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论文题目： Mechanistic Insights into pH-Dependent Catalytic Behavior and Enzyme Structural Responses during Toluene Biodegradation for Green Treatment of Contaminated Water

Abstract

Environmental pH is a critical operational parameter influencing microbial treatment of aromatic-hydrocarbon-contaminated water, yet its molecular effects on biodegradation remain insufficiently understood. Here, pH-dependent toluene biodegradation by *Pseudomonas* sp. BO3-4 was investigated by integrating biodegradation kinetics, GC–MS metabolite profiling, recombinant enzyme assays, and computational protonation-state analysis. BO3-4 completely degraded 50 mg·L⁻¹ toluene within 18 h across pH 5.0–9.0, with the highest apparent first-order rate constants observed at pH 8.0–9.0 (0.233–0.235 h⁻¹). GC–MS analysis revealed a marked redistribution of pathway-associated metabolites, with TDO-associated aromatic-ring oxidation signals increasing from 44.5% at pH 5.0 to 75.3% and 78.9% at pH 7.0 and 9.0, respectively. Consistently, the apparent catalytic activity of the recombinant Tod system increased by 1.44- and 1.61-fold at pH 7.0 and 9.0 relative to pH 5.0. Protonation-state analysis further identified His385, His390, and Glu424 as distal pH-responsive residues in TodC1, suggesting that pH-dependent changes in its electrostatic microenvironment may contribute to the catalytic response of the Tod system. These findings deepen the mechanistic understanding of pH-regulated microbial degradation and provide a scientific basis for improving the efficiency, stability, and sustainability of biological treatment of aromatic-hydrocarbon-contaminated environments.

Keywords: Toluene biodegradation; *Pseudomonas*; pH regulation; toluene dioxygenase; enzyme protonation

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1 Introduction

Petrochemical and chemical industries are important sources of aromatic hydrocarbon contamination in wastewater, soil, and groundwater, among which benzene, toluene, ethylbenzene, and xylenes (BTEX) are of particular concern because of their toxicity, mobility, and widespread occurrence. Recent field investigations indicate that this problem is both regionally prevalent and locally severe. A survey of 372 surface-soil samples and 96 soil profiles from 33 industrial parks in the Yangtze River Delta detected monoaromatic hydrocarbons in all surface samples; BTEXS showed detection frequencies of 90.9–100% and accounted for more than 78.2% of the total monoaromatic hydrocarbon burden (Zhu et al., 2023). At a chemical industrial site in southern China, groundwater benzene was detected in 61% of samples and reached a maximum of $340 \text{ mg}\cdot\text{L}^{-1}$, while toluene and xylenes reached 4.07 and $6.0 \text{ mg}\cdot\text{L}^{-1}$, respectively (Huang et al., 2024). Similarly, groundwater beneath a petrochemical site near the Yangtze River contained $644 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ benzene, $208 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ ethylbenzene, and $161 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ m/p-xylene at a contaminated location (Chen et al., 2022). In response to these risks, China has tightened regulation and source control of industrial water and groundwater pollution. Since 2021, the Ministry has conducted groundwater surveys in chemical parks, enhanced monitoring of key sources, and issued zoning guidelines. Recently, action plans on soil pollution prevention and new pollutant treatment have further strengthened source control, lifecycle management, and cross-media coordination. Together, these measures

indicate that pollution control in chemical industries is shifting from end-of-pipe treatment toward systematic, source-oriented, green and low-carbon environmental management.

Within this policy and engineering context, biological treatment is attractive because microorganisms can transform or mineralize aromatic contaminants through enzyme-catalyzed reactions under relatively mild conditions, potentially reducing chemical consumption and energy demand. This advantage is increasingly important because intensified physicochemical treatment can carry a considerable carbon penalty. For example, upgrading a large petrochemical wastewater treatment plant significantly improved the removal of organic micropollutants, but electricity consumption increased by 44.1%, resulting in higher indirect carbon emissions (Wu et al., 2018). A major challenge, however, lies in translating high biodegradation potential observed under controlled laboratory conditions into stable performance under fluctuating industrial environments. In a full-scale petrochemical biofilter, elevated pH and solids reduced COD removal from 50.1% to 30.0% and suppressed *Pseudomonas* and other key genera (Wang et al., 2024). Among these environmental factors, pH is particularly important because it simultaneously influences whole-cell physiology and molecular catalysis. Experimental evidence confirms this sensitivity: *Stenotrophomonas maltophilia* T3-c maintained toluene-degrading activity mainly within pH 5–8, while mixed consortia also exhibited clear pH-dependent changes in toluene biodegradation kinetics (Lee et al., 2002). These findings suggest that pH is not merely a routine operating parameter, but a key factor determining the robustness of aromatic-hydrocarbon biodegradation.

Despite this importance, most environmental studies have treated pH primarily as an empirical optimization variable, focusing on bacterial growth, removal efficiency, or an “optimal pH,” while the molecular basis of pH-dependent catalytic behavior remains poorly resolved. This gap is particularly relevant to aerobic BTEX biodegradation, in which the initial oxygenation reaction often constitutes a decisive step linking environmental conditions to downstream metabolic flux. In the classical *tod* pathway of *Pseudomonas*, toluene dioxygenase initiates aromatic-ring oxidation, followed by formation of 3-methylcatechol and subsequent ring-cleavage reactions (Zylstra et al., 1988). Toluene dioxygenase belongs to the Rieske non-heme iron oxygenase family and functions through coordinated electron transfer involving a reductase, ferredoxin, a Rieske [2Fe–2S] center, and a mononuclear iron catalytic site (Friemann et al., 2009; Jiang et al., 1999). Such a multicomponent catalytic system may be sensitive to its acid–base microenvironment because environmental pH can alter the protonation states of ionizable amino-acid residues and thereby influence electrostatic interactions, substrate binding, proton transfer, conformational stability, and catalytic kinetics. Studies of protein protonation and enzyme catalysis have consistently shown that pH-dependent changes in protonation can regulate protein structure, ligand binding, and reaction efficiency (Schönichen et al., 2013; Silverstein, 2021). Therefore, the unresolved scientific problem is no longer simply whether pH affects BTEX degradation, but how environmental pH is translated into changes in enzyme structure, catalytic activity, and metabolic pathway utilization, and ultimately into altered pollutant removal performance. Establishing this structure–activity–

performance relationship is essential for moving from empirical pH adjustment toward mechanism-guided regulation of biological treatment.

To resolve this mechanistic gap, a model system is required in which both aromatic-ring and side-chain oxidation can be examined under the same environmental perturbation. *Pseudomonas* sp. BO3-4 was selected as a representative field-derived aromatic-hydrocarbon degrader with complementary *tod*- and *xyl*-associated catabolic pathways. Using toluene as a model BTEX compound, this study investigates how environmental pH regulates biodegradation across kinetic, enzymatic, structural, and metabolic levels. Rather than merely identifying an empirical optimum, the study seeks to determine whether and how pH-induced changes in enzyme structure and catalytic activity are coupled to pathway utilization and overall degradation performance. By establishing a mechanistic linkage between environmental pH, enzyme response, catalytic behavior, and biodegradation efficiency, this work aims to define a robust operating pH window and provide a scientific basis for more stable and low-carbon biological treatment of aromatic-hydrocarbon-contaminated water in chemical industries.

