and PPK2) enzymes and one exopolyphosphatase (PPX) were identified via genome annotation. PPK could catalyze the reversible transfer of the terminal phosphate of ATP to form a long-chain polyphosphate (poly-P) while PPX could catalyze the liberation of phosphate from polyphosphate (Li et al., 2021; Andreeva et al., 2019). Polyphosphate glucokinase (PPGK) could catalyze the phosphorylation of glucose using poly-P as the phosphoryl donor. The phosphorylation of glucose plays a significant role in the creation of intermediates for oligosaccharide synthesis.

Biological nitrogen removal has been recognized as the most efficient and economical approach for nitrogen removal. Great efforts have been made on the isolation of functional microorganisms, investigation of related mechanisms and application in wastewater treatment. Investigations mainly focused on ammonium removal through heterotrophic nitrification, by which ammonium can be oxidized to nitrate via nitrite. Nitrate could supply as the electronic receptor and generate nitrogen-containing gas via aerobic denitrification pathways (Chen et al., 2016). Although nitrogen is one of the crucial factors for cell growth, the investigation of nitrogen removal via ammonium assimilation is still underrepresented while some recent works have demonstrated that heterotrophic ammonium assimilation should be considered as an important driving force for aerobic denitrification. Meanwhile, study has

shown that α-ketoglutarate produced by carbon source metabolism is an important substrate for glutamate biosynthesis since α-ketoglutarate provides a carbon skeleton for microbial ammonium assimilation (Meng et al., 2016). For the simultaneous removal of DEHP and nitrogen by strain RL-GZ01, carbon source provided by DEHP could be supplied as carbon skeleton and used for the biosynthesis of glutamate with ammonium. Furthermore, it has been reported that some heterotrophic denitrifiers can store polyphosphate under either aerobic/anoxic conditions. These microbes are termed as denitrifying polyphosphate accumulating organisms (dPAOs) and are capable of deriving energy from oxidation of external carbon sources unlike PAOs (Li et al., 2015; Zhou et al., 2010). Hence, strain RL-GZ01 can be defined as a typical dPAO which could perform simultaneous nitrogen and phosphorus removal by the utilization of external carbon sources (DEHP and other organic compounds), consequently facilitating COD/BOD removal and DEHP elimination as well. Owing to its ability to simultaneously remove PAEs, nitrogen and phosphorus, strain RL-GZ01 has great application potential in the treatment of PAEs containing wastewater.

3.5 Simultaneous removal of nitrogen and DEHP from municipal wastewater

To evaluate the potential of strain RL-GZ01 in wastewater treatment, the simultaneous removal of nitrogen and DEHP from municipal wastewater was conducted (Fig. 5). As shown in Table S4, the total nitrogen content (74.51 mg/L) in municipal wastewater was mainly due to NH_4^+-N (70.91 mg/L) while the COD value of wastewater was 622.4 mg/L. The concentration of DEHP in original wastewater was detected as 0.13 mg/L and additional DEHP was supplemented with a final concentration of 5.17 mg/L. After 84 h of treatment, the residual concentrations of TN and NH_4^+-N were 50.31 mg/L and 49.77 mg/L in the wastewater of control treatment. When strain RL-GZ01 was inoculated into the wastewater, the residual concentrations of TN and NH_4^+-N were reduced to 4.40 mg/L and 3.72 mg/L, respectively. The COD value showed similar tendency with TN and NH_4^+-N. Meanwhile, after inoculation of strain RL-GZ01, 5.17 mg/L of DEHP in the wastewater was completely degraded while the residual concentration of DEHP in the control treatment was 4.65 mg/L after 36 h. Since the concentration of NO_3^--N and NO_2^--N were very low, their contribution to TN and COD was limited and therefore insignificant. Thus, the total removal percentages of DEHP, TN, NH_4^+-N, phosphorus, and COD in municipal wastewater were 100%, 93.82%, 94.75%, 76.3%, and 83.77% while the strain RL-GZ01 accounted for 89.94%, 64.45%, 64.94%, 76.3%, and 63.23%, respectively. These results also indicated that some indigenous microbes in municipal wastewater might be involved in the removal of DEHP and nitrogen. Overall, strain RL-

GZ01 exhibited excellent DEHP, nitrogen and phosphorus removal ability in the treatment of DEHP-containing municipal wastewater as that in the test media.

