

Article

Harnessing *Bacillus thuringiensis* and *Bacillus cereus* for Effective Biodegradation of Endocrine Disruptor 4-Nonylphenol

Lian Yang, Fanglian Lu, Deqin Luo and Ranran Dong *

College of Animal Science, Guizhou University, Guiyang 550025, China; gs.lianyang22@gzu.edu.cn (L.Y.); gs.fllu23@gzu.edu.cn (F.L.); gs.dqluo24@gzu.edu.cn (D.L.)

* Correspondence: rrdong@gzu.edu.cn; Tel.: +86-18286079830

Abstract

4-Nonylphenol (4-NP), an important fine chemical precursor, can cause endocrine disruption, resist natural degradation, and bioconcentrate. Biodegradation is an effective and environmentally sustainable approach to its remediation. This study employed a mixture comprising equal proportions of six non-pathogenic *Bacillus* strains to screen and identify strains capable of degrading 4-NP, and degradation rate was measured using an ELISA kit, and metabolomic analyses and whole-genome sequencing were used to investigate the response of *Bacillus* to 4-NP and elucidate pathways involved in 4-NP degradation. The results revealed DY and LY strains isolated at 500 µg/L 4-NP. The DY strain was identified as *Bacillus thuringiensis*, and the LY strain was identified as *Bacillus cereus* via physiological, biochemical and PCR analyses. The degradation efficiency of a DY and LY strain mixture was 79.45% after 7 days. At 1000 µg/L 4-NP, only the LY strain was successfully isolated. Whole-genome sequencing indicated that the LY strain (accession number: CRA021210) shares the highest homology with *B. cereus* strain FORC-047. Notably, it showed a degradation rate of 86.34% after 7 days. Metabolomics analysis indicates that 4-NP affects the degradation pathways of aromatic compounds and benzoic acid in *B. cereus*. Combined with genome data, it is hypothesized that the 4-NP degradation pathway involves its conversion to p-hydroxybenzoic acid, catalyzed by monooxygenases, dioxygenases and oxidases. Subsequently, p-hydroxybenzoic acid degrades via one of two potential pathways: it produces phenol through decarboxylase or is oxidized to benzoic acid by monooxygenase. In summary, the DY and LY strains are capable of degrading 4-NP. Furthermore, we postulate potential 4-NP degradation pathways, providing insights for the remediation of 4-NP in aquatic environments.

Keywords: 4-Nonylphenol; biodegradation; *Bacillus*; metabolomics; whole-genome sequencing; bioremediation



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1. Introduction

4-Nonylphenol (4-NP) is an environmental endocrine disruptor, classified as an emerging pollutant [1]. It is widely used in the manufacturing of various pesticides, industrial chemicals, and additives due to its cost-effectiveness and high efficiency [2]. Its widespread presence in urban, industrial, and domestic wastewater systems has significant toxic effects on aquatic organisms and human health, including endocrine disruption, decreased fecundity and carcinogenesis [3,4]. Consequently, the remediation of 4-NP-contaminated environments is of critical importance.

Remediation strategies for 4-NP include physicochemical and biological techniques [5]. Physicochemical methods such as adsorption, membrane filtration, and advanced oxidation processes are effective but often face limitations, including high cost, the generation of toxic by-products, and limited recyclability [6–11]. In contrast, bioremediation, particularly microbial degradation, is efficient, economical, and sustainable [12]. To date, approximately 37 NP-degrading bacterial strains have been isolated from aquatic environments [5]. However, a significant limitation of the currently available strains is that many are either pathogenic (e.g., *Shigella*, *Aeromonas* and *Shewanella*) or conditionally pathogenic (e.g., *Enterobacter*, *Acinetobacter* and *Pseudomonas*), which raises safety concerns for their application in environmental remediation [13–15]. Furthermore, the degradation mechanisms of non-pathogenic strains remain insufficiently researched, and comprehensive studies integrating multi-omics approaches to elucidate these pathways are still lacking.

To address these gaps, we screened and identified non-pathogenic *Bacillus* strains with high 4-NP degradation efficiency and investigated their degradation mechanism. We evaluated six non-pathogenic *Bacillus* strains isolated from fish—*B. cereus*, *Bacillus licheniformis*, *B. Thuringiensis*, *Bacillus tropicus*, *Bacillus wiedmannii*, *Bacillus amyloliquefaciens*—for their capacity to degrade 4-NP via stepwise acclimation. Efficient degraders were identified through physiological, biochemical, and molecular assays. More importantly, the degradation pathway of 4-NP was elucidated for the first time in these non-pathogenic *Bacillus* strains through an integrative analysis combining whole-genome sequencing and metabolomics. Our findings provide novel insights into the use of safe bacterial agents and their mechanistic basis for the bioremediation of 4-NP in aquatic environments.

2. Materials and Methods

2.1. Preliminary Bioinformatics Screening of 4-NP-Degrading Bacteria

Genomic sequences of *Bacillus* were obtained from the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) for analysis of the enzymes associated with degradation.

2.2. Screening of 4-NP-Degrading *Bacillus*

The *B. Cereus*, *Bacillus licheniformis*, *B. Thuringiensis*, *Bacillus tropicus*, *Bacillus wiedmannii* and *Bacillus amyloliquefaciens* used in this study were previously isolated from southern catfish. The analytical standard nonylphenol (NP, C₁₅H₂₄O; purity: 93.8%; molecular weight 220.35 kg/mol; CAS: 84852-15-3) was purchased from Sigma-Aldrich. Strains tolerant to 4-NP were screened on Tryptic Soy Agar (TSA) medium with a pH of 7.3, using a stepwise increase in the 4-NP concentration. Plates were coated 10 µg/L, 100 µg/L, 300 µg/L, 400 µg/L, 500 µg/L, 600 µg/L, 800 µg/L and 1000 µg/L 4-NP, with TSA medium without 4-NP as a control. A 100 µL logarithmic-phase bacterial culture was inoculated into TSA medium. Each group was established in triplicate. First, the bacteria were streaked and inoculated separately onto 4-NP tryptone soy agarose (TSA) plates, progressively increasing the concentration from 100 to 500 µg/L to select for tolerant strains (Steps 1–3). Then, 1 mL of selected isolates was cultivated in liquid Tryptic Soy Broth (TSB) with 4-NP to confirm growth (Steps 4 and 5). Subsequently, the strains' ability to utilize 4-NP as a sole carbon source was tested on a minimal salt medium (MSM). As no growth occurred, glucose was added as an auxiliary carbon source. The isolates grew successfully on the amended medium with 4-NP concentrations of up to 500 µg/L (Steps 6–9). The domesticated and screened strains were preliminarily identified through 16S rDNA sequencing (universal bacterial primers: F:5'-AGAGTTTGATCATGGCTCAG-3', R:5'-ACGGTTA CCTTGTTACGACTT-3') [16]. The PCR products were purified and sequenced by Sanger sequencing on an ABI 3730XL DNA Analyzer (Thermo Fisher Scientific, Vacaville, CA, USA).

