

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

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## 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),  $\text{NH}_4^+\text{-N}$  (70.9 mg/L),  $\text{PO}_4^{3-}\text{-P}$  (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.

## 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 %) [1]. Since most of these plastic products are used once, large quantities of plastics are discharged as wastes which might finally enter aquatic ecosystems. In 2016, approximately twenty-three Mt. of plastic wastes entered aquatic environments which has been recognized to

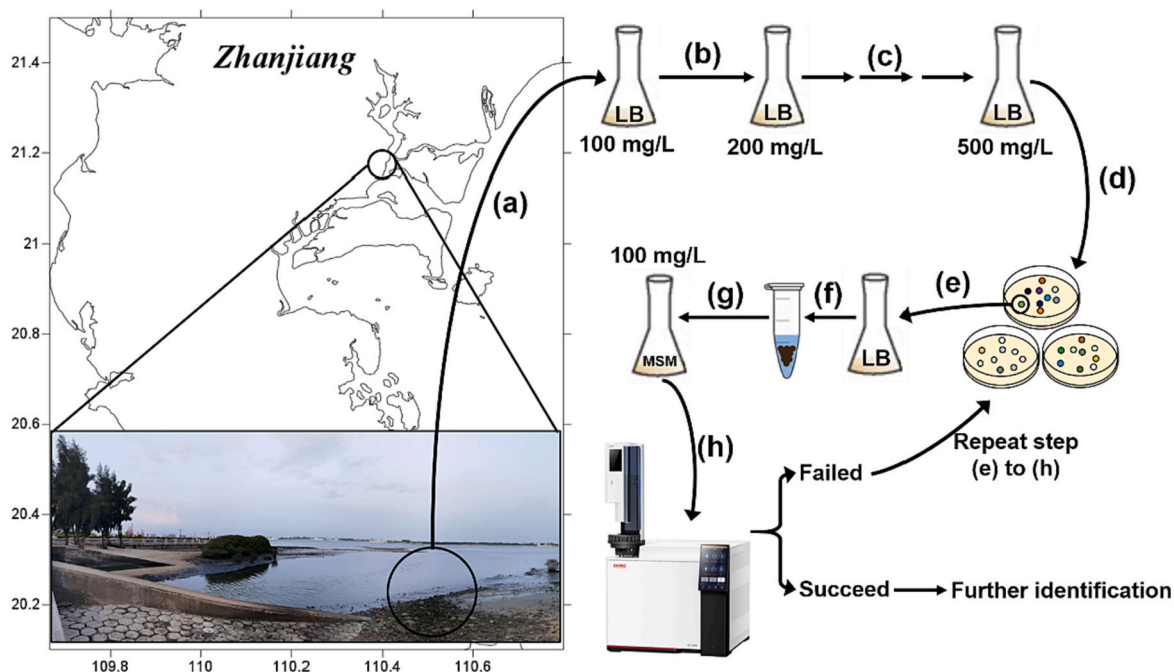
affect the global aquatic ecosystem [2]. When plastic wastes are released into environments, UV radiation mediated photo-oxidation makes the plastics become brittle, causing the generation of plastic debris [3]. Further, with the impact of wind, wave action, abrasion etc., plastic fragments will be degraded into micro-plastics (MPs) (0.1  $\mu\text{m}$  - 5 mm diameter) and potentially nano-plastics (NPs) ( $\leq 0.1 \mu\text{m}$  diameter) [4]. Recently, wastewater treatment plants (WWTPs) have been proposed as significant sources of MPs and NPs in freshwater and marine environments [5]. Meanwhile, "Occurrence, fate, detection, and removal of microplastics in wastewater treatment" was listed as the Top 1 of

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

research fronts in field of ecological and environmental sciences in 2020 (<http://english.casid.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. During the decomposition of plastics, plastic additives might be released from polymers [6].

Plasticizers are widely used as plastic additives which are applied to enhance the flexibility and durability of products. Since the global consumption of plasticizers in 2020 has already exceeded 10 Mt. (<https://www.plasticisers.org/plasticisers> and <https://www.statista.com>), the elimination of plasticizers from different environments, including WWTPs, becomes urgent. The global plasticizer market was dominated by phthalic acid esters (PAEs) with a market share of 55.0 % [7]. PAEs are known as external plasticizer which might be released from plastics during decomposition. Thus, PAEs are ubiquitously detected in various environments and have been recognized as one of the typical emerging pollutants in municipal wastewater [8]. The composition, distribution and concentration of PAEs in WWTPs have been systematically investigated (Table S1). The toxicological evaluation of PAEs has been extensively conducted and many reports demonstrated that PAEs exhibited carcinogenic, developmental and reproductive toxicity [9,10]. Therefore, six kinds of PAEs have been listed as the priority pollutants by US-EPA, European Union, and China National Environmental Monitoring Center [11–13]. 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. Among these methods, bacteria-mediated degradation has been recognized as the most ecological method for PAEs elimination. PAEs-degrading bacterial strains have been extensively isolated and investigated, and some of these isolates have been applied for the bioremediation of PAEs-contaminated environments [7]. However, the application of bacterial isolates for the elimination of PAEs from wastewater is rarely reported. Meanwhile, nitrogen and phosphate are the leading causes of water eutrophication, and the removal of nitrogen and phosphate is recognized as the emphasis in wastewater treatment field for eutrophication control while the removal of nitrogen and phosphate is mainly achieved

via biological treatment. Conventionally, alternative anaerobic/aerobic conditions are needed in the biological removal of nitrogen and phosphate process which makes the treatment system complicated and energy-intensive [14]. Further, different functional microorganisms are needed for the biological treatment process, such as nitrifying bacteria, denitrifying bacteria, phosphorus-accumulating organisms, and emerging pollutants degraders [15]. Hence, acquiring denitrifying and phosphorus-accumulating functional microorganisms capable of degrading PAEs is highly advantageous for wastewater treatment since these microbes are conducive to simplify the wastewater treatment and decrease the operating costs.

In the present work, a novel PAEs-degrading bacterium RL-GZ01, with capacity of simultaneous removal of nitrogen, phosphate and di-(2-ethylhexyl) phthalate (DEHP) was isolated and the identification of strain RL-GZ01 was done via 16S rRNA gene analysis, physio-biochemical characterization, and genome-based average nucleotide identity (ANI) calculation. The substrate range of strain RL-GZ01 was determined with different PAEs. Environmental factors potentially affected the degradation of DEHP were characterized. Further, the degrading intermediates of DEHP were analyzed through ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis and the metabolic pathway was proposed thereafter. Genes and gene clusters contributed to the degradation of DEHP and removal of nitrogen and phosphate were identified through whole genome sequencing and analysis. Eventually, strain RL-GZ01 was applied to municipal wastewater for simultaneous removal of DEHP, nitrogen and phosphate to evaluate its application potential. This is the first report that *Rhodococcus pyridinivorans* bacteria with the capability of simultaneously removing PAEs, nitrogen and phosphorus from municipal wastewater.