To improve existing wastewater treatment systems, the focus is to develop biological treatment processes for the concurrent removal of conventional pollution (essentially nitrogen and phosphorus) and emerging pollutants including microplastics. The majority of the existing biological wastewater treatment systems are not designed for the removal of emerging pollutants. The current status highlights the urgent need to develop methods capable of simultaneously removing nutrients and emerging pollutants. Zhao et al. (2021) developed intermittently-aerated subsurface flow constructed wetlands for the simultaneous removal of nitrogen and dimethyl phthalate from low-carbon wastewaters which provided novel insights into DMP removal mechanism and useful guidance for the practical application of constructed wetlands for treating wastewater containing phthalates. Hong et al. (2022) isolated a novel Zn(II)-resistant *Pseudomonas stutzeri* KY-37 which was capable of eliminating nitrogen and bisphenol A from wastewater at the same time. Gani et al. (2020) investigated the effects of treatment configuration on the efficient removal of nitrogen and priority PAEs from municipal wastewater in an integrated biofilm activated sludge system which demonstrated that treatment configuration and F/M ratio might be one of the guiding parameters. Cao et al. (2014) achieved the simultaneous removal of DEHP and nitrogen in a laboratory-scale pre-denitrification biofilter system and suggested that biodegradation plays a key role in DEHP elimination. Xu et al. (2021) fabricated a novel biological carrier combining sponge and modified walnut shell biochar with Fe_3O_4 which was used to remove nitrate and DEP simultaneously. However, microbes capable of simultaneously removing PAEs, nitrogen and phosphorus were rarely reported. Therefore, the isolation, investigation and application of strain RL-GZ01 provide novel potential for the treatment of PAEs containing municipal wastewater.

4. Conclusions

In this study, simultaneous removal of nitrogen and DEHP was achieved with a newly isolated bacterial strain *Rhodococcus pyridinivorans* RL-GZ01. Strain RL-GZ01 showed preference to alkaline conditions and good tolerance to salinity and organic solvents. The kinetics analysis of DEHP degradation by strain RL-GZ01 indicated that the process was well fitted with the modified Gompertz model ($R^2 = 0.9985$). The metabolic pathway of DEHP in strain RL-GZ01 was deduced by metabolites identification. Prior to de-esterification, DEHP was transformed into DEP via β-oxidation while DEP was further hydrolyzed into PA. PA was transformed into catechol via benzoate while catechol was

utilized for cell growth through catechol branch of the β-ketoadipate pathway. Genes and gene clusters involved in the degradation of DEHP were identified via genome sequencing analysis. Analysis of the KEGG nitrogen metabolism pathway indicated that nitrogen removal was achieved via ammonium assimilation. Finally, the application of strain RL-GZ01 in municipal wastewater treatment was conducted for simultaneous removal of nitrogen and DEHP. The removal percentages of DEHP, TN, NH_4^+-N and COD by strain RL-GZ01 in municipal wastewater were 89.94%, 64.45%, 64.94%, and 63.23%, respectively, suggesting excellent application potential of RL-GZ01 in treating PAEs containing wastewater.

Acknowledgements

The authors acknowledge financial support from the National Natural Science Foundation of China (31800109), Natural Science Foundation of Guangdong Province (2022A1515012128), Shenzhen Science and Technology R&D Fund (project number: KCXFZ202002011011057), South China Sea Scholar of Guangdong Ocean University (002029002004), and Program for Scientific Research start-up Funds of Guangdong Ocean University (R18013).

References

Allenby, N.E.E., O'Connor, N., Prágai, Z., Ward, A.C., Wipat, A., Harwood, C.R., 2005. Genome-wide transcriptional analysis of the phosphate starvation stimulon of *Bacillus subtilis*. J. Bacteriol., 187, 8063-8080.

Amir, S., Hafidi, M., Merlina, G., Hamdi, H., Jouraiphy, A., El Gharous, M., Revel, J.C., 2005. Fate of phthalic acid esters during composting of both lagooning and activated sludges. Process Biochem., 40, 2183-2190.

Andreeva, N., Ledova, L., Ryazanova, L., Tomashevsky, A., Kulakovskaya, T., Eldarov, M., 2019. Ppn2 endopolyphosphatase overexpressed in *Saccharomyces cerevisiae*: Comparison with Ppn1, Ppx1, and Ddp1 polyphosphatases. Biochimie, 163, 101-107.

APHA. Standard Methods for the Examination of Water and Wastewater. 2017, American Public Health Association, Washington, D.C., USA.