2.3. Effects of 4-NP on the Morphology of *Bacillus*

Well-established *Bacillus* cultures were collected and prepared for scanning electron microscopy by sequential processing: primary fixation with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) for structural preservation, secondary fixation with 1% osmium tetroxide for membrane stabilization and contrast enhancement, and stepwise dehydration through a graded acetone series (30–100%). After critical point drying and gold-palladium sputter-coating, the samples were examined using a JEM-1400FLASH scanning electron microscope (Japan Electronics Co., Ltd., Tokyo, Japan) at an accelerating voltage of 5–15 kV to visualize morphological changes before and after 4-NP exposure.

2.4. Morphological, Physiological and Biochemical Identification

The selected strains were inoculated individually onto TSA and TSB (Tryptic Soy Broth) media, followed by incubation at 36 °C for 18 to 24 h. Initial identification was conducted by observing the colony size, color, and shape on solid TSA plates. Gram staining was used to observe the morphological features and staining characteristics. Subsequently, physiological and biochemical analyses included tests for carbohydrate fermentation (Xylose, Sorbitol, Adonitol, Raffinose, Glucose), enzyme activity and metabolic traits (Peptone water, Gluconate, Urea, Hydrogen sulfide, Phenylalanine deaminase, Ornithine decarboxylase, Lysine decarboxylase), and substrate utilization (Citrate, Semi-solid agar for motility). Analyses were performed in accordance with the instructions provided with the commercial biochemical identification tubes used, which were purchased from Hangzhou Microbial Reagent Co., Ltd. (Hangzhou, China). The antibiotic sensitivity discs were obtained from Changde Biocomma Biotechnology Co., Ltd. (Changde, China).

2.5. Whole-Genome Sequencing to Identify 4-NP-Degrading Bacteria

The 4-NP-degradation bacteria, harvested from the TSB culture medium during the logarithmic growth phase, were subjected to centrifugation at 8000 rpm, flash-frozen in liquid nitrogen, and subsequently transported to Guangdong Magigene Biotechnology Co., Ltd. (Zhaoqing, China). for sequencing analysis. A sequencing library was constructed using the SQK-LSK109 ligation kit, following the manufacturer's protocol, including DNA damage repair, end repair and A-tailing, and adapter ligation. The resulting libraries were loaded onto R9.4.1 flow cells and sequenced on the Nanopore PromethION platform. Base calling was performed using Guppy (v3.2.6), with the high-accuracy model. Raw reads were filtered using NanoFilt (v2.8.0) to generate subreads, which comprised reads longer than 2000 bp and served as the input for genome assembly with Canu v1.5 [17] and wtdbg2 v2.2 [18]. Functional annotation of the genes was performed using NCBI (<ftp.ncbi.nlm.nih.gov>), KEGG (<http://www.kegg.jp>), and CARD (<https://card.mcmaster.ca/>).

2.6. Determination of 4-NP Concentration

A degradation experiment was conducted with a 1:1 mix of *B. thuringiensis* and *B. cereus*, as well as *B. cereus* alone, in an oscillating incubator at 30 °C and 150 rpm for 7 days. The experiment was carried out in a defined inorganic salt medium (Minimal Salt Medium, MSM) with the following composition (g/L): Na₂HPO₄ 2.5, KH₂PO₄ 1.5, (NH₄)₂SO₄ 0.5, MgSO₄ 0.1, CaCl₂ 0.04, EDTA-2Na 0.005, FeSO₄·7H₂O 0.002. The pH was adjusted to 7.0 and the medium was sterilized by autoclaving at 115 °C for 15 min. Inorganic salt cultures were collected on days 1, 3, and 7, and 4-NP concentration was measured using an ELISA kit (Shenzhen Rongjin Technology Co., Ltd., Shenzhen, China, Catalog No. RNC95011). The assay was performed strictly according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader, and the concentration of 4-NP in the samples was determined by interpolating from the standard curve.

2.7. Metabolomics Analysis

The experiment was divided into two groups: the 4-NP group, containing 1 mg/L 4-NP and 1 mg/L glucose, and the GL group, containing 1 mg/L glucose in the inorganic salt medium. Each group was incubated in a shaker at 150 rpm and 37 °C. After 72 h, the broth culture was collected and centrifuged at 3000 rpm and 4 °C for 10 min to obtain the supernatant. Six parallel samples from each group ($n = 6$) were subsequently sent to Beijing Omicson Gene Technology Co., Ltd. (Beijing, China). for non-targeted metabolomic analysis. The analysis was performed using ultra-high performance liquid chromatography (UHPLC) coupled to a Thermo Scientific Q-Exactive HF-X Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, California, USA).

Raw data were processed using Compound Discoverer software (Version 3.1, Thermo Fisher Scientific) for peak alignment, retention time correction, and peak area extraction. Metabolites were identified by querying the mzCloud and HMDB databases with a mass tolerance of 5 ppm. Multivariate statistical analysis, including orthogonal partial least squares-discriminant analysis (OPLS-DA), was performed using the ropls R package (Version 1.6.2). Differential metabolites were screened with variable importance in projection (VIP) > 1.0 and p -value < 0.05 from Student's t -test. Pathway analysis of differential metabolites was conducted using KEGG (<http://www.kegg.jp>) and MetaboAnalyst (Version 6.0) (<http://www.metaboanalyst.ca/>).