## 2. Materials and methods

### 2.1. Chemicals and media

The detailed information of PAEs and chemicals used in this study was presented in Table S2. The mother solution of PAE was prepared in menthol (20,000 mg/L). Biological reagents were purchased from

Sangon Biotech (Shanghai, China) while chemicals were purchased from Sinopharm Chemical Reagent (Shanghai, China). Media used in this study were prepared as previously described [16,17] and the components of each medium were presented in Table S3.

## 2.2. Isolation and identification of DEHP-degrading strain

The characteristics of sediment were shown in Table S4. The enrichment, domestication and isolation were conducted as previously reported [18] and the schematic diagram was shown in Fig. 1. Briefly, 1 g of sample was inoculated into 10 mL mineral salt medium (MSM) containing 100 mg/L of DEHP (Fig. 1, step a). Flasks were incubated in a constant shaker under 30 °C (160 rpm) for 5 d. Serial inoculation, incubation and transferring were conducted until the concentration of DEHP reached 500 mg/L (Fig. 1, step b to c). Subsequently, cells were harvested by centrifugation (4000 g, 6 min) and washing (Tris-HCl buffer, 0.1 M, pH 7.2). Cells were resuspended in MSM medium and spread on solid MSM medium supplemented with DEHP (500 mg/L) as carbon sources. The incubation of plates was conducted with a constant temperature (30 °C) in dark (Fig. 1, step d). All plates were checked every day to obtain colonies with hydrolytic halos. The target colony was inoculated into LB medium and incubated under constant shaking (180 rpm, 30 °C). Cell pellets were harvested as described above (Fig. 1, step e) and the DEHP-degrading capacity was confirmed followed by inoculation, incubation and residual quantification (Fig. 1, step f to h). The isolation process (Fig. 1, steps a to h) was repeated to obtain DEHP-degrading bacterial strains. Meanwhile, the nitrogen and phosphate removal capacity of isolated DEHP-degrading strain was verified with nitrification medium (NM, 100 mg/L of  $\text{NH}_4^+\text{-N}$ ), denitrification medium (DM, 100 mg/L of  $\text{NO}_3^-\text{-N}$  or  $\text{NO}_2^-\text{-N}$ ) and phosphate uptake medium (PUM, 20 mg/L of  $\text{PO}_4^{3-}\text{-P}$ ), separately. Finally, isolated strains capable of simultaneously eliminating of DEHP, nitrogen and phosphate were selected for further investigation.

## 2.3. Identification of isolated strain

Firstly, the 16S rRNA gene of target strain was amplified with universal primers of 27F and 1492R. Fragments were purified and inserted into pMD-19 T vector for sequencing. The obtained sequence was applied for phylogenetic analysis. The phylogenetic tree was established using MEGA 11.0. Secondly, specific physio-biochemical characteristics of target strain were determined according to previous reports [19]. Thirdly, 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 identification of target strain via ANI calculation. 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 bacterial cells for inoculation were prepared in LB medium. Cultures of target strain were prepared in LB medium by inoculation and incubation under constant shaking (160 rpm, 30 °C) for 1 d. Culture was applied to centrifugation (4000 g, 4 min) and the cells were washed by Tris-HCl buffer (0.1 M, pH 7.2). Cell pellets were resuspended in MSM liquid medium with a  $\text{OD}_{600}$  value of 0.8 ( $\sim 5.6 \times 10^7$  cell/mL) after three times centrifugation and washing. The obtained cell suspension was used as inoculants and the inoculation ratio was set as 1.0 % (v/v).

The catabolic capacity of different PAEs was determined to have an insight into the substrate range of target strain. 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

conducted in flasks with 10 mL of MSM liquid medium containing one kind of PAEs (100 mg/L), and the cell suspension of target strain was prepared and inoculated as described above. Cultures without supplementation of cell suspension was chosen as abiotic control and the incubation of all flasks were performed 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 (from 4.0 to 10.0 with an interval of 1.0), temperature (from 10 °C to 50 °C with an interval of 10 °C), salinities (from 4.0 %, to 10.0 % with an interval of 2.0 %, w/v), and organic solvents (*p*-xylene, toluene, ethyl acetate, and biphenyl, 500 mg/L of each). Same cultures without supplementation of cell suspension were selected as abiotic control. Thereafter, the DEHP content was quantified under the optimized condition and further applied for the kinetics analysis using a first-order decay model (Eq. (1)) and a modified Gompertz model (Eq. (2)) [20].

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

The modified Gompertz model :  $S$

$$= S_0 - A \cdot \exp \left\{ - \exp \left[ \frac{V_m \cdot e}{A} (L - t_1) + 1 \right] \right\} \quad (2)$$

where  $S$  means the concentration of DEHP;  $S_0$  is the original concentration of DEHP;  $A$  represents the degradation potential;  $V_m$  is the maximum biodegradation rate; and  $L$  means the lag phase.

All cultures above were incubated in a shaker (180 rpm, 30 °C). Samples were collected at a time interval of 6 h and the residual DEHP concentration was determined by gas chromatograph (GC). All experimental assays were accomplished in three replicates.

## 2.5. Identification of DEHP intermediates and metabolic pathways

The identification of DEHP intermediates was accomplished through ultra-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS, Agilent, 1290–6470) analysis. Further, the metabolic pathway of DEHP in the target strain was proposed according to the information of intermediates. Cell suspension was supplemented into MSM medium (100 mg/L of DEHP) and cultures were withdrawn at a time interval of 4 h. For the collected cultures, *n*-hexane was employed for extraction by adding equal volume of solvents into the sample and mixing with a vortex for 30 s. After that, the mixture was fully extracted by ultrasound (40 kHz, 10 min) under 4 °C. The organic phase was collected for the identification of metabolic intermediates and the catabolic pathway of DEHP were proposed thereafter.