Bai, F., Tian, H., Ma, J., 2020. Landfill leachate treatment through the combination of genetically engineered bacteria *Rhodococcus erythropolis* expressing Nirs and AMO and membrane filtration processes. Environ. Pollut., 263, 114061.

Banerjee, A., Shelver, W.L., 2021. Micro- and nanoplastic induced cellular toxicity in mammals: A review. Sci. Total Environ., 755, 142518.

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G.H., Hilleary, M.A., Eriksen, M., Possingham, H.P., De Frond, H., Gerber, L.R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M., Rochman, C.M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. Science, 369, 1515-1518.

Cao, L., Zhang, J., Zhao, R., Deng, Y., Liu, J., Fu, W., Lei, Y., Zhang, T., Li, X., Li, B., 2019. Genomic characterization, kinetics, and pathways of sulfamethazine biodegradation by *Paenarthrobacter* sp. A01. Environ. Int., 131, 104961.

Cao, X., Ai, N., Meng, X., 2014. Simultaneous removal of di-(2-ethylhexyl) phthalate and nitrogen in a laboratory-scale pre-denitrification biofilter system. Bioresource Technol., 156, 29-34.

Chen, J., Gu, S., Hao, H., Chen, J., 2016. Characteristics and metabolic pathway of *Alcaligenes* sp. TB for simultaneous heterotrophic nitrification-aerobic denitrification. Appl. Microbiol. Biotechnol., 100, 9787-9794.

Chen, X., Zhang, X., Yang, Y., Yue, D., Xiao, L., Yang, L., 2015. Biodegradation of an endocrine-disrupting chemical di-*n*-butyl phthalate by newly isolated *Camelimonas* sp. and enzymatic properties of its hydrolase. Biodegradation, 26, 171-182.

Deng, D., Tang, J., Liu, Z., Tian, Z., Song, M., Cui, Y., Rong, T., Lu, H., Yu, M., Li, J., Pang, R., Ma, X., 2022. Functional characterization and whole-genome analysis of an aflatoxin-degrading *Rhodococcus pyridinivorans* strain. Biology, 11, 774.

Dueholm, M.S., Albertsen, M., D'Imperio, S., Tale, V.P., Lewis, D., Nielsen, P.H., Nielsen, J.L., 2014. Complete genome of *Rhodococcus pyridinivorans* SB3094, a methyl-ethyl-ketone-degrading bacterium used for bioaugmentation. Genome Announc., 2, e00525-14.

EPA, U., 1992. Code of federal regulations. 40 CFR, Part.

European, U., 1993. Council Regulation (EEC), No 793/93 of 23 March 1993 on the Evaluation and Control of the Risks of Existing Substances (OJ L84, 5 April 1993). European Union, Brussels.

Gani, K.M., Nazir, F.U., Kumari, S., Bux, F., Kazmi, A.A., 2020. Role of treatment configuration in simultaneous removal of priority phthalic acid esters and nitrogen in a post anoxic integrated biofilm activated sludge system. Sci. Total Environ., 702, 134733.

George,M.G., Don, J.B., Noel, R.K., James T.S., David R.B., RichardW.C., 2001. Bergey's Manual of

Systematic Bacteriology. 2nd edition. Springer Publishing Press, New York.

Hasan, S.A., Wietzes, P., Janssen, D.B., 2012. Biodegradation kinetics of 4-fluorocinnamic acid by a consortium of *Arthrobacter* and *Ralstonia* strains. Biodegradation, 23, 117-125.

Hermabessiere, L., Dehaut, A., Paul-Pont, I., Lacroix, C., Jezequel, R., Soudant, P., Duflos, G., 2017. Occurrence and effects of plastic additives on marine environments and organisms: A review. Chemosphere, 182, 781-793.

Hong, P., Zhang, K., Dai, Y., Yuen, C.N.T., Gao, Y., Gu, Y., Mei Yee Leung, K., 2022. Application of aerobic denitrifier for simultaneous removal of nitrogen, zinc, and bisphenol A from wastewater. Bioresour. Technol., 354, 127192.

Horn, O., Nalli, S., Cooper, D., Nicell, J., 2004. Plasticizer metabolites in the environment. Water Res., 38, 3693-3698.

Jin, D., Bai, Z., Chang, D., Hoefel, D., Jin, B., Wang, P., Wei, D., Zhuang, G., 2012. Biodegradation of di-n-butyl phthalate by an isolated *Gordonia* sp. strain QH-11: Genetic identification and degradation kinetics. J. Hazard. Mater., 221-222, 80-85.