2 Materials and Methods

2.1 Chemicals and culture media

Analytical-grade toluene was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *N,N*-Dimethylformamide (DMF), ethyl acetate, anhydrous sodium sulfate, acetonitrile, and *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from commercial suppliers and used without further purification. Toluene stock solution ($20 \text{ g}\cdot\text{L}^{-1}$) was prepared in DMF and stored in an amber glass bottle. The stock solution was diluted as required to obtain an initial toluene concentration of $50 \text{ mg}\cdot\text{L}^{-1}$ in the biodegradation experiments. A synthetic salt-defined medium (SSDM) was used for biodegradation experiments and contained (g L^{-1}): NH_4Cl , 0.20; NaCl , 7.947; $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, 0.77; $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 0.490; CaCl_2 , 0.076; NaHCO_3 , 0.010; KCl , 0.22; and NaBr , 0.026, supplemented with K_2HPO_4 and trace elements (Chen et al., 2023). LB medium ($10 \text{ g}\cdot\text{L}^{-1}$ tryptone, $5 \text{ g}\cdot\text{L}^{-1}$ yeast extract, and $10 \text{ g}\cdot\text{L}^{-1}$ NaCl) was used for routine cultivation of *Pseudomonas* sp. BO3-4. Media were adjusted to the required pH using 1 M HCl or NaOH and sterilized at 121°C for 20 min.

2.2 Strain and cultivation conditions

Pseudomonas sp. BO3-4, an indigenous aromatic-hydrocarbon-degrading bacterium previously isolated from long-term contaminated groundwater at a petrochemical site, was used in this study (Ouyang et al., 2025). The groundwater at the isolation site had a pH of approximately 6.8–7.4, and BO3-4 was obtained through enrichment with a mixture of BTEX and naphthalene. Previous genomic characterization identified complementary *tod*- and *xyl*-

associated catabolic gene clusters, which are involved in aromatic-ring dioxygenation and methyl side-chain oxidation, respectively. BO3-4 was therefore used as a model strain to investigate the pH-dependent catalytic response of alternative toluene degradation routes. The morphology of BO3-4 was characterized by scanning electron microscopy (SEM) following a previously described procedure (Czerwińska-Główka & Krukiewicz, 2021). Prior to biodegradation experiments, BO3-4 was activated in 150 mL of LB medium supplemented with 50 mg·L⁻¹ toluene and incubated at 20 °C and 150 rpm for 12 h (Pan et al., 2024). Cells were harvested by centrifugation at 8,000 rpm for 5 min and washed three times with sterile physiological saline (0.9%, w/v NaCl) to remove residual nutrients and toluene. The washed cells were subsequently resuspended in sterile mineral medium and adjusted to an initial optical density (OD₆₀₀) of 0.20 for subsequent experiments.

2.3 pH-dependent biodegradation experiments

The effects of environmental pH on toluene biodegradation by *Pseudomonas* sp. BO3-4 were investigated at initial pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. Experiments were conducted in 150-mL serum bottles containing 20 mL of sterile mineral medium. The medium pH was adjusted using 1 M HCl or 1 M NaOH and verified with a calibrated pH meter. Washed BO3-4 cells were inoculated to an initial OD₆₀₀ of 0.20, followed by the addition of toluene to an initial concentration of 50 mg·L⁻¹. The bottles were immediately sealed with PTFE-lined aluminum caps and incubated in the dark at 20 °C and 180 rpm. Uninoculated controls containing the same medium and 50 mg·L⁻¹ toluene, but without BO3-4, were incubated under otherwise identical conditions to account for abiotic toluene losses, including volatilization.

Samples were collected at 3-h intervals for determination of residual toluene concentrations and bacterial growth. All treatments were conducted in triplicate.

2.4 Determination and kinetic analysis of toluene biodegradation

Residual toluene concentrations were determined at 3-h intervals using headspace gas chromatography. Toluene concentrations were quantified using an external calibration curve prepared from standard solutions of known concentrations. The biodegradation efficiency at time t was calculated as:

$$R_t = \frac{C_0}{C_0 - C_t} \times 100\%$$

where C_0 and C_t represent the toluene concentrations ($\text{mg}\cdot\text{L}^{-1}$) at the beginning of the experiment and at time t , respectively.

Toluene degradation kinetics were evaluated using an apparent first-order kinetic model:

$$\ln\left(\frac{C_0}{C_t}\right) = -kt$$

where k is the apparent first-order degradation rate constant (h^{-1}) and t is the incubation time (h). The rate constant was obtained from the slope of the linear regression of $\ln(C_t/C_0)$ against time during the active degradation period. Data from 0–12 h were used for kinetic fitting because toluene concentrations approached the analytical detection limit at later sampling points.

2.5 GC–MS identification of pathway-associated metabolites

To characterize pH-associated differences in the metabolite profiles of toluene biodegradation, intermediate products were analyzed by gas chromatography–mass

spectrometry (GC–MS). Samples were collected at 3-h intervals from biodegradation systems maintained at pH 5.0, 6.0, 7.0, 8.0, and 9.0. Immediately after collection, biodegradation was terminated by acidification with HCl. Samples collected at different time points under the same pH condition were pooled to obtain a composite sample representing metabolites accumulated over the biodegradation period.

The pooled samples were extracted with 100 mL of ethyl acetate by ultrasonication at 40 kHz and 200 W for 30 min. The organic phase was separated and dried over 4–6 g of anhydrous Na₂SO₄. The dried extract was concentrated using a rotary evaporator (R-100, BUCHI, Switzerland) and redissolved in acetonitrile. For derivatization, 750 µL of the concentrated extract was mixed with 450 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and incubated at 60 °C for 1 h. The derivatized samples were subsequently analyzed using GC–MS (7890A–5975C, Agilent Technologies, USA). Metabolites were identified by comparison of their mass spectra with the NIST mass spectral library (Ouyang et al., 2025). Relative chromatographic peak areas in the pooled samples were used to compare the overall distribution of pathway-associated metabolites among pH treatments. Because samples from different time points were pooled prior to extraction, the resulting profiles represent integrated metabolite signals over the biodegradation period rather than time-resolved metabolic dynamics.

2.6 pH-dependent catalytic activity of key degradation enzymes

The complete *todC1C2BAD* gene cluster was PCR-amplified from the genomic DNA of *Pseudomonas* sp. BO3-4 and cloned into the *E. coli* expression vector pTarget, yielding the recombinant plasmid pTarget-*tod*. The recombinant plasmid was transformed into *Escherichia*

coli BL21(DE3) competent cells, while cells carrying the empty pTarget vector were used as the negative control (Liu et al., 2025). For heterologous expression, recombinant *E. coli* cells were cultured in LB medium to an OD_{600nm} of approximately 1.0, and protein expression was induced with 0.1 mM IPTG at 20 °C for 72 h. Cells were subsequently harvested by centrifugation, washed, and resuspended in buffer. The cell suspensions were disrupted by ultrasonication on ice, followed by centrifugation at 8000 g for 5 min at 4 °C to remove cell debris. The resulting supernatant was collected as the crude enzyme extract, and total protein concentration was determined using the Bradford assay (Xiao et al., 2025).

To evaluate the pH-dependent catalytic activity of the recombinant Tod enzyme system, enzymatic reactions were conducted at pH 5.0, 7.0, and 9.0. Each 10-mL reaction contained 9 mL of PBS at the designated pH, 825 µL of crude enzyme extract, 150 µL of NADH solution (10 mM final concentration), and 25 µL of toluene stock solution, giving an initial toluene concentration of 50 mg L⁻¹. Reaction vessels were immediately sealed to minimize volatilization and incubated at 20 °C with shaking at 150 rpm. Samples were collected at 8 h, and residual toluene concentrations were determined by GC as described in Section 2.4. Crude extracts prepared from *E. coli* carrying the empty pTarget vector were assayed under identical conditions at each pH as negative controls. All assays were performed in triplicate.