## 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 phosphate removal in the isolated strain, whole genome sequencing and analysis were conducted. Cells of target strain were prepared in LB medium and harvested by centrifugation and washing as described above. The obtained bacterial cells were applied to genome extraction. DNA integrity, quality and concentration were determined by agarose gel electrophoresis, a Qubit fluorometer and a NanoDrop 2000 spectrophotometer, respectively. Complete genome sequencing of target strain 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 PGAP ([https://www.ncbi.nih.gov/genome/annotation\\_prok/](https://www.ncbi.nih.gov/genome/annotation_prok/)) for automatic gene prediction and in-depth annotation. In addition, the predicted coding sequences were searched against the eggNOG Database (<http://eggnoг.embl.de>) and KEGG database (<http://www.genome.jp/kegg/>) to analyze the gene function and metabolic pathways, respectively. The map of genome and

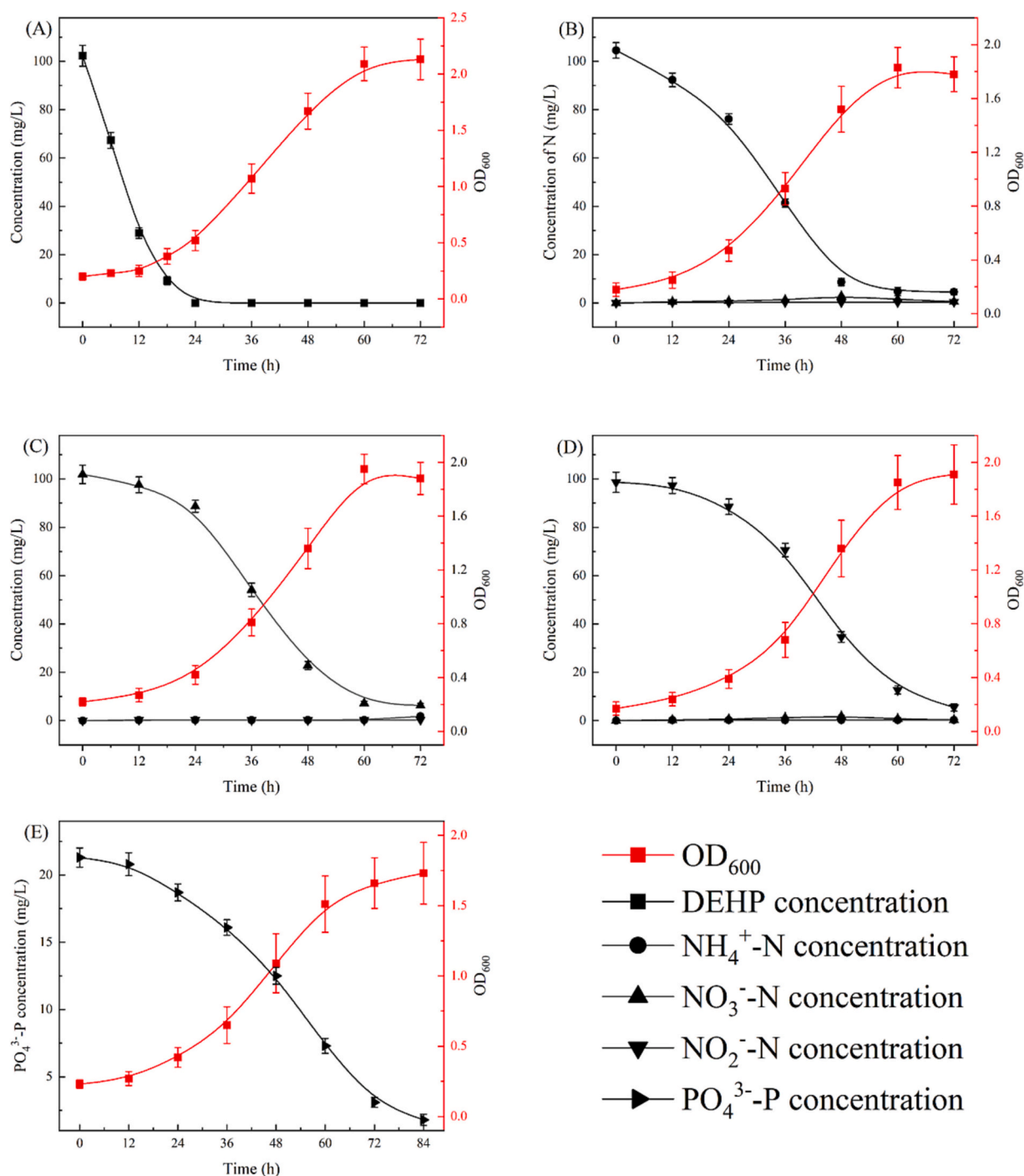


Fig. 2. The degradation of (A) DEHP, (B) NH<sub>4</sub><sup>+</sup>-N, (C) NO<sub>3</sub><sup>-</sup>-N, (D) NO<sub>2</sub><sup>-</sup>-N, and (E) PO<sub>4</sub><sup>3-</sup>-P by strain RL-GZ01. Values are means  $\pm$  SD for three replicates.

plasmids was produced by *circos* (version 0.69). Genes potentially contributed to the metabolism of DEHP and removal of nitrogen and phosphate 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 were presented in Table S4. DEHP was added into 10 mL wastewater in a 100 mL flask with a final concentration of 5 mg/L. The cells of target strain

were prepared and inoculated as described above, and the same wastewater containing DEHP (5 mg/L) without supplementation of cell suspension was chosen as control. All treatments were performed in three replicates. These flasks were kept in a constant shaker (180 rpm, 30 °C) and the cultures were withdrawn every 4 h. The residual concentration of DEHP, NH<sub>4</sub><sup>+</sup>-N, NO<sub>3</sub><sup>-</sup>-N, NO<sub>2</sub><sup>-</sup>-N, total nitrogen (TN), PO<sub>4</sub><sup>3-</sup>-P, and chemical oxygen demand (COD) were determined.

## 2.8. Analytic methods

Cell growth was determined through the detection of OD<sub>600</sub> values with a spectrophotometer (P4PC, MAPADA, Shanghai, China) while the