Katsikantami, I., Sifakis, S., Tzatzarakis, M.N., Vakonaki, E., Kalantzi, O., Tsatsakis, A.M., Rizos, A.K., 2016. A global assessment of phthalates burden and related links to health effects. Environ. Int., 97, 212-236.

Ke, X., Liu, C., Tang, S., Guo, T., Pan, L., Xue, Y., Zheng, Y., 2022. Characterization of *Acinetobacter* indicus ZJB20129 for heterotrophic nitrification and aerobic denitrification isolated from an urban sewage treatment plant. Bioresour. Technol., 347, 126423.

Kim, D., Choi, K.Y., Yoo, M., Zylstra, G.J., Kim, E., 2018. Biotechnological potential of *Rhodococcus* biodegradative pathways. J. Microbiol. Biotechnol., 28, 1037-1051.

Kimber, I., Dearman, R.J., 2010. An assessment of the ability of phthalates to influence immune and allergic responses. *Toxicolo.*, 271, 73-82.

Koelmans, A.A., Besseling, E., Foekema, E.M., 2014. Leaching of plastic additives to marine organisms. Environ. Pollut., 187, 49-54.

Kriszt, B., Tancsics, A., Cserhati, M., Toth, A., Nagy, I., Horvath, B., Nagy, I., Tamura, T., Kukolya, J., Szoboszlay, S., 2012. De novo genome project for the aromatic degrader *Rhodococcus pyridinivorans* strain AK37. J. Bacteriol., 194, 1247-1248.

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., Marra, M.A.,

2009. Circos: an information aesthetic for comparative genomics. Genome Res., 19, 1639-45.

Kundu, D., Hazra, C., Dandi, N., Chaudhari, A., 2013. Biodegradation of 4-nitrotoluene with biosurfactant production by *Rhodococcus pyridinivorans* NT2: metabolic pathway, cell surface properties and toxicological characterization. Biodegradation, 24, 775-793.

Li, B., Jing, F., Wu, D., Xiao, B., Hu, Z., 2021. Simultaneous removal of nitrogen and phosphorus by a novel aerobic denitrifying phosphorus-accumulating bacterium, *Pseudomonas stutzeri* ADP-19. Bioresour. Technol., 321, 124445.

Li, C., Yang, J., Wang, X., Wang, E., Li, B., He, R., Yuan, H., 2015. Removal of nitrogen by heterotrophic nitrification–aerobic denitrification of a phosphate accumulating bacterium *Pseudomonas stutzeri* YG-24. Bioresour. Technol., 182, 18-25.

Lin, K.D., Yuan, D.X., 2005. Degradation kinetics and products of triazophos in intertidal sediment. J. Environ. Sci., 17, 933-6.

Lu, H., Wang, X., Zang, M., Zhou, J., Wang, J., Guo, W., 2019. Degradation pathways and kinetics of anthraquinone compounds along with nitrate removal by a newly isolated *Rhodococcus pyridinivorans* GF3 under aerobic conditions. Bioresour. Technol., 285, 121336.

Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R., Langlois, V.S., 2016. Influence of lipophilicity on the toxicity of bisphenol a and phthalates to aquatic organisms. B. Environ. Contam. Tox., 97, 4-10.

Meng, L., Li, W., Zhang, S., Wu, C., Jiang, W., Sha, C., 2016. Effect of different extra carbon sources on nitrogen loss control and the change of bacterial populations in sewage sludge composting. Ecol. Eng., 94, 238-243.

Monds, R.D., Newell, P.D., Schwartzman, J.A., O'Toole, G.A., 2006. Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. Appl. Environ. Microb., 72, 1910-1924.

Mu, D., Gao, F., Fan, Z., Shen, H., Peng, H., Hu, J., 2015. Levels of phthalate metabolites in urine of pregnant women and risk of clinical pregnancy loss. Environ. Sci. Technol., 49, 10651-10657.

Nahurira, R., Ren, L., Song, J., Jia, Y., Wang, J., Fan, S., Wang, H., Yan, Y., 2017. Degradation of di(2-ethylhexyl) phthalate by a novel *Gordonia alkanivorans* strain YC-RL2. Curr. Microbiol., 74, 309-319.

Nalli, S., Cooper, D.G., Nicell, J.A., 2006. Interaction of metabolites with *R. rhodochrous* during the biodegradation of di-ester plasticizers. Chemosphere, 65, 1510-1517.