To account for differences in crude-extract protein concentration, catalytic activity was normalized to the total amount of protein added to each reaction. The amount of toluene consumed during the reaction was calculated as:

$$\Delta n = (C_0 - C_t)V$$

where C_0 and C_t are the initial and residual toluene concentrations ($\text{mmol}\cdot\text{L}^{-1}$), respectively, and V is the reaction volume (L). The apparent catalytic rate was calculated as $v=\Delta n/t$ and the protein-normalized specific catalytic activity was calculated as $A= v/ m_{\text{protein}}$, where m_{protein} is the total amount of crude-extract protein added to the reaction. One unit (U) was defined as the amount of crude enzyme preparation catalyzing the conversion of 1 μmol of toluene per minute under the specified assay conditions. The protein-normalized apparent specific catalytic activity was expressed as U mg^{-1} protein.

2.7 Computational analysis of the pH-dependent protonation response of TodC1

The three-dimensional structure of the toluene dioxygenase large subunit TodC1 from *Pseudomonas* sp. BO3-4 was predicted using AlphaFold3 and prepared in PyMOL by removing solvent molecules and non-protein heteroatoms. pH-dependent protonation states of ionizable residues were subsequently predicted using the H++ web server based on continuum electrostatics calculations. Calculations were performed at pH 5.0, 7.0, and 9.0, corresponding to the experimental catalytic conditions, with a salinity of 0.15 M and internal and external dielectric constants of 10 and 80, respectively. Protein net charge and residue-specific titration profiles, including predicted $\text{pK}_{1/2}$ values, were obtained to identify pH-responsive ionizable residues. Changes in the protonation states of selected residues were further mapped onto the predicted TodC1 structure to evaluate their spatial relationships with the catalytic and metal-binding regions (Anandakrishnan et al., 2012).

2.8 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD) of three replicate experiments.

Data processing and visualization were performed using Microsoft Excel 2020 and Origin 2021.

3 Results and Discussion

3.1 Morphological and cultivation characteristics of *Pseudomonas* sp. BO3-4

The cultivation and morphological characteristics of *Pseudomonas* sp. BO3-4 are shown in *Fig. 1*. After streaking on LB agar and incubation at 20 °C for 20 h, BO3-4 formed colonies with relatively uniform morphological characteristics (*Fig. 1a*). The colonies were circular, pale yellow, opaque, and slightly convex, with smooth surfaces, and no morphologically distinct colonies were observed under the tested cultivation conditions. Scanning electron microscopy further revealed a relatively homogeneous population of rod-shaped cells with a length of approximately 2.4 μm (*Fig. 1b*). These colony and cellular characteristics were consistent with the morphology of the previously identified BO3-4 culture, with no obvious morphological evidence of contamination. Following 12 h of cultivation in LB medium, the culture changed from transparent yellow to visibly turbid yellow (*Fig. 1c*), indicating substantial biomass accumulation. The OD₆₀₀ of the 20-fold diluted culture reached 0.169, corresponding to an estimated OD₆₀₀ of 3.38 for the undiluted suspension. The resulting biomass was subsequently harvested and standardized for the pH-dependent toluene biodegradation experiments.

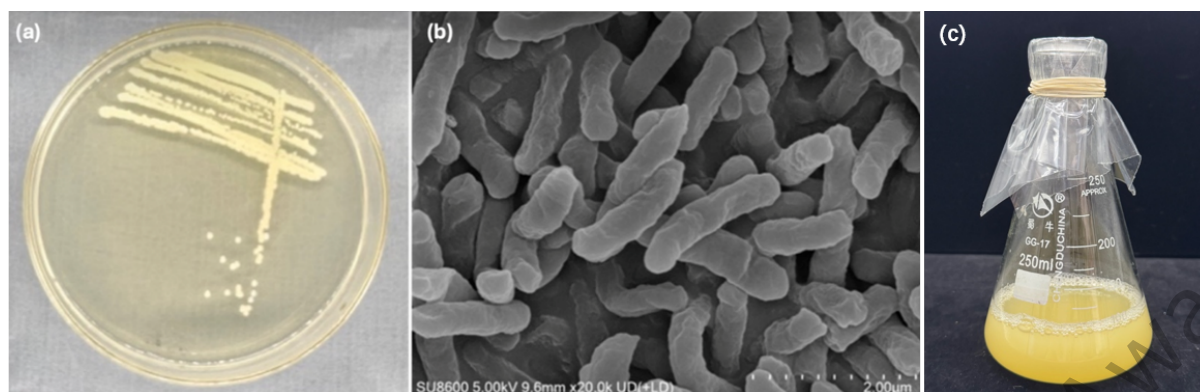


Fig.1 Morphological and cultivation characteristics of *Pseudomonas* sp. BO3-4. (a) Colony morphology on LB agar; (b) cell morphology observed by scanning electron microscopy (SEM); and (c) appearance of the BO3-4 culture after 12 h of incubation in LB medium.

3.2 pH-dependent toluene biodegradation kinetics and growth of *Pseudomonas* sp. BO3-4

The effects of environmental pH on toluene biodegradation by *Pseudomonas* sp. BO3-4 were evaluated over a pH range of 5.0–9.0 (*Fig. 2a*). BO3-4 exhibited efficient toluene removal under all tested conditions, with 50 mg L⁻¹ toluene being completely degraded within 18 h. However, distinct differences in degradation rates were observed among the pH treatments. Toluene depletion was slower under acidic conditions (pH 5.0–6.0), whereas accelerated removal occurred at neutral to alkaline pH, particularly at pH 8.0 and 9.0. These results indicate that BO3-4 maintained biodegradation capacity over a relatively broad pH range, while mildly alkaline conditions favored more rapid toluene removal.

To quantitatively compare biodegradation performance, toluene concentration data during the active degradation period (0–12 h) were fitted using an apparent first-order kinetic model (*Fig. 2b and Table 1*). The apparent degradation rate constant increased with pH from 5.0 to 8.0, reaching a maximum of 0.235 h⁻¹ at pH 8.0. At pH 9.0, the rate constant was 0.233 h⁻¹, only 0.002 h⁻¹ lower than that at pH 8.0 and within the experimental variability of both

treatments. Therefore, rather than indicating a single optimum at pH 8.0, these results suggest a high-activity window under mildly alkaline conditions (pH 8.0–9.0). The growth of the strain was demonstrated in *Fig.2c*. During the first 3 h, OD_{600nm} decreased slightly from 0.20 to approximately 0.17–0.19 across all pH treatments. This initial decrease may reflect cellular adjustment following transfer to the toluene-containing mineral medium. Toluene is a hydrophobic organic solvent capable of partitioning into bacterial membranes and perturbing membrane organization, while *Pseudomonas* sp. employ multiple adaptive responses, including membrane remodeling and solvent-efflux systems, to tolerate such stress (Ai et al., 2023). Because OD_{600nm} measures culture turbidity rather than cell viability, however, the initial decrease cannot be attributed specifically to cell death or solvent toxicity. From 3 to 12 h, OD_{600nm} increased concurrently with rapid toluene removal, consistent with utilization of toluene as a growth substrate. After approximately 12 h, when most of the toluene had been depleted, OD_{600nm} no longer increased and subsequently declined.