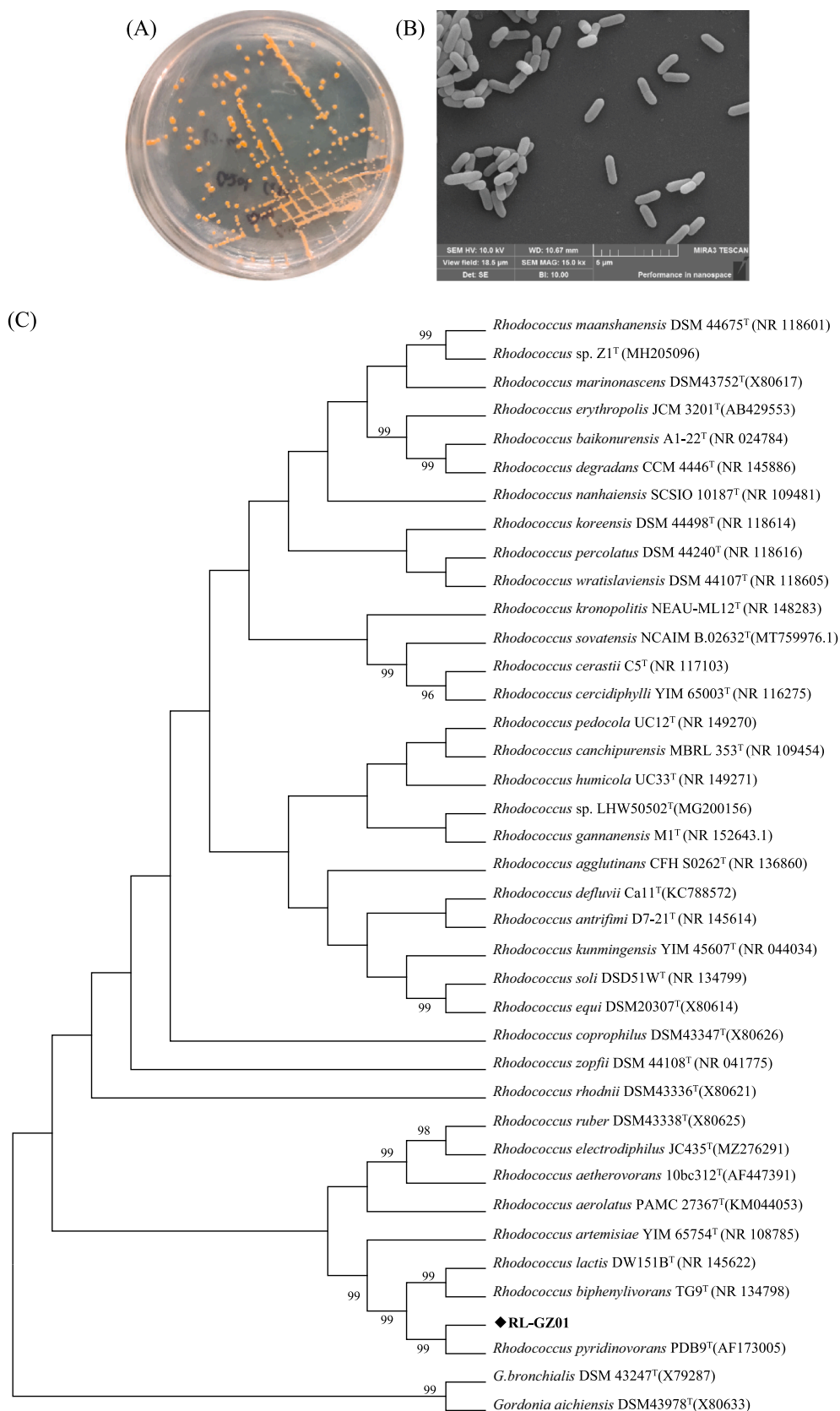


Fig. 3. Identification of strain RL-GZ01 by (A) colony morphology, (B) cell morphology, and (C) phylogenetic analysis of 16S rRNA gene.

cell density was quantified and presented as colony-forming units (CFUs). Depending on the standard methods, the concentration of COD,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$ , TN and TP were measured by the potassium dichromate method, Nessler's reagent spectrophotometry, the ultraviolet spectrophotometric method,  $\alpha$ -naphthylamine spectrophotometry, and antimony molybdenum blue spectrophotometry, respectively [21].

The nitrogen and phosphate 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,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ ,  $\text{NO}_2^-\text{-N}$  or  $\text{PO}_4^{3-}\text{-P}$ , respectively.

PAEs was quantified with a GC system (GC-2010 pro, Suzhou, China) as described by Wang et al. [18]. The recovery rates of PAEs were all above 97.0 %. The standard curves of selected substrates were calculated (Table S5). The metabolic intermediates of DEHP were identified using a UHPLC-MS/MS platform in Bionovogene Co. Ltd. (Suzhou, China) as described by Ren et al. [17]. The calculation of degradation percentage was performed with Eq. (3).

## 2.9. Data accessibility

Strain RL-GZ01 is accessible in Guangdong Microbial Culture Collection Center under accession number 62401. The 16S rRNA gene of strain RL-GZ01 has been deposited in GenBank with accession number OP320921. Genome information for the chromosome and plasmids of strain RL-GZ01 were deposited in GenBank under the accession numbers CP103308, CP103309, CP103310 and CP103311, respectively.

## 3. Result and discussion

### 3.1. Strain RL-GZ01, capable of degrading DEHP and removing nitrogen and phosphate

One bacterium (RL-GZ01), exhibited the capability of utilizing DEHP for cell growth, was obtained from the sediment samples and 100 mg/L of DEHP was entirely eliminated within 24 h with a significant increase of  $\text{OD}_{600}$  value (Fig. 2A). 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. 2. When  $(\text{NH}_4)_2\text{SO}_4$  was supplied as the sole nitrogen source, no obvious lag phase of cell growth was observed and 95.6 % of  $\text{NH}_4^+\text{-N}$  was reduced with a maximum removal rate of 2.85 mg/L/h between 24 h and 36 h (Fig. 2B). With a short time (12h) lag phase,  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  were rapidly removed between 24 h and 48 h and the removal percentages were 93.8 % and 94.4 % within 72 h, respectively (Fig. 2C and D). Phosphate could be rapidly removed by strain RL-GZ01 with 91.5 % removal percentages (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. 3A). The scanning electron microscope (SEM) image showed that strain RL-GZ01 was rod-shaped without flagellum (Fig. 3B). The 16S rRNA gene sequence of strain RL-GZ01 was amplified and sequenced with a length of 1484 bp. The phylogenetic analysis of 16S rRNA gene indicated that strain RL-GZ01 has the closest relationship with the species of *Rhodococcus pyridinivorans* (Fig. 3C). The physio-biochemical properties of strain RL-GZ01 were presented in Table S6. The ANI value between the genome of RL-GZ01 and reference genome of *Rhodococcus pyridinivorans* (NC\_023150) is 98.68 % (Fig. S1). Consequently, strain RL-GZ01 was identified as *Rhodococcus pyridinivorans* based on 16S rRNA gene analysis, physio-biochemical characterization and ANI calculation. The genus *Rhodococcus* is a phylogenetically and catabolically diverse group that are frequent

**Table 1**

Summaries of reported xenobiotics-degrading strains of *Rhodococcus pyridinivorans*.