Nas, B., Ateş, H., Dolu, T., Yel, E., Argun, M.E., Koyuncu, S., Kara, M., Dinç, S., 2022. Evaluation of

occurrence, fate and removal of priority phthalate esters (PAEs) in wastewater and sewage sludge by advanced biological treatment, waste stabilization pond and constructed wetland. Chemosphere, 295, 133864.

Navacharoen, A., Vangnai, A.S., 2011. Biodegradation of diethyl phthalate by an organic-solvent-tolerant *Bacillus subtilis* strain 3C3 and effect of phthalate ester coexistence. Int. Biodeter. & Biodegr., 65, 818-826.

Nawong, C., Umsakul, K., Sermwittayawong, N., 2018. Rubber gloves biodegradation by a consortium, mixed culture and pure culture isolated from soil samples. Braz. J. Microbiol., 49, 481-488.

Net, S., Sempéré, R., Delmont, A., Paluselli, A., Ouddane, B., 2015. Occurrence, fate, behavior and ecotoxicological state of phthalates in different environmental matrices. Environ. Sci. Technol., 49, 4019-4035.

Peng, F., Ye, M., Liu, Y., Liu, J., Lan, Y., Luo, A., Zhang, T., Jiang, Z., Song, H., 2022. Comparative genomics reveals response of *Rhodococcus pyridinivorans* B403 to phenol after evolution. Appl. Microbiol. Biotechnol., 106, 2751-2761.

Pirsaheb, M., Nouri, M., Hossini, H., 2022. Advanced oxidation processes for the removal of phthalate esters (PAEs) in aqueous matrices: a review. Rev. Environ. Health. DOI: 10.1515/reveh-2022-0001.

PlasticEurope. Plastics-the facts 2018: an analysis of European plastics production, demand and waste data. 2018, https://www.plasticseurope.org.

Ren, L., Jia, Y., Ruth, N., Qiao, C., Wang, J., Zhao, B., Yan, Y., 2016. Biodegradation of phthalic acid esters by a newly isolated *Mycobacterium* sp. YC-RL4 and the bioprocess with environmental samples. Environ. Sci. Pollut. Res., 23, 16609-16619.

Ren, L., Lin, Z., Liu, H., Hu, H., 2018. Bacteria-mediated phthalic acid esters degradation and related molecular mechanisms. Appl. Microbiol. Biotechnol., 102, 1085-1096.

Ren, L., Wang, G., Huang, Y., Guo, J., Li, C., Jia, Y., Chen, S., Zhou, J.L., Hu, H., 2021. Phthalic acid esters degradation by a novel marine bacterial strain *Mycolicibacterium phocaicum* RL-HY01: Characterization, metabolic pathway and bioaugmentation. Sci. Total. Environ., 791, 148303.

Rout, P.R., Bhunia, P., Dash, R.R., 2017. Simultaneous removal of nitrogen and phosphorous from domestic wastewater using *Bacillus cereus* GS-5 strain exhibiting heterotrophic nitrification, aerobic denitrification and denitrifying phosphorous removal. Bioresource Technol., 244, 484-495.

Sakshi, Singh, S.K., Haritash, A.K., 2021. Catabolic enzyme activities during biodegradation of three-

ring PAHs by novel DTU-1Y and DTU-7P strains isolated from petroleum-contaminated soil. Arch. Microbiol., 203, 3101-3110.

Stojanoska, M.M., Milosevic, N., Milic, N., Abenavoli, L., 2017. The influence of phthalates and bisphenol A on the obesity development and glucose metabolism disorders. Endocrine, 55, 666-681.

Wang, H., Hu, J., Xu, K., Tang, X., Xu, X., Shen, C., 2018. Biodegradation and chemotaxis of polychlorinated biphenyls, biphenyls, and their metabolites by *Rhodococcus* spp. Biodegradation, 29, 1-10.

Wang, L., Gan, D., Gong, L., Zhang, Y., Wang, J., Guan, R., Zeng, L., Qu, J., Dong, M., Wang, L., 2022. Analysis of the performance of the efficient di-(2-ethylhexyl) phthalate-degrading bacterium *Rhodococcus pyridinovorans* DNHP-S2 and associated catabolic pathways. Chemosphere, 306, 135610.

Wang, W., Li, Q., Zhang, L., Cui, J., Yu, H., Wang, X., Ouyang, X., Tao, F., Xu, P., Tang, H., 2021. Genetic mapping of highly versatile and solvent-tolerant *Pseudomonas putida* B6-2 (ATCC BAA-2545) as a 'superstar' for mineralization of PAHs and dioxin-like compounds. Environ. Microbiol., 23, 4309-4325.