Among the tested conditions, pH 8.0 showed the highest time-averaged OD_{600nm} (0.414), consistent with the favorable biodegradation performance observed under mildly alkaline conditions. Environmental pH can influence bacterial physiology by affecting cytoplasmic pH homeostasis, transmembrane electrochemical gradients, and proton-motive-force-dependent transport and energy conservation. Recent analyses of bacterial physicochemical homeostasis emphasize that maintenance of intracellular pH requires coordinated regulation of proton and ion transport rather than a simple increase in ATP expenditure as extracellular pH deviates from neutrality (Poolman, 2023). Thus, the lower OD_{600nm} and degradation rates observed at acidic

pH are consistent with less favorable cellular and catalytic conditions, whereas the high activity at pH 8.0–9.0 indicates that BO3-4 maintained favorable biodegradation performance under mildly alkaline conditions. Nevertheless, OD_{600nm} alone cannot distinguish cellular responses to environmental pH from direct pH-dependent changes in the catalytic performance of the toluene-degrading enzyme system, necessitating further investigation at the metabolic and enzymatic levels.

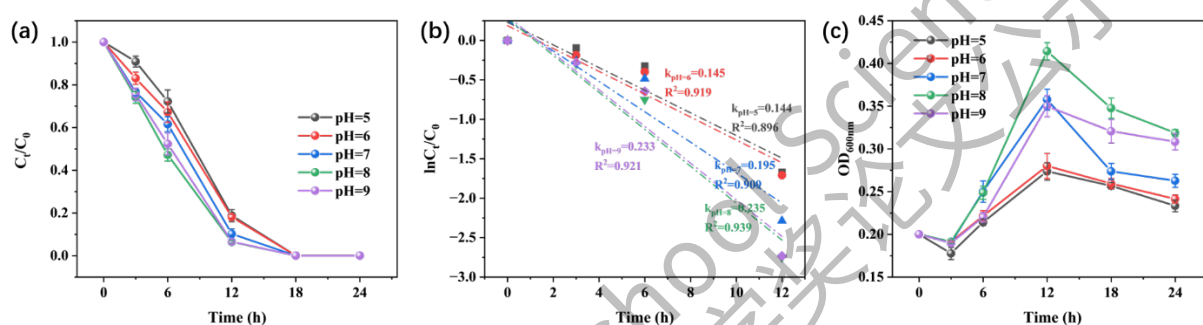


Fig.2 Toluene biodegradation and growth characteristics of *Pseudomonas* sp. BO3-4 under different pH conditions. (a) Toluene biodegradation by *Pseudomonas* sp. BO3-4; (b) first-order kinetic fitting of toluene biodegradation; and (c) growth curves of BO3-4.

Table 1. Apparent first-order kinetic parameters for toluene biodegradation by *Pseudomonas* sp. BO3-4 under different pH conditions.

pH	Average rate constant k (h^{-1})	Standard deviation	Coefficient of Determination (R^2)
5.0	0.144	0.014	0.894
6.0	0.145	0.007	0.919
7.0	0.195	0.020	0.908
8.0	0.235	0.009	0.939
9.0	0.233	0.015	0.921

3.3 pH-dependent redistribution of toluene biodegradation pathways

GC–MS analysis revealed multiple intermediate products during toluene biodegradation by *Pseudomonas* sp. BO3-4, with distinct metabolite profiles observed under different pH conditions (Fig. 3). Based on comparison of the EI mass spectra with the NIST spectral library, the detected compounds were tentatively identified as several hydroxylated aromatic products, including o-cresol, p-cresol, and 3-methylcatechol, as well as benzyl alcohol and its derivatives. Importantly, the number and composition of detected metabolites differed among the tested pH conditions. At pH 7.0, a broader range of hydroxylated aromatic products was detected, including both o-cresol and p-cresol, together with a methylcatechol-related TMS derivative. In comparison, the metabolite profile at pH 5.0 contained a greater representation of benzyl alcohol-related products, whereas fewer ring-hydroxylation-associated products were observed. At pH 9.0, the overall number and chromatographic abundance of the selected detectable intermediates were lower than those at pH 5.0 and 7.0. These differences demonstrate that environmental pH was associated not only with the degradation kinetics of toluene but also with the composition of intermediates accumulated during biodegradation.

The identified metabolites were further classified according to the two proposed toluene oxidation routes of BO3-4 (Fig. 3). In the *tod*-associated aromatic-ring oxidation route, toluene undergoes initial ring dioxygenation followed by the formation of cis-toluene-2,3-dihydrodiol and subsequent conversion to 3-methylcatechol before ring cleavage (Finette & Gibson, 1988; Zylstra et al., 1988). Accordingly, the methylcatechol-related TMS derivative and related hydroxylated aromatic products detected in the present study were considered TDO-associated

metabolites. In contrast, benzyl alcohol and its derivatives were associated with oxidation of the methyl side chain and were therefore assigned to the XMO-associated route (Harayama et al., 1986; Worsey & Williams, 1975). The simultaneous detection of metabolites related to both routes indicates that BO3-4 possesses alternative metabolic strategies for toluene transformation, consistent with the complementary *tod*- and *xyl*-associated catabolic systems identified in this strain.

Comparison of the relative chromatographic peak areas further revealed a pronounced pH-associated redistribution of pathway-associated metabolites. For this analysis, TDO-associated signals included cresol-related products, which were interpreted as products associated with aromatic-ring dioxygenation followed by dehydration/aromatization, together with the derivatized methylcatechol product, whereas benzyl alcohol and its TMS derivative were assigned to the XMO-associated methyl side-chain oxidation route. At pH 5.0, XMO-associated metabolites accounted for approximately 55.5% of the selected GC–MS peak-area signal, compared with 44.5% for TDO-associated products, indicating a slightly greater relative representation of methyl side-chain oxidation under acidic conditions. As pH increased to 7.0, the distribution shifted markedly toward aromatic-ring oxidation, with TDO-associated products increasing to 75.3% of the selected signal and XMO-associated products decreasing to 24.7%. This trend was maintained at pH 9.0, where TDO-associated products accounted for 78.9% of the selected signal, whereas XMO-associated products represented only 21.1%. Thus, increasing pH from acidic to neutral–alkaline conditions was associated with a progressive increase in the relative representation of TDO-associated aromatic-ring oxidation products.

Notably, the increased relative representation of TDO-associated products at pH 9.0 did not correspond to greater overall intermediate accumulation. Instead, the total chromatographic abundance of the selected intermediates decreased, while the apparent first-order degradation rate constant remained high (0.233 h^{-1}), nearly identical to the maximum value observed at pH 8.0 (0.235 h^{-1}). The coexistence of rapid substrate removal, a high relative proportion of TDO-associated products, and low overall intermediate accumulation suggests that the lower metabolite abundance at pH 9.0 was unlikely to result from inhibition of the initial aromatic-ring oxidation process. Rather, it may reflect a shift in the balance between intermediate formation and subsequent conversion, whereby initially formed metabolites underwent more rapid downstream transformation and therefore accumulated to lower detectable levels. Because the GC–MS samples were pooled over the biodegradation period, however, intermediate formation and consumption rates could not be independently resolved.

Taken together, the metabolite and kinetic profiles point to the initial aromatic-ring dioxygenation step as a potential pH-responsive control point in toluene biodegradation by BO3-4. Increasing pH from acidic to neutral conditions was accompanied by a greater relative representation of TDO-associated metabolites, whereas at pH 9.0, intermediate accumulation decreased despite the maintenance of a high toluene degradation rate. Toluene dioxygenase (TDO), encoded by the *tod* system, catalyzes the initial incorporation of molecular oxygen into the aromatic ring and therefore occupies a critical position at the entrance of this degradation route (Ferraro et al., 2005). The contrasting responses of intermediate accumulation and overall

substrate removal further suggest that changes in metabolite abundance alone cannot reveal the catalytic state of TDO.