2.8. Statistical Analysis

All experiments were conducted in at least three independent replicates. Data are presented as mean $\pm$ standard deviation (SD) value. Statistical significance between groups was assessed using an unpaired two-tailed Student's t -test, and a p -value of less than 0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics 27.0.1 software.

3. Results

3.1. Primary Screening of 4-NP-Degrading Bacteria

Analysis of the *Bacillus* strains' genomes from the NCBI database revealed that they possess a variety of benzene ring degradation-related enzymes including PabB, YhbJ, YhcA, YhcB, MenC, DhbA, CatD and YhjG (Table 1), suggesting their potential for 4-NP degradation.

Table 1. Bioinformatics analysis of degradation-related enzymes in *Bacillus*.

Protein Abbreviation	Accessing Number	Function
PabB	NP_387955.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_387955.1%20Bacillus accessed on 12 February 2021	p -aminobenzoic acid synthetase
YhbJ	NP_388781.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388781.1+Bacillus accessed on 12 February 2021	Enzymes associated with benzoic acid degradation
YhcA	NP_388782.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388782.1+Bacillus accessed on 12 February 2021	Enzymes associated with benzoic acid degradation

Table 1. Cont.

Protein Ab- breviation	Accessing Number	Function
YhcB	NP_388783.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388783.1+Bacillus accessed on 12 February 2021	Quinone oxidoreductase related to benzoic acid
MenC	NP_390956.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388783.1+Bacillus accessed on 12 February 2021	O-Succinylbenzoate-CoA Synthase
DhbA	NP_391080.2 https://www.ncbi.nlm.nih.gov/gene/?term=NP_391080.2+Bacillus accessed on 12 February 2021	Benzoate dehydrogenase
CatD	NP_388704.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388704.1+Bacillus accessed on 12 February 2021	Oxygenase
YhjG	NP_388931.1 https://www.ncbi.nlm.nih.gov/gene/?term=NP_388931.1+Bacillus accessed on 12 February 2021	Aromatic compounds monooxyge- nase/hydroxylase

3.2. Screening of *Bacillus* Under 4-NP

By screening bacterial growth under adverse conditions, LY was ultimately determined to be the dominant bacterium (Table 2). With increase in 4-NP concentration, two dominant strains, DY and LY, were isolated at a 4-NP concentration of 500 µg/L. A dominant strain, LY, was isolated at a concentration of 1000 µg/L.

Table 2. Screening of degrading bacteria.

Steps	Screening Medium	Screening NP Concentration (µg/L)	Inoculation Regimes	Results
1	TSA solid medium	10, 100, 1000	Streaking	Growth at 100
2	TSA solid medium	100, 300, 600	Transferred all colonies from Step 1 plate	Growth at 300
3	TSA solid medium	300, 400, 500, 600, 700, 800	Washed all colonies from Step 3 plate with saline	Growth at 500
4	TSB liquid medium	100	Inoculated from Step 1; washed with saline	Turbid, growth
5	TSB liquid medium	500	Inoculated from Step 4; washed with saline	Turbid, growth
6	Inorganic salt solid medium	100, 300, 500	Transferred all colonies from Step 4 plate; inoculated with broth from Step 5	No growth
7	Inorganic salt solid medium	50, 100, 200, 300	Spread plate (1 µL, 10 µL, 100 µL); washed with saline	No growth
8	Inorganic salt solid medium	10, 50, 100, 300	Transferred all colonies from Step 4 plate; washed with saline	Growth at 300
9	Inorganic salt solid medium	300, 400, 500	Transferred all colonies from Step 8 plate	Growth at 500
10	Inorganic salt solid medium	500, 600, 700, 800	Transferred all colonies from Step 9 plate	Growth at 800
11	Inorganic salt solid medium	800, 900, 1000	Transferred all colonies from Step 10 plate	Growth at 1000

Note: At the end of step 9, there are two dominant bacteria, DY and LY, in the medium. By the end of step 11, LY has become the dominant bacterium on the medium.

3.3. Effect of 4-NP on *Bacillus* Morphology

Morphological alterations in the bacteria pre- and post-screening were examined using scanning electron microscopy. The analysis revealed that the DY strain exhibited dimensions ranging from 2.8 to 4.5 μm in length and 0.42 to 0.56 μm in width prior to screening. The bacteria were rod-shaped, with a smooth surface and clear arrangement. (Figure 1A). Post-screening, they appeared clustered and adherent (Figure 1B,C). Before screening, the LY strain was 1.8 to 3.1 μm long, 0.77 to 1.1 μm wide, rod-shaped, with a smooth surface, and arranged in chains (Figure 1a). After screening, its length was 1.5 to 2.5 μm and its width was 0.52 to 0.84 μm , and it exhibited collapsed ends, a rough surface, and an irregular arrangement (Figure 1b,c).