Wang, Y., Miao, B., Hou, D., Wu, X., Peng, B., 2012. Biodegradation of di-*n*-butyl phthalate and expression of the 3, 4-phthalate dioxygenase gene in *Arthrobacter* sp. ZH2 strain. Process Biochem., 47, 936-940.

Wright, R.J., Bosch, R., Gibson, M.I., Christie-Oleza, J.A., 2020. Plasticizer degradation by marine bacterial isolates: A proteogenomic and metabolomic characterization. Environ. Sci. Technol., 54, 2244-2256.

Wright, S.L., Kelly, F.J., 2017. Plastic and human health: A micro issue? Environ. Sci. Technol., 51, 6634-6647.

Wu, X., Liang, R., Dai, Q., Jin, D., Wang, Y., Chao, W., 2010. Complete degradation of di-*n*-octyl phthalate by biochemical cooperation between *Gordonia* sp. strain JDC-2 and *Arthrobacter* sp. strain JDC-32 isolated from activated sludge. J. Hazard. Mater., 176, 262-268.

Xu, L., Su, J., Huang, T., Li, G., Ali, A., Shi, J., 2021. Simultaneous removal of nitrate and diethyl phthalate using a novel sponge-based biocarrier combined modified walnut shell biochar with Fe_3O_4 in the immobilized bioreactor. J. Hazard. Mater., 414, 125578.

Xu, X., Li, H., Gu, J., 2005. Biodegradation of an endocrine-disrupting chemical di-n-butyl phthalate

ester by *Pseudomonas fluorescens* B-1. Int. Biodeter. Biodegr., 55, 9-15.

Yang, T., Ren, L., Jia, Y., Fan, S., Wang, J., Wang, J., Nahurira, R., Wang, H., Yan, Y., 2018. Biodegradation of di-(2-ethylhexyl) phthalate by *Rhodococcus ruber* YC-YT1 in contaminated water and soil. Int. J. Env. Res. Pub. He., 15, 964.

Yoon, J.H., Kang, S.S., Cho, Y.G., Lee, S.T., Kho, Y.H., Kim, C.J., Park, Y.H., 2000. *Rhodococcus pyridinivorans* sp. nov., a pyridine-degrading bacterium. Int. J. Syst. Evol. Micr., 50, 2173.

Zeng, F., Cui, K., Li, X., Fu, J., Sheng, G., 2004. Biodegradation kinetics of phthalate esters by *Pseudomonas fluoresences* FS1. Process Biochem., 39, 1125-1129.

Zhang, H., Lin, Z., Liu, B., Wang, G., Weng, L., Zhou, J., Hu, H., He, H., Huang, Y., Chen, J., Ruth, N., Li, C., Ren, L., 2020. Bioremediation of di-(2-ethylhexyl) phthalate contaminated red soil by *Gordonia terrae* RL-JC02: Characterization, metabolic pathway and kinetics. Sci. Total. Environ., 733, 139138-139138.

Zhang, J., Zhao, R., Cao, L., Lei, Y., Liu, J., Feng, J., Fu, W., Li, X., Li, B., 2020. High-efficiency biodegradation of chloramphenicol by enriched bacterial consortia: Kinetics study and bacterial community characterization. J. Hazard. Mater., 384, 121344.

Zhao, H., Hu, R., Chen, X., Chen, X., Lü, H., Li, Y., Li, H., Mo, C., Cai, Q., Wong, M., 2018. Biodegradation pathway of di-(2-ethylhexyl) phthalate by a novel *Rhodococcus pyridinivorans* XB and its bioaugmentation for remediation of DEHP contaminated soil. Sci. Total. Environ., 640-641, 1121-1131.

Zhao, X., Wang, R., Dong, L., Li, W., Li, M., Wu, H., 2021. Simultaneous removal of nitrogen and dimethyl phthalate from low-carbon wastewaters by using intermittently-aerated constructed wetlands. J. Hazard. Mater., 404, 124130.

Zhou, Y., Ganda, L., Lim, M., Yuan, Z., Kjelleberg, S., Ng, W.J., 2010. Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs). Appl. Microbiol. Biotechnol., 88, 359-369.

Zou, Y., Ye, C., Pan, Y., 2021. Abundance and characteristics of microplastics in municipal wastewater treatment plant effluent: a case study of Guangzhou, China. Environ. Sci. Pollut. Res., 28, 11572-11585.