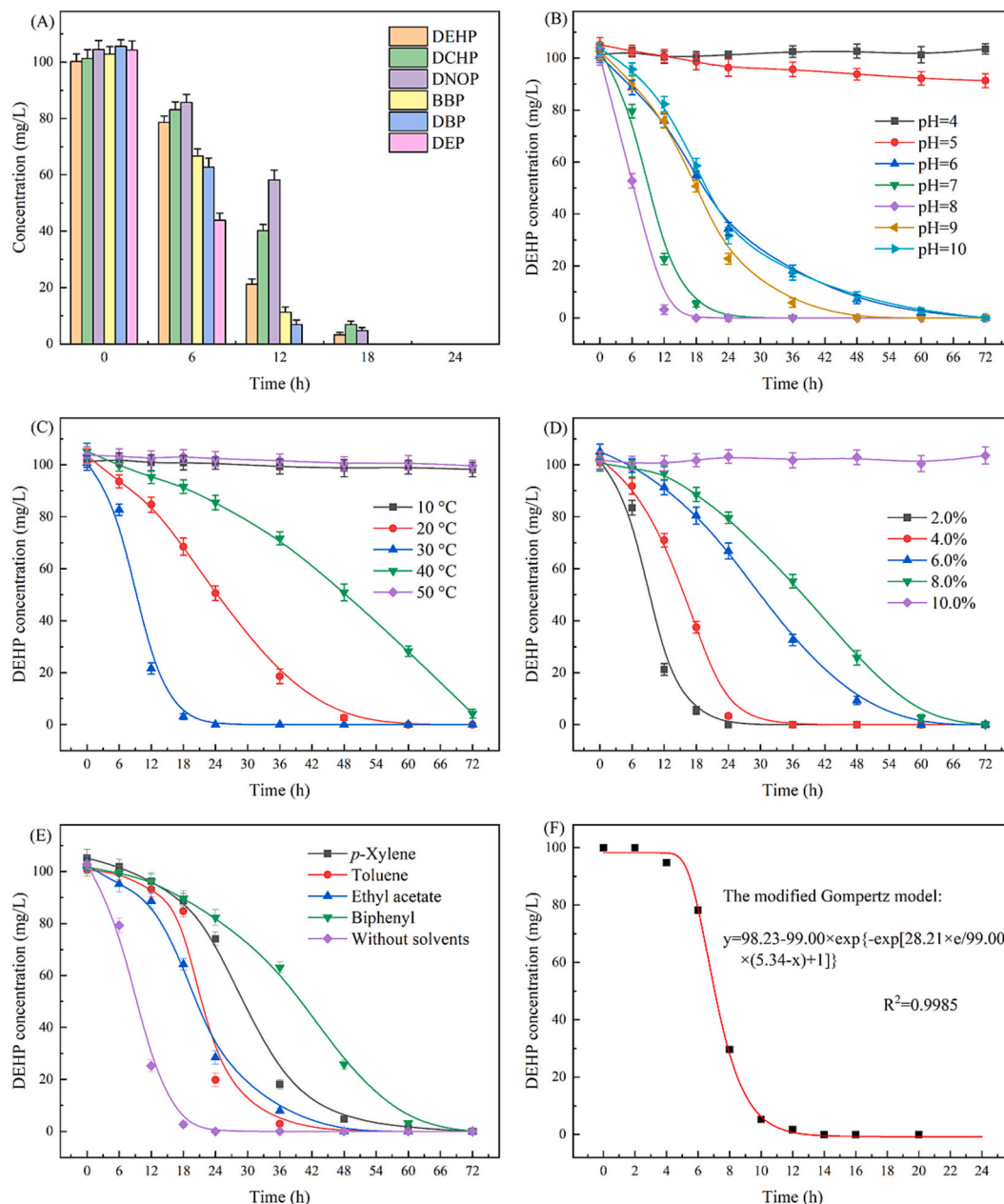
Strain	Matrix	Substrate	Reference
<i>Rhodococcus pyridinivorans</i> PDB9 <sup>T</sup>	Industrial wastewater	Pyridine	[23]
<i>Rhodococcus pyridinivorans</i> AK37	Crude oil-contaminated site	Aromatic compounds	[24]
<i>Rhodococcus pyridinivorans</i> NT2	Sediment	4-Nitrotoluene	[25]
<i>Rhodococcus pyridinivorans</i> SB3094	Bioaugmentation product	Methyl-ethyl-ketone	[26]
<i>Rhodococcus pyridinivorans</i> F5	Soil	Rubber	[27]
<i>Rhodococcus pyridinivorans</i> SS2	PCB-contaminated Soil	PCBs and biphenyls	[28]
<i>Rhodococcus pyridinivorans</i> GF3	Soil	Anthraquinone compounds	[29]
<i>Rhodococcus pyridinivorans</i> DTU-7P	Petroleum contaminated soil	PAHs	[30]
<i>Rhodococcus pyridinivorans</i> 4-4	Soil	Aflatoxin B1	[31]
<i>Rhodococcus pyridinivorans</i> B403	Activated sludge	Phenolic pollutants	[32]
<i>Rhodococcus pyridinivorans</i> DNHP-S2	Plastic film covered soil	DEHP	[33]
<i>Rhodococcus pyridinivorans</i> XB	Activated sludge	DEHP	[34]
<i>Rhodococcus pyridinivorans</i> RL-GZ01	Sediment	DEHP	This study

(*Rhodococcus pyridinivorans* PDB9<sup>T</sup>: type strain of *Rhodococcus pyridinivorans*; PAHs: polycyclic aromatic hydrocarbons; PCBs: polychlorinated biphenyls)

components of microbial communities in diverse natural environments, including polar and alpine regions [22]. Numerous strains of *Rhodococcus* spp. were isolated for their versatility to decompose a wide range of natural and synthetic organic compounds [22]. Species *Rhodococcus pyridinivorans* is known as pyridine-degrading bacterium and several xenobiotics degrading strains of *Rhodococcus pyridinivorans* have been reported (Table 1). Among these strains, *Rhodococcus pyridinivorans* XB and *Rhodococcus pyridinivorans* DNHP-S2 were identified as PAEs-degrading isolates [33,34]. Although *Rhodococcus pyridinivorans* 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 suggested that strain RL-GZ01 is able to completely degrade all the selected PAEs within 24 h (Fig. 4A), 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 complete degradation of DEP is faster than the others as the complete degradation of DEHP was done in 12 h. Therefore, we speculate that the steric effect of side chains might affect the degradation efficiency of PAEs which is in consist with known reports [35,36]. Strain RL-GZ01 could degrade DEHP under a wide range of pH, although the data shown that strain RL-GZ01 preferred the alkaline environments (Fig. 4B). As shown in Fig. 4B, 100 mg/L of DEHP could be completely degraded within 18 h under pH 8.0 which was therefore the optimal pH. DEHP degradation by strain RL-GZ01 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. As to the influences of temperature, the concentration of DEHP were



**Fig. 4.** Characterization of strain RL-GZ01. (A: substrate profile assay, B to E: effects of initial pH, temperature, salinity, and organic solvents, and F: the best-fitted curve for DEHP degradation).