Table 2. GC–MS-detected metabolites associated with toluene biodegradation by *Pseudomonas* sp. BO3-4 under different pH conditions.

Metabolite	CAS No.	Proposed pathway association	pH	Retention time (min)	Library match (%)	Detection form
Benzyl alcohol	10-51-6	Side-chain oxidation / XMO-associated	5	11.226	96	Underivatized
			7	11.295	96	Underivatized
			9	11.335	92	Underivatized
<i>o</i> -Cresol (2-methylphenol)	95-48-7	Aromatic hydroxylation-associated	5	11.645	97	Underivatized
			7	11.660	96	Underivatized
			9	11.586	92	Underivatized
<i>p</i> -Cresol (4-methylphenol)	10-64-5	Aromatic hydroxylation-associated	7	11.435	70	Underivatized
			9	11.530	69	Underivatized
Cresol–TMS derivative†	95-48-7*	Aromatic hydroxylation-associated	5	13.681	93	TMS derivative
			7	13.675	94	TMS derivative
			9	13.651	95	TMS derivative
Benzyl alcohol–TMS derivative†	10-51-6*	Side-chain oxidation / XMO-associated	5	14.266	85	TMS derivative
			7	14.410	79	TMS derivative
			9	14.410	75	TMS derivative
3-Methylcatechol–TMS derivative†	48-17-5*	TDO-associated aromatic-ring dioxygenation	5	21.218	73	TMS derivative
			7	21.220	77	TMS derivative
			9	21.225	76	TMS derivative

* CAS numbers refer to the parent metabolites, rather than their trimethylsilyl derivatives.

† Hydroxyl-containing metabolites were derivatized with BSTFA before GC–MS analysis;

therefore, both underivatized compounds and their trimethylsilyl (TMS) derivatives may occur in the chromatograms.

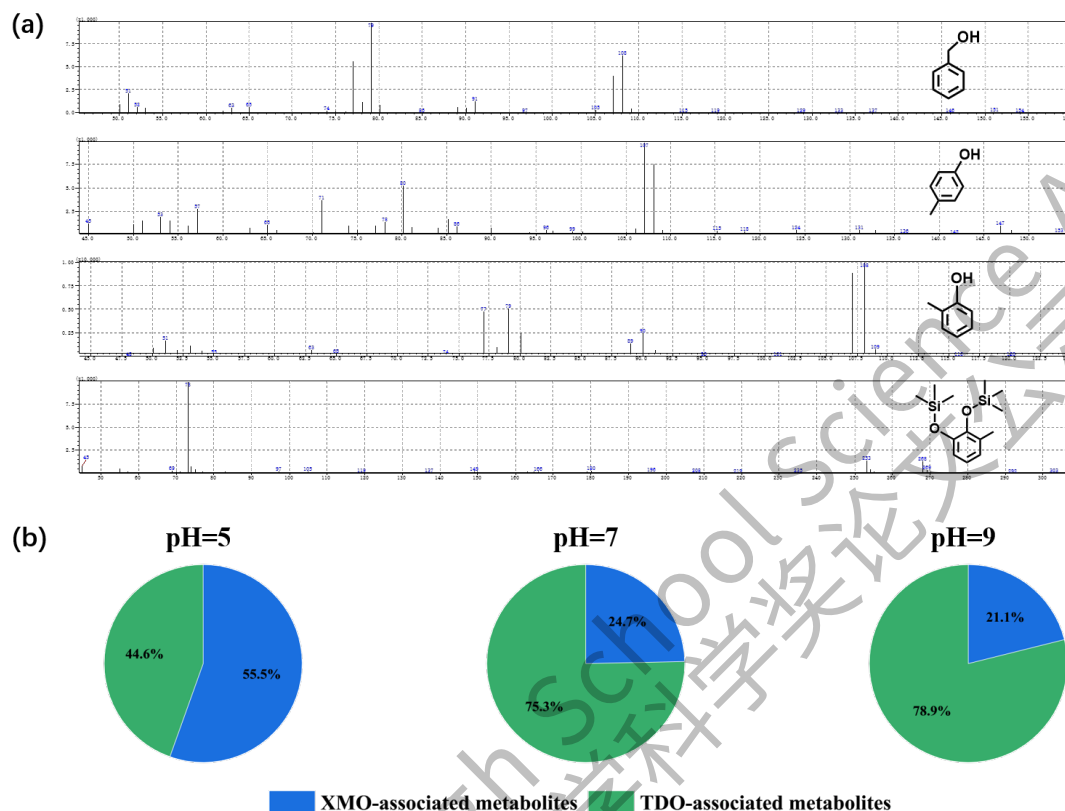


Fig.3 pH-dependent metabolite profiles during toluene biodegradation by *Pseudomonas* sp.

BO3-4. (a) GC-MS profiles of the major pathway-associated metabolites detected under different pH conditions; (b) relative chromatographic peak-area distributions of XMO-associated methyl side-chain oxidation products and TDO-associated aromatic-ring oxidation products at pH 5.0, 7.0, and 9.0.

3.4 pH-dependent catalytic activity of the recombinant Tod enzyme system

To directly evaluate the pH-dependent catalytic response of the Tod enzyme system, the complete *todC1C2BAD* gene cluster from BO3-4 was heterologously expressed in *E. coli* BL21(DE3), followed by crude enzyme extraction and catalytic assays at different pH values

(Fig. 4a). Following IPTG induction, the recombinant *E. coli* culture developed a pronounced dark-blue coloration (Fig. 4b). Similar coloration has been reported in *E. coli* expressing toluene dioxygenase and is generally associated with TDO-mediated oxidation of indole, derived from tryptophan metabolism in rich media, followed by the formation of the blue pigment indigo (Lee et al., 2005; Stephens et al., 1989). Therefore, although the pigment was not chemically identified in the present study, the observed color change provided qualitative evidence consistent with functional expression of the recombinant Tod system.

The recombinant Tod enzyme system exhibited a clear pH-dependent catalytic response (Fig. 4c). After 8 h of reaction, toluene removal efficiencies at pH 5.0, 7.0, and 9.0 were approximately 15.6%, 21.8%, and 26.5%, respectively, indicating progressively enhanced catalytic conversion from acidic to alkaline conditions. After normalization to the total protein content of the crude enzyme extracts, the apparent specific catalytic activities were 0.00307, 0.00442, and 0.00494 U mg⁻¹ protein at pH 5.0, 7.0, and 9.0, respectively. Thus, the activity at pH 7.0 and 9.0 was approximately 1.44- and 1.61-fold that at pH 5.0, whereas the additional increase from pH 7.0 to 9.0 was comparatively small. This pattern was consistent with the whole-cell biodegradation kinetics of BO3-4, which exhibited substantially higher apparent degradation rate constants under neutral-to-mildly alkaline conditions than under acidic conditions. Moreover, the increased Tod activity at higher pH was consistent with the greater relative representation of TDO-associated metabolites observed in Section 3.3, providing enzymatic evidence supporting the proposed pH-dependent redistribution of toluene oxidation routes. Notably, the combination of high Tod activity and rapid whole-cell degradation at pH

9.0, despite the lower accumulation of detectable intermediates, supports the interpretation that low intermediate abundance under alkaline conditions did not result from inhibition of the initial dioxygenation process. Collectively, these results suggest that the reduced biodegradation observed under acidic conditions was associated, at least in part, with lower catalytic performance of the Tod system.