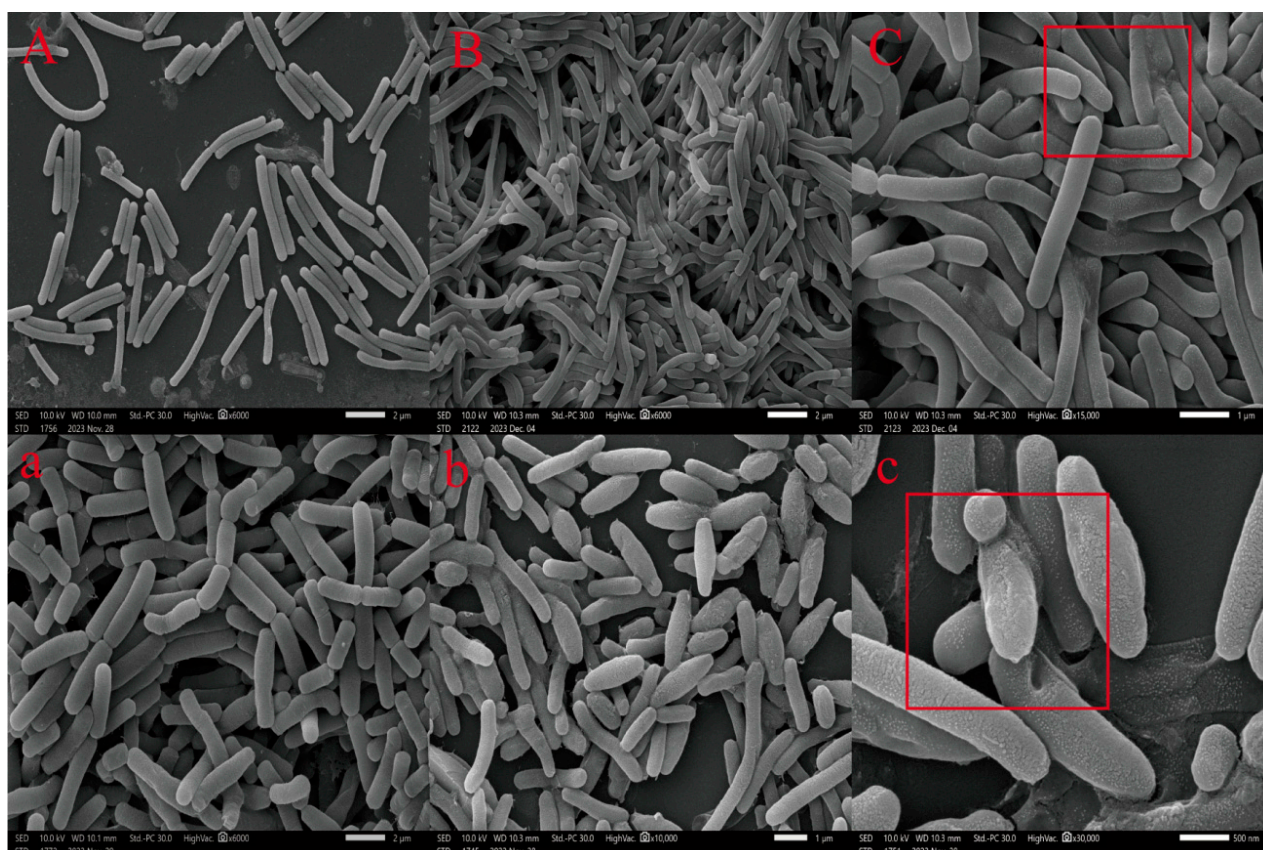


Figure 1. Scanning electron microscope images of *B. thuringiensis* strain DY and *B. cereus* strain LY before and after screening with 4-NP. (A) *B. thuringiensis* DY before screening; (B,C) *B. Thuringiensis* DY after screening at 500 $\mu\text{g/L}$ (B) and 1000 $\mu\text{g/L}$ (C) 4-NP. (a) *B. cereus* LY before screening; (b,c) *B. cereus* LY after screening at 500 $\mu\text{g/L}$ (b) and 1000 $\mu\text{g/L}$ (c) 4-NP. The red border in (C) showed that DY strain appeared clustered and adherent; The red border in c showed that LY strain exhibited a rough surface. All images were taken at a magnification of 5000 $\times$.

3.4. Morphological Identification Results for 4-NP Degrading Bacteria

After 18 h of incubation at 37 $^{\circ}\text{C}$, the DY colony had a diameter of 1 to 1.5 mm and appeared white and circular, with a rough, filamentous structure around the edge. In contrast, the LY colony, with a diameter of 1 to 2 mm, also appeared white and circular but had a relatively rough surface, low transparency, and a patchy texture. (Figure 2A,B). Both strains were identified as Gram-positive bacilli after Gram staining (Figure 2a,b).

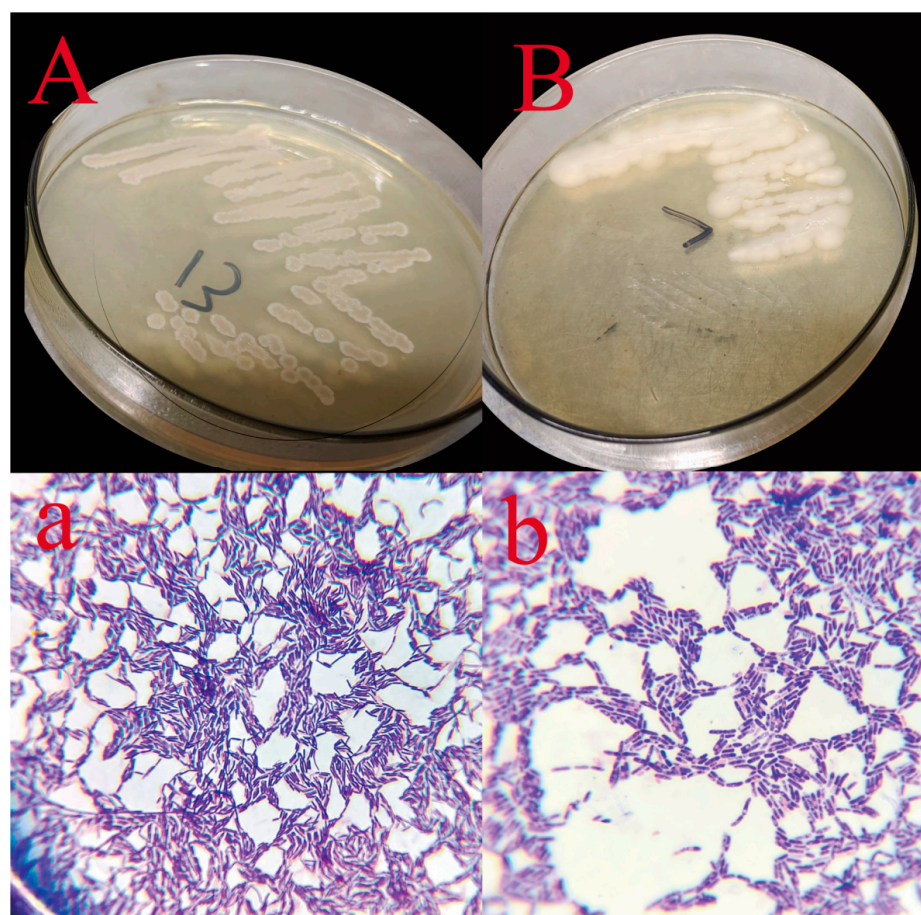


Figure 2. Colony morphology of *B. Thuringiensis* strain DY (A) and *B. cereus* strain LY (B), Gram staining of *B. Thuringiensis* strain DY (a) and *B. cereus* strain LY (b).