presented in Fig. 4C and the results suggested that relatively low (10 °C) or relatively 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. 4D). 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 where 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. 4E). 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 applied to kinetics analysis and the data shown that strain RL-GZ01 mediated DEHP degradation followed the modified Gompertz model ( $R^2 = 0.9985$ ) better compared with the first-order decay model ( $R^2 = 0.8689$ ). The best fitted curve for DEHP degradation was shown in Fig. 4F. The fitted curves and related parameters for the first-order decay model were shown in Fig. S2. The kinetics analysis demonstrated that RL-GZ01 is able to degrade DEHP with a  $V_m$  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

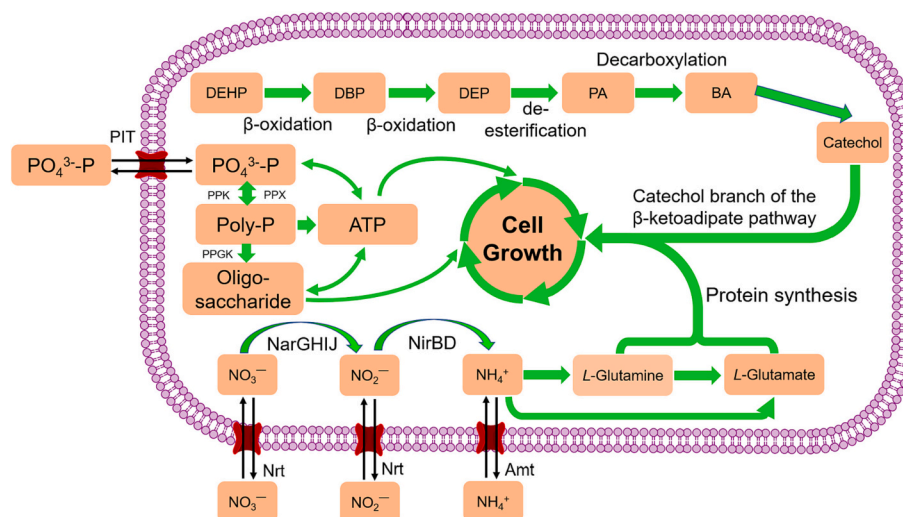


Fig. 5. Metabolic mechanisms of DEHP and nitrogen in strain RL-GZ01. (DEHP: di-(2-ethyl hexyl) phthalate, DBP: di-n-butyl phthalate; DEP: di-ethyl phthalate, PA: phthalic acid; PCA: protocatechuic acid).

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 [37,38], and the steric effect of long or complex side chain might be the reason [7,35,36]. To our knowledge, most of the known PAEs-degrading isolates showed preference to neutral or alkaline environments, such as *Arthrobacter* sp. ZH2, *Gordonia alkanivorans* strain YC-RL2 and *Rhodococcus ruber* YC-YT1 [39–41]. RL-GZ01 exhibited tolerance to high salinity and could efficiently degrade DEHP with salinities ranging from 2.0 % to 8.0 %. DEHP degradation was completely inhibited until the salinity reached 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 [17,41]. 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 of microbial-based remediation, especially for the elimination of hydrophobic compounds [18]. 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 isolates [20]. For the most studies of metabolic 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 [17,38,42].

### 3.3. Metabolic pathway of DEHP in strain RL-GZ01

The proposed metabolic pathway of DEHP in strain RL-GZ01 was presented in Fig. 5 and the data of metabolic intermediates was shown in Table S7 and Fig. S3. All these compounds were undetectable after 72 h, which indicated that they were entirely utilized by strain RL-GZ01. According to the identification of metabolic intermediates, we found that 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 hydrolyzation. Further, phthalic acid was converted 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 is consisted of two major steps: (i) transformation of PAEs into phthalic acid, and (ii) utilization of phthalic acid [7]. 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 [38,43,44]. Some other strains could reduce the side-chain's length prior to ester bond hydrolyzation [45]. Although most isolates are able to transform PAEs into PA, some of these are incapable of utilizing PA for growth, such as *Gordonia* sp. JDC-2 and *Camelimonas* sp. M11 [37,46]. Phthalic acid is the main intermediates of PAEs degradation. The catabolic pathway of PA has been extensively investigated while PA might be transformed into some certain intermediates like PCA, benzoate, catechol, and gentisate. Subsequently, these intermediates could be utilized for cell growth via specific metabolic pathway [7]. 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-hinrance 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 are known to be more toxic and be more recalcitrant for biodegradation, such as 2-ethylhexanol (side-chain of DEHP) [47,48]. 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 phosphate 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 %). The circular map of genome and plasmids was shown in Fig. S4. In total, 5081 genes have been predicted in the chromosome and plasmids of strain RL-GZ01. Among these genes, 4988 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 4235 (84.9 %) of the predicted CDSs were assigned to the eggNOG function classification (Fig. S5). The top three classifications with known function were transcription (357, 8.33 %), energy production and conversion (284, 6.62 %), and replication, recombination and