Previous studies demonstrate that aromatic-hydrocarbon oxygenases can exhibit substantial sensitivity to their catalytic environment. TDO activity in *Pseudomonas putida* F1 varies with physiological state, demonstrating that the catalytic capacity of this enzyme system is not invariant (Woo et al., 2000). More direct evidence for pH-dependent catalysis has been reported for the related multicomponent toluene/*o*-xylene monooxygenase. The wild-type hydroxylase exhibited a bell-shaped pH profile with apparent pK_a values of approximately 6.1 and 7.1, indicating the involvement of protonatable groups in catalysis. Mutation of a glutamine residue involved in the catalytic network shifted the pH dependence and resulted in pronounced uncoupling at lower pH, further demonstrating the importance of the local proton-transfer environment for catalytic performance (Liang et al., 2014). Although this monooxygenase is structurally distinct from Rieske-type TDO, these findings support the broader principle that protonation states and local electrostatic interactions can regulate aromatic-hydrocarbon oxygenase catalysis.

Such pH sensitivity is mechanistically plausible for the Tod system because toluene dioxygenase is a multicomponent electron-transfer system in which reducing equivalents from NADH are transferred through a reductase and a Rieske-type [2Fe–2S] ferredoxin to the

terminal oxygenase responsible for oxygen incorporation into the aromatic substrate (Ferraro et al., 2005; Zu et al., 2003). Consequently, changes in the protonation states of ionizable residues surrounding the electron-transfer components, mononuclear iron catalytic center, or substrate-binding pocket could alter local electrostatic interactions, substrate positioning, or catalytic geometry. The substantially lower catalytic activity observed at pH 5.0 therefore provides experimental evidence for acid-sensitive catalytic performance, but does not by itself demonstrate structural disruption of the Rieske cluster or oxygenase. The molecular basis of this response was therefore further examined using pH-specific protonation-state analysis of TodC1 to determine whether changes in residue protonation could provide a molecular explanation for the experimentally observed pH dependence.

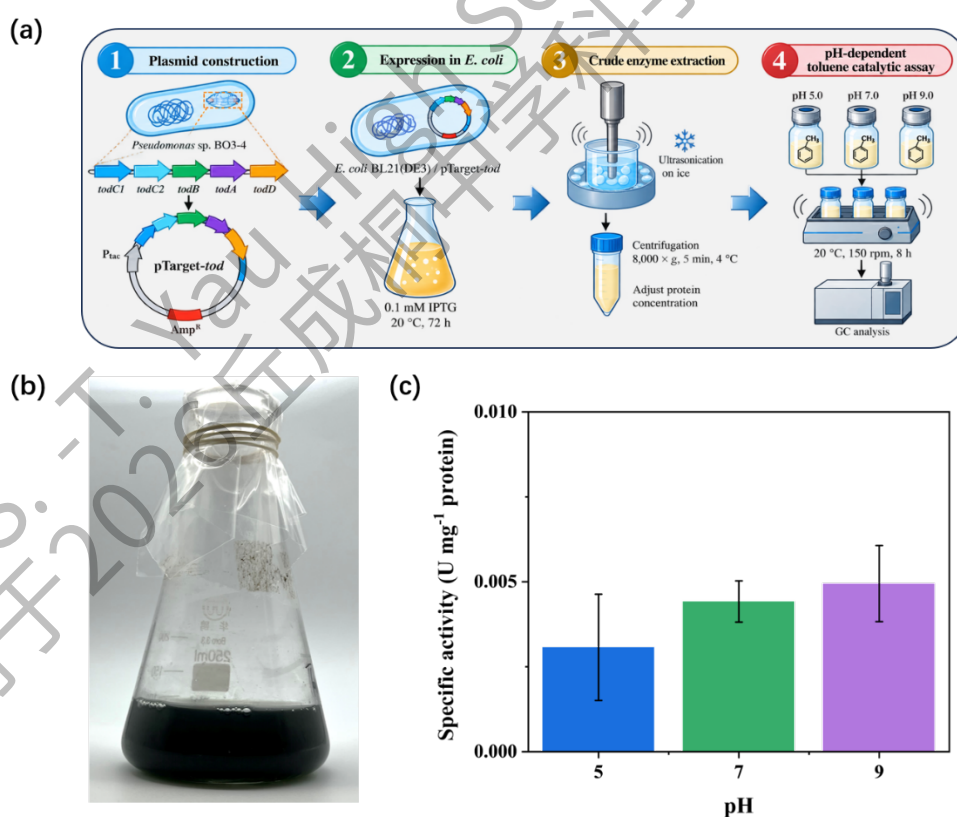


Fig. 4. Heterologous expression and pH-dependent catalytic activity of the recombinant Tod enzyme system. (a) Schematic workflow for construction, heterologous expression, crude enzyme extraction, and pH-dependent catalytic assays of the recombinant Tod system; (b) dark-blue coloration of the recombinant *E. coli* culture following IPTG induction of the *todC1C2BAD* gene cluster; and (c) apparent specific catalytic activity of the recombinant Tod system toward toluene at pH 5.0, 7.0, and 9.0.

3.5 pH-dependent protonation responses of toluene dioxygenase

To further explore the molecular basis underlying the pH-dependent catalytic behavior of the Tod enzyme system, computational protonation-state analysis was performed on the catalytic large subunit of toluene dioxygenase (TodC1) at pH 5.0, 7.0, and 9.0. The analysis identified several ionizable residues whose predicted protonation states changed substantially across the investigated pH range, indicating that environmental pH could markedly alter the local electrostatic properties of TodC1 (*Fig. 5*).

Among the pH-responsive residues, Glu424 exhibited a predicted $pK_{1/2}$ of approximately 5.15–5.20. At pH 5.0, approximately 54% of Glu424 was predicted to be protonated, partially neutralizing the negative charge normally associated with its deprotonated carboxylate group. As the pH increased to 7.0 and 9.0, Glu424 became predominantly deprotonated and therefore negatively charged. Pronounced pH-dependent changes were also predicted for His385 and His390. His390, with a predicted $pK_{1/2}$ of approximately 5.11, exhibited a protonated fraction of >55% at pH 5.0 but became predominantly deprotonated at higher pH. His385 showed an even stronger response because of its higher predicted $pK_{1/2}$ of

approximately 6.76, with >94% of the residue predicted to be protonated at pH 5.0, followed by progressive deprotonation as the pH increased. Thus, the transition from acidic to neutral conditions was accompanied by a pronounced redistribution of the predicted charge states of these ionizable residues.

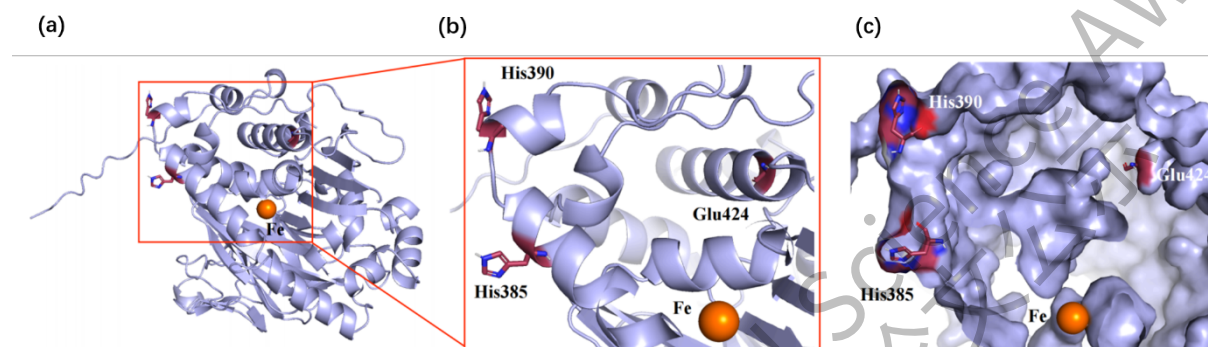


Fig. 5. Structural localization of distal pH-responsive residues in TodC1. (a) Overall predicted structure of TodC1; (b) enlarged view of His385, His390, and Glu424 relative to the mononuclear Fe catalytic center; and (c) surface representation showing the spatial distribution of the three pH-responsive residues relative to the catalytic region.