3.5. Physiological and Biochemical Identification Results

The DY strain tested negative for xylose, sorbose, adonitol, raffinose, peptone water, Simmons citrate, urea, hydrogen sulfide, phenylalanine deaminase, gluconate, ornithine decarboxylase, and lysine decarboxylase but positive for gas production from glucose, hydrogen sulfide, MR and VP, and motility in semi-solid agar. The LY strain had positive MR, VP, and agar test results, but all other tests were negative (Table 3). According to the *Manual of Common Bacterial Systematic Identification* [19] and *Bergey's Manual of Determinative Bacteriology* [20], these results are consistent with the biochemical characteristics of the *Bacillus*.

Table 3. Biochemical identification of DY and LY strains.

Test Item	Test Result		Test Item	Test Result	
	DY	LY		DY	LY
Xylose	—	—	Urea	—	—
Sorbitol	—	—	Hydrogen sulfide	+	—
Adonitol	—	—	Phenylalanine deaminase	—	—
Raffinose	—	—	Gluconate	—	—
Glucose	+	—	Ornithine decarboxylase	—	—
Peptone water	—	—	Lysine decarboxylase	—	—
Glufosinate water	+	+	Semi-solid agar	+	+
Citrate	—	—			

Note: “+” is positive; “—” is negative.

3.6. 16S rDNA Identification Results

The 16S rDNA sequencing results of the single colonies of DY and LY strains were compared using BLAST (Version 2.17.0) (Basic Local Alignment Search Tool). The results showed that the DY strain is 99.7% similar to *B. thuringiensis* strain IAM 12077, while the LY strain is 99.9% similar to *B. cereus* ATCC 14579.

3.7. Whole-Genome Sequencing Results for LY Bacteria

The LY Strain's genome sequence (accession number: CRA021210) was analyzed using BLAST, revealing that it exhibited the highest homology with *B. cereus* FORC_047 (Table 4). Further comparative genomic analysis using MUMmer software (version 3.23) confirmed strong collinearity between the LY and FORC_047 genomes (Figure 3), identifying the LY strain as *B. cereus*.

Table 4. Average Nucleic Acid Similarity Analysis.

Query ID	Reference ID	ANI	Mapped_Fragment	Query_Fragment	Taxon
LY	GCF_002220285	96.9227	1636	1785	<i>B. cereus</i> strain FORC_047

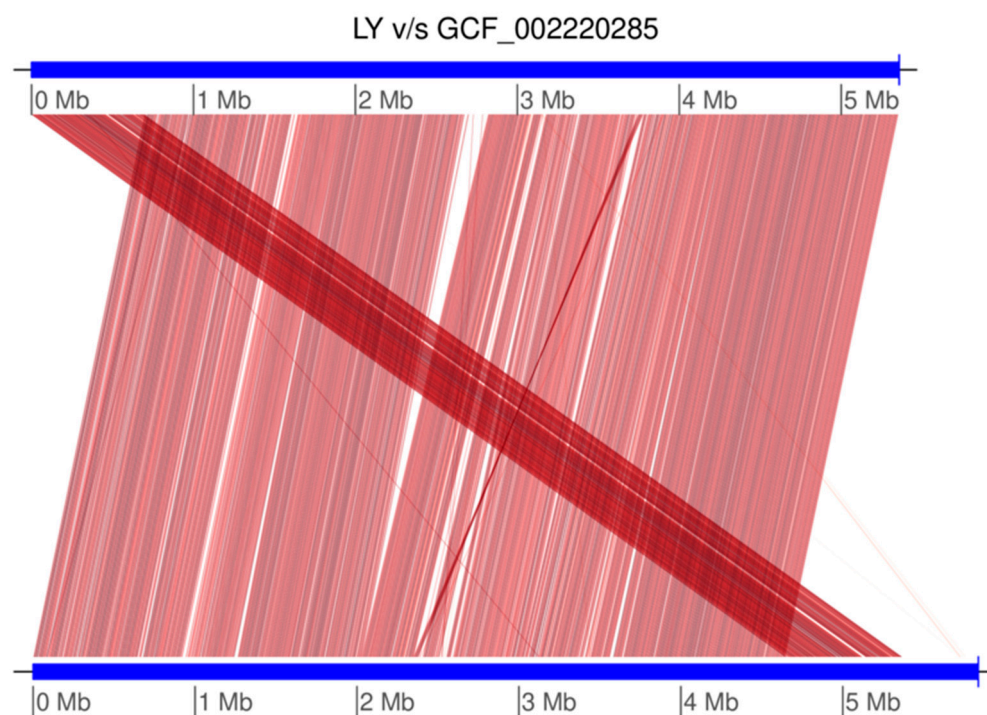


Figure 3. The collinearity diagram of LY strain and *B. cereus* FORC_047. Genomes depicted on the top and bottom horizontal bars, respectively. The horizontal bars represent the genomic sequences, with the scale in megabases (Mb), indicating the physical position along the chromosome. The diagram reveals a largely collinear genome architecture, suggesting a close phylogenetic relationship.

3.8. 4-NP Degradation Rate

During co-cultivation of the isolated strains *B. thuringiensis* DY and the isolated strains *B. cereus* LY, the degradation rate reached 79.45% over seven days at 500 µg/L 4-NP. However, when the 4-NP concentration was increased to 1000 µg/L, the degradation rate of the mixed culture decreased to 46.35%. In contrast, a monoculture of *B. cereus* LY showed a degradation rate of 59.45% within 7 days at 500 µg/L 4-NP, which increased to 86.34% at 1000 µg/L 4-NP (Figure 4). These results suggest that compared to the isolated strains *B. cereus* LY monoculture, the isolated strains *B. thuringiensis* DY and the *B. cereus* LY mixed

culture showed higher degradation efficiency at low concentrations. However, as *B. cereus* LY became the predominant degrading bacterium at high concentrations, it is possible that *B. thuringiensis* DY experienced inhibition, thereby impeding its growth and reducing the degradation rate.