**Table 2**

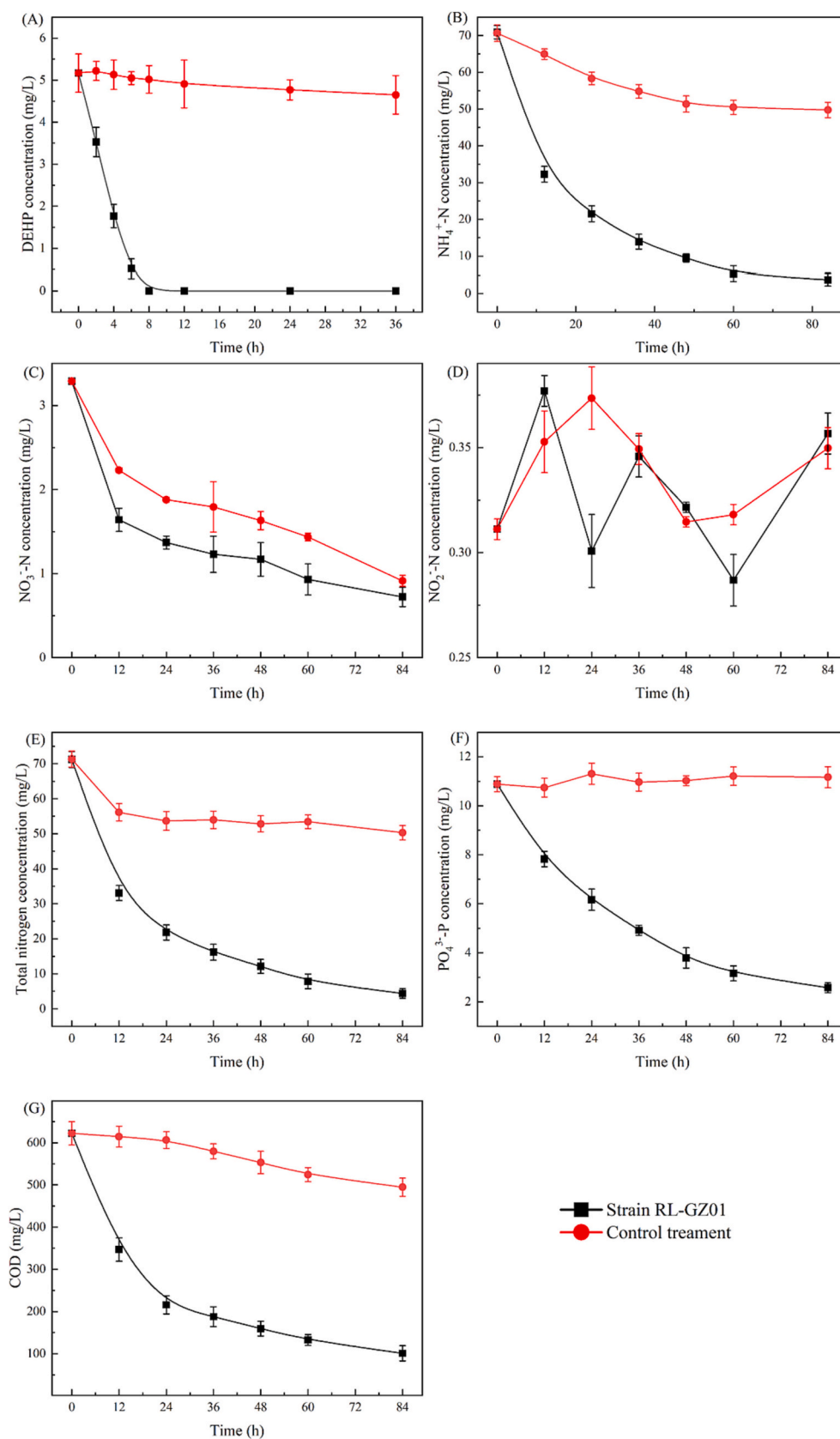
Enzymes involved in the metabolism of DEHP, nitrogen and phosphorus by RL-GZ01.

Function	Gene	Enzyme	Locus_tag
Phosphorus sensing and accumulating	<i>phoB</i>	Phosphate regulon transcriptional regulatory protein	NXT08_21505
	<i>phoR</i>	Phosphate regulon sensor protein (EC 2.7.13.3)	NXT08_21500
	<i>phoU</i>	Phosphate transport system regulatory protein	NXT08_11930
	<i>pit</i>	Low-affinity inorganic phosphate transporter	NXT08_21720
	<i>ppk</i>	Polyphosphate kinase (EC 2.7.4.1)	NXT08_22005
	<i>ppk2</i>	Polyphosphate kinase 2 (EC 2.7.4.1)	NXT08_22145 NXT08_09165
	<i>ppx</i>	Exopolyphosphatase (EC 3.6.1.11)	NXT08_21520
	<i>ppgk</i>	Polyphosphate glucokinase (EC 2.7.1.63)	NXT08_05565
Nitrate and nitrite ammonification	<i>naRas</i>	Assimilatory nitrate reductase large subunit (EC:1.7.99.4)	NXT08_12460
	<i>narI</i>	Nitrate reductase gamma chain (EC 1.7.99.4)	NXT08_04370
	<i>narJ</i>	Nitrate reductase delta chain (EC 1.7.99.4)	NXT08_04375
	<i>narH</i>	Nitrate reductase beta chain (EC 1.7.99.4)	NXT08_04380
	<i>narG</i>	Nitrate reductase alpha chain (EC 1.7.99.4)	NXT08_04385
	<i>nirB</i>	Nitrite reductase large subunit	NXT08_21235
	<i>nirD</i>	Nitrite reductase small subunit	NXT08_21240
	<i>nrt</i>	Nitrate/nitrite transporter	NXT08_04390
Ammonia assimilation	<i>gsl</i>	Glutamine synthetase type I (EC 6.3.1.2)	NXT08_22905
	<i>gslIII</i>	Glutamine synthetase type III (EC 6.3.1.2)	NXT08_05165
	<i>glnE</i>	Glutamine synthetase adenyl-L-tyrosine phosphorylase (EC 2.7.7.42)	NXT08_22910
	<i>glnD</i>	[Protein-P <sub>II</sub> ] uridylyltransferase (EC 2.7.7.59)	NXT08_04870
	<i>gogdp1</i>	Glutamate synthase large chain (EC 1.4.1.13)	NXT08_13240
	<i>gogdp2</i>	Glutamate synthase small chain (EC 1.4.1.13)	NXT08_13235
	<i>glxB</i>	Glutamine amidotransferase protein (EC 2.4.2.-)	NXT08_05170
	<i>glxC</i>	Glutamate synthase (EC 1.4.1.13)	NXT08_05175
	<i>gdh</i>	Glutamate dehydrogenase (EC:1.4.1.2)	NXT08_05385
	<i>gdh1</i>	Glutamate dehydrogenase (EC:1.4.1.3)	NXT08_06705
	<i>gdh2</i>	Glutamate dehydrogenase (EC:1.4.1.4)	NXT08_05450
	<i>amt</i>	Ammonium transporter	NXT08_04880
	<i>mehpH</i>	Mono-alkyl phthalates hydrolase	NXT08_24015
	<i>benD</i>	Dihydroxycyclohexadiene carboxylate dehydrogenase (EC:1.3.1.25)	NXT08_15320
	<i>benA</i>	Benzoate 1,2-dioxygenase subunit $\beta$ (EC:1.14.12.10)	NXT08_15330
De-esterification Utilization of phthalic acid	<i>benB</i>	Benzoate 1,2-dioxygenase subunit $\alpha$ (EC:1.14.12.10)	NXT08_15335
	<i>catA</i>	Catechol 1,2-dioxygenase (EC 1.13.11.1)	NXT08_15380
	<i>catB</i>	Muconate cycloisomerase (EC 5.5.1.1)	NXT08_15385
	<i>catC</i>	Muconolactone isomerase (EC 5.3.3.4)	NXT08_15390
	<i>catD</i>	Beta-ketoadipate enol-lactone hydrolase (EC 3.1.1.24)	NXT08_15490
	<i>catEA</i>	3-Oxoadipate CoA-transferase subunit A (EC 2.8.3.6)	NXT08_16810
	<i>catEB</i>	3-Oxoadipate CoA-transferase subunit B (EC 2.8.3.6)	NXT08_16805

repair (269, 6.27 %). The alignment of predicted CDSs with KEGG database revealed that 1973 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. 5).