Importantly, structural mapping showed that Glu424, His385, and His390 were not located directly within the Rieske [2Fe–2S] center, the mononuclear iron catalytic site, or the substrate-binding pocket. Their pH-dependent protonation is therefore unlikely to regulate dioxygenation through direct coordination of the catalytic metal centers or direct interaction with the substrate. Instead, these residues may represent distal pH-responsive sites that modulate the electrostatic microenvironment of TodC1. Protonation of Glu424 under acidic conditions reduces its negative-charge contribution, whereas increased protonation of His385 and His390 increases the local positive-charge contribution. Such charge redistribution may modify local electrostatic interactions and the stability of neighboring hydrogen-bonding or

ionic interaction networks, thereby influencing regional conformational stability and indirectly contributing to the catalytic response of TodC1. Because the present computational analysis was limited to protonation-state prediction, however, long-range propagation of these local changes to the catalytic center cannot be directly established and would require further molecular dynamics or experimental structural analyses.

Despite this limitation, the predicted protonation response showed a notable correspondence with the experimentally determined catalytic behavior of the recombinant Tod system. The largest protonation changes occurred during the transition from pH 5.0 to 7.0, particularly for His385, His390, and Glu424. Over the same pH interval, the apparent specific catalytic activity increased from 0.00307 to 0.00442 U·mg⁻¹ protein, representing an approximately 1.44-fold increase. A further increase in pH to 9.0 resulted in a comparatively smaller increase in catalytic activity to 0.00494 U·mg⁻¹ protein, approximately 1.61-fold that at pH 5.0. This correspondence suggests that the major recovery of Tod catalytic activity from acidic to neutral conditions coincides with substantial deprotonation of pH-sensitive residues, whereas the smaller activity change from pH 7.0 to 9.0 occurs after these residues have largely transitioned toward their higher-pH protonation states. Collectively, these results support a model in which pH-dependent protonation of distal ionizable residues may alter the electrostatic microenvironment of TodC1 and may contribute to the reduced catalytic performance observed under acidic conditions, providing a molecular-level explanation consistent with the pH-dependent biodegradation and enzyme-activity patterns observed experimentally.

Conclusion

Understanding how environmental conditions regulate microbial catalytic performance is essential for improving the stability and efficiency of bioremediation in contaminated environments. This study demonstrated that *Pseudomonas* sp. BO3-4 efficiently degraded toluene across pH 5.0–9.0, with the highest apparent degradation rates occurring at pH 8.0–9.0. Increasing pH was accompanied by a marked redistribution toward TDO-associated metabolites, whose relative signals increased from 44.5% at pH 5.0 to 75.3% and 78.9% at pH 7.0 and 9.0, respectively. Consistently, the apparent catalytic activity of the recombinant Tod system increased by 1.44- and 1.61-fold at pH 7.0 and 9.0 relative to pH 5.0. Protonation analysis further identified His385, His390, and Glu424 as distal pH-responsive residues in TodC1, suggesting that changes in its electrostatic microenvironment may contribute to the catalytic response. Together, these findings connect environmental pH with enzyme-level responses, pathway redistribution, and biodegradation performance, providing a mechanistic basis for more stable and low-carbon biological treatment of aromatic-hydrocarbon-contaminated water.

References

- Ai, L., Mei, F., Peng, R. 2023. An overview of the role of membrane proteins in microbial solvents tolerance. *Protein Pept Lett*, **30**(2), 137-145.
- Anandakrishnan, R., Aguilar, B., Onufriev, A.V. 2012. H++ 3.0: automating pK prediction and the preparation of biomolecular structures for atomistic molecular modeling and simulations. *Nucleic Acids Res*, **40**(Web Server issue), W537-41.
- Chen, X., Shan, G.B., Shen, J.M., Zhang, F., Liu, Y.D., Cui, C.Z. 2023. In situ bioremediation of petroleum hydrocarbon-contaminated soil: isolation and application of a *Rhodococcus* strain. *Int Microbiol*, **26**(2), 411-421.
- Chen, X., Zhang, S., Yi, L., Liu, Z., Ye, X., Yu, B., Shi, S., Lu, X. 2022. Evaluation of biodegradation of BTEX in the subsurface of a petrochemical site near the Yangtze River, China. *Int J Environ Res Public Health*, **19**(24), 16449.
- Czerwińska-Główka, D., Krukiewicz, K. 2021. Guidelines for a morphometric analysis of prokaryotic and eukaryotic cells by scanning electron microscopy. *Cells*, **10**(12), 3304.
- Ferraro, D.J., Gakhar, L., Ramaswamy, S. 2005. Rieske business: Structure–function of Rieske non-heme oxygenases. *Biochem Biophys Res Commun*, **338**(1), 175-190.
- Finette, B.A., Gibson, D.T. 1988. Initial studies on the regulation of toluene degradation by *Pseudomonas putida* F1. *Biocatalysis*, **2**(1), 29-37.
- Friemann, R., Lee, K., Brown, E.N., Gibson, D.T., Eklund, H., Ramaswamy, S. 2009. Structures of the multicomponent Rieske non-heme iron toluene 2,3-dioxygenase enzyme system. *Acta Crystallogr D Biol Crystallogr*, **65**(Pt 1), 24-33.

- Harayama, S., Leppik, R.A., Rekik, M., Mermoud, N., Lehrbach, P.R., Reineke, W., Timmis, K.N. 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the xylA product. *J Bacteriol*, **167**(2), 455-61.
- Huang, Y., Chen, Y., Wu, Q., Shi, P., Yang, B., Xie, Y. 2024. Evaluation of BTEX pollution and health risk for sustainable use of a typical chemical pesticide industrial site. *Sustainability*, **16**(15), 6494.
- Jiang, H., Parales, R.E., Gibson, D.T. 1999. The alpha subunit of toluene dioxygenase from *Pseudomonas putida* F1 can accept electrons from reduced FerredoxinTOL but is catalytically inactive in the absence of the beta subunit. *Appl Environ Microbiol*, **65**(1), 315-318.
- Lee, E.Y., Jun, Y.S., Cho, K.S., Ryu, H.W. 2002. Degradation characteristics of toluene, benzene, ethylbenzene, and xylene by *Stenotrophomonas maltophilia* T3-c. *J Air Waste Manag Assoc*, **52**(4), 400-6.
- Lee, K., Friemann, R., Parales, J.V., Gibson, D.T., Ramaswamy, S. 2005. Purification, crystallization and preliminary X-ray diffraction studies of the three components of the toluene 2,3-dioxygenase enzyme system. *Acta Crystallogr Sect F Struct Biol Cryst Commun*, **61**(Pt 7), 669-672.
- Liang, A.D., Wrobel, A.T., Lippard, S.J. 2014. A flexible glutamine regulates the catalytic activity of toluene o-xylene monooxygenase. *Biochemistry*, **53**(22), 3585-3592.

Liu, H., Xu, Z.L., Dai, W.J., Chen, W.J., Song, H.R., Huang, Y.H., Zhang, L.H., Chen, S.H.