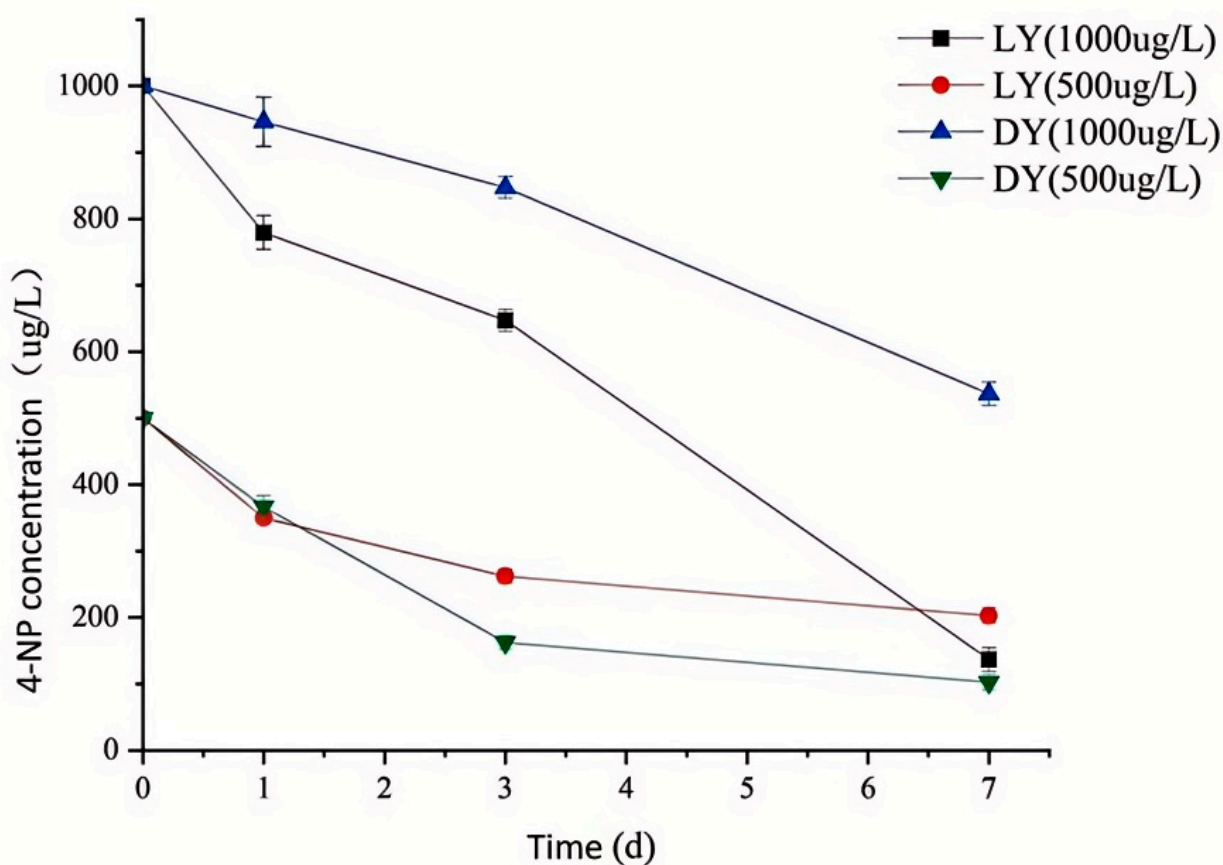


Figure 4. Detection of 4-NP concentration. ■ and ● degradation curves of *B. cereus* strain LY and *B. thuringiensis* strain DY under 1000 µg/L 4-NP. ▲ and ▼ degradation curves of *B. cereus* strain LY and *B. thuringiensis* strain DY under 500 µg/L 4-NP.

3.9. Effect of 4-NP on Metabolites of *B. cereus*

Six hundred forty-five metabolites were detected from the sample metabolites (Table 5). By combining univariate analysis (Student's *t*-test) and multivariate analysis (OPLS-DA), calculating variable importance in projection (VIP) values, and integrating *p*-values or fold change values from the univariate analysis, differential metabolites were further screened [21]. A total of 120 differential metabolites were identified. Notable examples include key intermediates in aromatic compound degradation such as *p*-hydroxybenzoic acid and catechol, as well as amino acids like L-glutamate and sugars such as D-ribose, which are indicative of broader metabolic shifts. Correlation analysis was performed on significant differential metabolites (Figure 5), revealing that the majority of differential metabolites showed strong positive correlations.

Table 5. Metabolite number statistics.

Mode	Total Ion Number	After Pre-Processing	Ratio (%)
merge	645	645	100.00%

Note: Databases include KEGG Database, Human Metabolome Database (HMDB), and METLIN database.