The genes and gene clusters potentially contributed to the metabolism of DEHP, nitrogen and phosphate are listed in Table 2. 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 nitrate 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  $\beta$ -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 phosphate, 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 [49]. Phosphate transport system regulatory protein (PhoU) could regulate the transport of extracellular phosphate into cells while the transportation was mediated by a low-affinity inorganic phosphate transporter (PIT). Two poly phosphate kinases (PPK and PPK2) and an 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 [50]. 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 [51]. 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 one of the major driving forces for aerobic denitrification [52]. Meanwhile, study has demonstrated that  $\alpha$ -ketoglutarate generated by carbon source metabolism is an important substrate for glutamate biosynthesis since  $\alpha$ -ketoglutarate provides a carbon skeleton for microbial ammonium assimilation [53]. For the simultaneous elimination 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 [14,54,55]. These microbes are termed as denitrifying polyphosphate accumulating organisms (dPAOs) and are able to derive energy from utilization of external carbon sources unlike PAOs [56]. Hence, strain RL-GZ01 can be defined as a typical dPAO which could simultaneously remove nitrogen and phosphorus 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



**Fig. 6.** Simultaneous removal of DEHP, nitrogen and phosphorus from municipal wastewater (A: DEHP, B:  $\text{NH}_4^+\text{-N}$ , C:  $\text{NO}_3^-\text{-N}$ , D:  $\text{NO}_2^-\text{-N}$ , E: total nitrogen, F:  $\text{PO}_4^{3-}\text{-P}$ , G: COD).

phosphate, strain RL-GZ01 has great application potential in the treatment of PAEs containing wastewater.

### 3.5. Simultaneous removal of nitrogen, phosphate and DEHP from municipal wastewater

To evaluate the potential of strain RL-GZ01 in wastewater treatment, the simultaneous removal of nitrogen, phosphate and DEHP from municipal wastewater was conducted (Fig. 6). As shown in Table S4, the total nitrogen content (74.51 mg/L) in municipal wastewater was mainly due to  $\text{NH}_4^+\text{-N}$  (70.91 mg/L) while the concentration of  $\text{PO}_4^{3-}\text{-P}$  and COD value of wastewater were 10.89 mg/L and 622.4 mg/L, respectively. 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, concentrations of TN and  $\text{NH}_4^+\text{-N}$  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  $\text{NH}_4^+\text{-N}$  were reduced to 4.40 mg/L and 3.72 mg/L, respectively. The COD value showed similar tendency with TN and  $\text{NH}_4^+\text{-N}$ . Specifically, no obvious changes of  $\text{PO}_4^{3-}\text{-P}$  concentration was observed in the control treatment while the concentration of  $\text{PO}_4^{3-}\text{-P}$  was reduced to 2.58 mg/L with inoculation of strain RL-GZ01. Meanwhile, after inoculation of strain RL-GZ01, DEHP (5.17 mg/L) in the wastewater was completely degraded while the DEHP concentration in the control treatment was decreased to 4.65 mg/L after 36 h. Since the concentration of  $\text{NO}_3^-\text{-N}$  and  $\text{NO}_2^-\text{-N}$  were very low, their contribution to TN and COD was limited and therefore insignificant. Thus, the total removal percentages of DEHP, TN,  $\text{NH}_4^+\text{-N}$ ,  $\text{PO}_4^{3-}\text{-P}$ , 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 %, 76.30 %, 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 phosphate removal ability in the treatment of DEHP-containing municipal wastewater as that in the test media.

To improve existing wastewater treatment systems, the opportunities is to develop biological treatment processes for the concurrent removal of conventional pollutants (essentially nitrogen and phosphorus) and emerging pollutants. Most of the existing biological wastewater treatment systems are not designed for the elimination of emerging pollutants. The current status highlights the urgent need to develop methods capable of simultaneously removing nutrients and emerging pollutants. Zhao et al. [57] 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. [58] reported a novel Zn (II)-resistant *Pseudomonas stutzeri* KY-37 which was capable of eliminating nitrogen and bisphenol A from wastewater at the same time. Both strain RL-GZ01 and strain KY-37 can remove nitrogen and emerging pollutants from wastewater. Specifically, strain KY-37 was identified as a Zn(II)-resistance aerobic denitrifier which suggested that strain KY-37 harbored great application potential in some specific situation (wastewater contaminated with nitrogen, BPA-like endocrine-disrupting chemicals, and metals). In contrast, strain RL-GZ01 would be more applicable for the treatment of municipal wastewater since PAEs has been identified as a widely distributed emerging pollutants in municipal wastewater (Table S1). Gani et al. [59] 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. Xu et al. [60] fabricated a novel biological carrier combining sponge and modified walnut shell biochar with  $\text{Fe}_3\text{O}_4$  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, phosphate and DEHP was achieved by strain *Rhodococcus pyridinivorans* RL-GZ01. Strain RL-GZ01 showed preference to alkaline conditions and good tolerance to salinity and organic solvents. The kinetics analysis demonstrated that the metabolism process of DEHP in strain RL-GZ01 was well fitted with the modified Gompertz model ( $R^2 = 0.9985$ ). The catabolic pathway of DEHP in strain RL-GZ01 was deduced by metabolites identification. Prior to de-esterification, DEHP was transformed into di-(2-ethyl hexyl) phthalate (DEP) via  $\beta$ -oxidation while DEP was further de-esterized into phthalic acid. Phthalic acid was transformed into catechol via benzoate while catechol was utilized for cell growth through catechol branch of the  $\beta$ -ketoadipate pathway. Genes involved in the degradation of DEHP were identified via genome sequencing analysis. The KEGG nitrogen metabolism pathway indicated that nitrate was transformed into nitrite by a respiratory nitrate reductase while nitrite was further transformed into ammonium through a nitrite reductase. Ammonium was used for the biosynthesis of L-glutamine and L-glutamate via glutamine synthetase and glutamate synthase, respectively. Strain RL-GZ01 was further identified as a dPAO for its capacity of phosphate accumulation. Finally, the application of strain RL-GZ01 in municipal wastewater treatment was conducted for simultaneous removal of nitrogen, phosphate and DEHP. The removal percentages of DEHP, TN,  $\text{NH}_4^+\text{-N}$ ,  $\text{PO}_4^{3-}\text{-P}$  and COD by strain RL-GZ01 in municipal wastewater were 89.94 %, 64.45 %, 64.94 %, 76.30 % and 63.23 %, respectively, suggesting excellent application potential of RL-GZ01 in treating PAEs containing wastewater.

## 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

I have shared the link to my data.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jwpe.2023.103532>.

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