2025. Mechanistic insights into chlorpyrifos biodegradation mediated by las quorum sensing system in *Pseudomonas aeruginosa* and its potential use for environmental remediation. *Chem Eng J*, **523**, 168754.

Ouyang, J., Pan, L.X., Gu, F., Lei, S.M., Chen, X., Zhou, Y., Zhu, X., Cui, C.Z. 2025. In-situ bioaugmentation with indigenous *Pseudomonas* sp. BO3-4 for the remediation of organic-contaminated groundwater in an operating petrochemical enterprise. *Chem Eng J*, **520**, 166498.

Pan, L.X., Yuan, B., Li, Q.Q., Ouyang, J., Yang, J., Zhou, Y., Cui, C.Z. 2024. Efficient biodegradation of chlorobenzene via monooxygenation pathways by *Pandoraea* sp. XJJ-1 with high potential for groundwater bioremediation. *Int Microbiol*, **28**, 355-364.

Poolman, B. 2023. Physicochemical homeostasis in bacteria. *FEMS Microbiol Rev*, **47**(4).

Schönichen, A., Webb, B.A., Jacobson, M.P., Barber, D.L. 2013. Considering protonation as a posttranslational modification regulating protein structure and function. *Annu Rev Biophys*, **42**, 289-314.

Silverstein, T.P. 2021. The proton in biochemistry: Impacts on bioenergetics, biophysical chemistry, and bioorganic chemistry. *Front Mol Biosci*, **8**, 764099.

Stephens, G.M., Sidebotham, J.M., Mann, N.H., Dalton, H. 1989. Cloning and expression in *Escherichia coli* of the toluene dioxygenase gene from *Pseudomonas putida* NCIB11767. *FEMS Microbiol Lett*, **57**(3), 295-300.

- Wang, H., Dai, B., Gao, F., Cao, X., Ji, X., Zuo, L., Cheng, C., Jiang, L. 2024. Petrochemical wastewater treatment in a full-scale biological aerated filter system: Insight into the mechanism of performance deterioration. *Chem Eng J*, **488**, 151089.
- Woo, H., Sanseverino, J., Cox, C.D., Robinson, K.G., Sayler, G.S. 2000. The measurement of toluene dioxygenase activity in biofilm culture of *Pseudomonas putida* F1. *J Microbiol Methods*, **40**(2), 181-191.
- Worsey, M.J., Williams, P.A. 1975. Metabolism of toluene and xylenes by *Pseudomonas* (putida (arvilla) mt-2: Evidence for a new function of the TOL plasmid. *J Bacteriol*, **124**(1), 7-13.
- Wu, C., Li, Y., Zhou, Y., Li, Z., Zhang, S., Liu, H. 2018. Upgrading the Chinese biggest petrochemical wastewater treatment plant: Technologies research and full scale application. *Sci Total Environ*, **633**, 189-197.
- Xiao, Y.F., Yang, B.B., Wang, S.Y., Zhao, M.H., Li, M., Li, J., Zhang, H., Wu, X. 2025. Integrated transcriptomic and enzymatic characterization of the molecular insights into nicosulfuron biodegradation by *Enterobacter ludwigii* ES2. *J Hazard Mater*, **497**, 139641.
- Zhu, H., Chen, Y., Ruan, Z., Wang, J., Liu, D., Wang, H., Zhao, M. 2023. Occurrence, seasonal variations, distribution patterns, and risk assessment of volatile monoaromatic hydrocarbons in soils of industrial parks in Yangtze River Delta, China. *Sci Total Environ*, **892**, 164511.

Zu, Y., Couture, M.M.-J., Kolling, D.R.J., Crofts, A.R., Eltis, L.D., Fee, J.A., Hirst, J. 2003.

Reduction potentials of rieske clusters: Importance of the coupling between oxidation state and histidine protonation state. *Biochemistry*, **42**(42), 12400-12408.

Zylstra, G.J., McCombie, W.R., Gibson, D.T., Finette, B.A. 1988. Toluene degradation by

Pseudomonas putida F1: genetic organization of the *tod* operon. *Appl Environ Microbiol*, **54**(6), 1498-1503.

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This study aims to study the biodegradation mechanism of toluene by *Pseudomonas* sp. The effect of pH on BO3-4. The research consisted of several steps such as literature search, experimental design, data collection and analysis, elucidation of metabolic pathways, recombinant enzymology experiments, and report writing. This study was initiated from a field remediation internship carried out by student Yin Zirui at a contaminated site that was previously owned by Suzhou Steel (Su-Gang) in 2024, where the main contaminants were found to be aromatic compounds. The overall research topic and framework were proposed and continuously improved by Yin Zirui, and the experimental procedures and analyses were conducted under the guidance of Professor Cui Changzheng from the East China University of Science and Technology (ECUST). Professor Cui offered free advice and suggested that the key rate-limiting enzymes, such as the toluene dioxygenase TodC1C2BAD system, should be studied and changes in the protonation network and metabolic flux diversion should be considered at various pH conditions. This strategy has enabled the development of an integrated research approach that integrates kinetics, metabolomics, in vitro recombinant enzymology and computational biology.

Yin Zirui was involved in the design, execution and optimization of the overall experimental design and procedures for the degradation of toluene at different pH levels, for metabolite analysis, for recombinant enzymology experiments, and for subsequent

computational analysis. Professor Cui gave valuable advice on the strategy of recombinant plasmid construction, the scientific rigor of in vitro enzyme activity assays, and experimental safety, which helped to improve the experimental plan. GC-MS analysis was performed with the help of the graduate students from ECUST, Lei Shiming and Ouyang Ji, who helped with sample injection and raw spectral data acquisition. They also helped and assisted with the cold field-emission scanning electron microscopy (SEM) experiments, which involved gold-sputter sample preparation and image acquisition. After data acquisition, Yin Zirui independently completed the GC-MS library matching, characteristic ion abundance calculation and quantitative metabolic flux analysis. In the data processing and analysis stage, Yin Zirui organized the experimental data, carried out kinetic calculations, and comprehensively analyzed the changes in metabolite profiles; based on this, he analyzed the effects of different pH values on the degradation rate of toluene, metabolic flux partitioning, and the protonation state of key enzymes. A number of changes and optimisations were made to overcome key issues that arose during the research. First, frequent sampling was a concern because toluene is very volatile and could lead to abiotic loss, which would affect degradation kinetics analysis. To counter this, an experimental system was set up under supervision using airtight serum bottles with PTFE lined aluminum caps, sterile parallel negative controls were added for each pH condition, and the reliability of the kinetic data was improved by baseline correction of abiotic losses. Second, the variety of the toluene metabolites created problems, especially in the area of incomplete derivatization and low signals for some of the polar metabolites. Yin Zirui optimized sample pretreatment and derivatization protocols independently and used

characteristic mass spectrometry ions to confirm specific metabolites, such as o-/ p-cresol, 3-methylcatechol, and benzyl alcohol, as a basis for metabolic flux analysis. To reduce the batch-to-batch variability, we optimized the concentration of IPTG induction and the expression temperature in the multicomponent TodC1C2BAD recombinant expression system and normalized the protein level by the Bradford assay. Finally, the professor suggested using AlphaFold 3 to build a structural model, which was combined with H++ analysis to identify the protonation states of titratable residues at various pH levels, and key residues (including His385, His390, and Glu424) were identified that are likely to play a role in the pH response.

Yin Zirui prepared the research report and created all the figures and mechanistic flowcharts independently. Professor Cui Changzheng checked the manuscript in the process of preparation and gave detailed suggestions for revision on the structural rigor, the uniformity of terminology, English academic expression and the depth of the discussion.

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