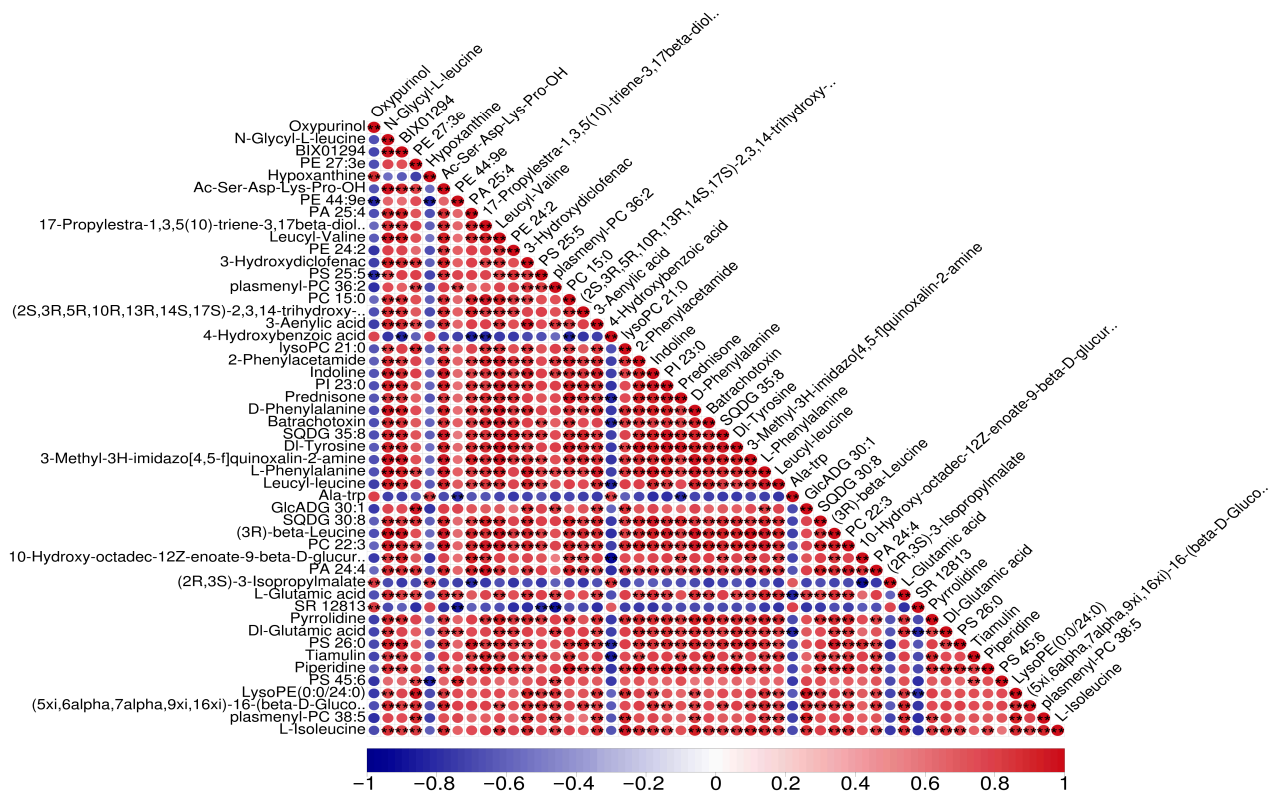


Figure 5. Differential metabolite correlation heat map. The heatmap displays Pearson correlation coefficients among the identified differential metabolites (VIP > 1.0 and *p*-value < 0.05). Each row and column represent a single metabolite. The color scale ranges from blue (strong negative correlation, *r* = −1) to red (strong positive correlation, *r* = +1). Metabolites and clusters were grouped by hierarchical clustering. Distinct blocks of high positive correlation (red squares) suggest co-regulated metabolic pathways, while strong negative correlations (blue squares) indicate potential inverse relationships in their abundances.

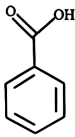
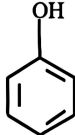
3.10. Degradation-Related Pathways in Metabolome

From the KEGG results, pathways associated with the degradation of 4-NP were screened, identifying two potential degradation pathways: the degradation of aromatic compounds and the degradation of benzoic acid. The primary metabolic products identified were p-hydroxybenzoic acid, benzoic acid, and phenol (Table 6). Moreover, p-hydroxybenzoic acid interconverts with benzoic acid and phenol (Figure 6A,B), indicating that these three metabolites may serve as intermediate products in 4-NP degradation.

Table 6. Possible degradation pathways of 4-NP by metabolites KEGG annotation.

Metabolite	Structural Formula	Pathway
4-Hydroxybenzoic acid	<chem>O=C(O)c1ccc(O)cc1</chem> C00156	Degradation of aromatic compounds Benzoate degradation

Table 6. Cont.

Metabolite	Structural Formula	Pathway
Benzoic acid	 C00180	Degradation of aromatic compounds Benzoate degradation
Phenol	 C00146	Degradation of aromatic compounds Benzoate degradation

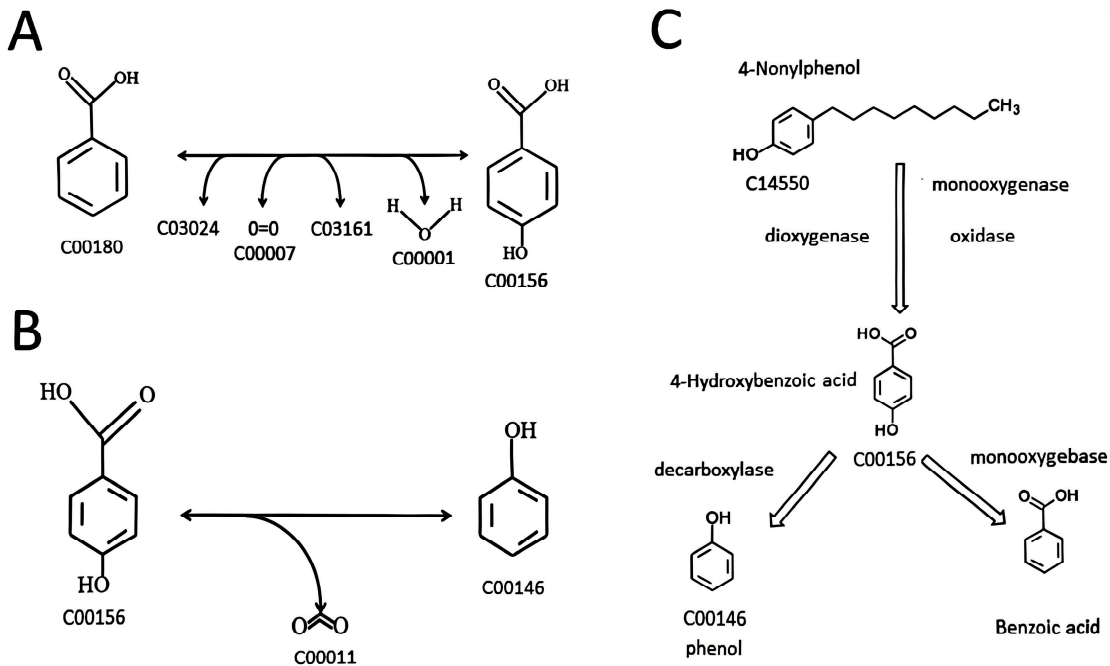


Figure 6. (A) Conversion between benzoic acid and p-hydroxybenzoic acid by KEGG. C00180 is benzoic acid; C00156 is p-hydroxybenzoic acid. (B) Conversion between p-hydroxybenzoic acid and phenol by KEGG; C00146 is phenol. (C) Possible pathways for LY strain to degrade 4-NP. The NP degradation pathway in *B. cereus* involves an initial step where 4-NP is oxidized to p-hydroxybenzoic acid, likely catalyzed by dioxxygenases or monooxygenases. Subsequently, p-hydroxybenzoic acid is transformed into phenol (via decarboxylation) or benzoic acid (through oxidative decarboxylation), reactions mediated by decarboxylases or monooxygenases, respectively.

3.11. Possible Pathways for 4-NP Biodegradation

The prospective 4-NP degradation pathway of *B. cereus* was inferred from the metabolomic and whole-genome analyses (Figure 6C). It is hypothesized that the degradation pathway involves 4-NP’s conversion to p-hydroxybenzoic acid, catalyzed by monooxygenases, dioxxygenases, and oxidases. Subsequently, p-hydroxybenzoic acid undergoes one of two potential degradation pathways: it produces phenol through decarboxylase, or is oxidized to benzoic acid by monooxygenase.

4. Discussion

4.1. 4-NP Is Degraded by *B. thuringiensis* and *B. cereus*

The bioremediation of 4-NP in aquatic environments has been demonstrated using bacteria such as *Enterobacteriaceae*, *Pseudomonas* and *Aeromonas* [22–24]. However, using these bacteria in aquatic environmental remediation poses a risk of toxic by-products [25]. Moreover, non-pathogenic bacteria exhibit limited competitiveness against indigenous pathogens for survival, resulting in a scarcity of suitable non-pathogenic degraders for bioremediation applications [5]. In contrast to these limitations, our study successfully isolated and identified the non-pathogenic bacteria *B. thuringiensis* and *B. cereus* through successive domestication, offering a safer and more robust alternative for bioremediation. *Bacillus*, comprising aerobic or facultative anaerobic Gram-positive bacteria, is ubiquitous in diverse environments including air, water, and soil [26,27]. Notably, *Bacillus* species demonstrate remarkable resilience under harsh and arid conditions [27] and are known for their ability to adsorb and reduce toxic substances [28]. Consequently, *Bacillus* are frequently utilized in probiotic development [29]. Furthermore, because *B. cereus* reduces pathogens in the intestine and enhances host immunity it is widely employed as a microecological agent and feed additive in aquaculture [24,30], underscoring its environmental compatibility. Our findings confirm the degradative prowess of these two strains. While *B. cereus* has been reported to degrade phenol efficiently [31], this study demonstrates its high efficacy against 4-NP, achieving a degradation efficiency of 86.34%. This performance surpasses literature values for other documented degraders, such as *Enterobacter asburiae* (>50%) and *Pseudomonas putida* (75.28%) [22,23]. Both *B. thuringiensis* and *B. cereus* are known to inhibit harmful microorganisms like *Vibrio*, *Pseudomonas*, and *Aeromonas* [32–34]. By integrating this high degradation efficiency with their inherent environmental hardiness and established safety profile, *B. thuringiensis* and *B. cereus* offer a comprehensive and promising solution for the bioremediation of 4-NP.

4.2. Possible Degradation Pathways of *B. cereus*

By combining metabolomics and whole-genome data, we propose a putative pathway for 4-NP degradation in *B. cereus*, involving its transformation into p-hydroxybenzoic acid, benzoic acid, and phenol, likely catalyzed by oxidases or oxygenases. This hypothesis is consistent with previous reports identifying p-hydroxybenzoic acid as a key intermediate during 4-NP degradation under electrical stimulation [35] and is analogous to the pathway of malachite green degradation by *B. cereus* [36]. While these similarities support the plausibility of our proposed pathway, a critical distinction in our work is the use of an integrated multi-omics approach to investigate this pathway specifically in a non-pathogenic *Bacillus* strain, as many prior mechanistic studies have relied on single-method analyses or focused on pathogenic genera. These findings suggest that *B. cereus* primarily degrades aromatic compounds via hydroxylation and oxidation reactions, involving the benzoic acid degradation pathway. The metabolite p-hydroxybenzoic acid is generally considered harmless and can even inhibit bacterial growth [37]. Benzoic acid, a common environmental pollutant, has been extensively studied, and its degradation is well-understood [38]. In comparison with 4-NP, phenol is a less toxic organic compound for which mature degradation technologies are available [39]. It has been hypothesized that in the *Pseudomonas putida* MT4, NP is first converted to a carboxylic acid via an alkane hydroxylase. This is followed by the cleavage of the aromatic ring, generating straight-chain carboxylic acids that enter the tricarboxylic acid (TCA) cycle and are completely oxidized to CO₂ and H₂O [40]. This proposed terminal step, wherein the intermediates enter the TCA cycle, is consistent with our own findings. In summary, the degradation of 4-NP by *Bacillus* is effective and can harm and improve polluted aquatic environments. However, it should be noted that the

proposed pathway remains hypothetical. In the future, degradation experiments will be conducted using carbon-13-labeled 4-NP. Intermediate products will be tracked by mass spectrometry to determine the pathway of 4-NP degradation. In addition, we will use heat-killed bacteria as control to definitively confirm biodegradation over adsorption, which represents a limitation of the present study.

5. Conclusions

In this study, we successfully screened and isolated two non-pathogenic *Bacillus* strains, *B. thuringiensis* and *B. cereus*, capable of degrading 4-NP via stepwise acclimation. Integrated metabolomic and genomic analyses proposed that *B. cereus* degrades 4-NP through hydroxylation and oxidation reactions, situating the process within the benzoic acid degradation pathway. Our findings provide not only promising, environmentally safe microbial resources for 4-NP bioremediation but also offer novel mechanistic insights into the metabolic capabilities of non-pathogenic *Bacillus* strains. Future work should focus on the degradation of carbon-13-labeled 4-NP to track intermediate products and test the efficacy of these strains in contaminated aquatic environments. The use of non-pathogenic *Bacillus* strains, which are compatible with aquatic ecosystems and even beneficial as probiotics in aquaculture, addresses a key safety challenge that has hindered the practical application of many previously reported degrading bacteria, highlighting the potential of our findings for developing safe bioremediation agents.

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