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Time-Resolved Resorcinol Biodegradation by *Pseudomonas putida* R1: Linking Substrate Removal, Bacterial Growth, and Differential Expression of Aromatic Catabolic Genes

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Abstract

Resorcinol is an industrial phenolic pollutant whose biological removal is important for environmental biotechnology. However, the temporal relationships among substrate removal, bacterial growth, and aromatic catabolic gene expression remain insufficiently characterized. This study investigated these relationships in the activated sludge-derived strain *Pseudomonas putida* R1.

The bacterium was cultivated in Bushnell–Haas minimal medium containing 100 mg/L resorcinol as the sole carbon source. Bacterial growth, residual resorcinol, and relative expression of phenol hydroxylase and catechol 1,2-dioxygenase gene targets were monitored over 32 h using optical density measurements, high-performance liquid chromatography, and reverse transcription quantitative PCR.

P. putida R1 achieved 94.55% resorcinol removal within 32 h, with concentrations decreasing from 100.00 ± 4.09 to 5.45 ± 0.59 mg/L. Optical density at 600 nm increased from 0.08 ± 0.02 to 0.65 ± 0.03. The highest average removal rate, 6.07 mg/L/h, occurred between 14 and 24 h. Relative to time-matched glucose-grown controls, phenol hydroxylase expression peaked at 14 h (543.072 ± 11.353-fold). Catechol 1,2-dioxygenase showed an earlier response at 10 h (9.883 ± 0.227-fold) and peaked at 24 h (26.630 ± 2.007-fold).

These findings reveal distinct yet temporally associated patterns of growth, substrate removal, and gene expression during resorcinol biodegradation. They characterize the physiological and transcriptional responses of *P. putida* R1 without establishing a specific degradation pathway and support its potential for biological treatment of resorcinol-containing wastewater.

Keywords: *Pseudomonas putida* R1; Resorcinol biodegradation; Phenol hydroxylase; Catechol 1,2-dioxygenase; RT-qPCR; Gene expression.

1. Introduction

Phenolic compounds are among the major organic pollutants found in wastewater generated by petrochemical operations, coke and coal processing, resin and polymer manufacturing, textile and dye industries, pharmaceutical production, and other chemical processes. Their structural diversity, toxic effects, and potential to inhibit microbial activity above certain concentrations make the effective removal of phenolic pollutants from environmental matrices particularly important. As alternatives to physical and chemical treatment methods, biological processes have attracted attention because they enable the biotransformation and, under suitable conditions, mineralization of phenolic compounds, while offering relatively low costs and a reduced potential for secondary pollution (Panigrahy et al., 2022; Wang et al., 2024).

Resorcinol (1,3-dihydroxybenzene) is a dihydric phenol bearing two hydroxyl groups in the meta positions of the benzene ring. It is used in the rubber industry and wood adhesive systems, as well as in dyes, pharmaceuticals, specialty chemicals, and various organic synthesis processes. Its physicochemical properties and industrial applications may facilitate its release into the environment, prompting investigations into its environmental fate and toxicological effects. A World Health Organization assessment comprehensively evaluated the available evidence on the industrial uses of resorcinol and its implications for human health and the environment (WHO/IPCS, 2006). Tamura et al. (2013) also investigated the acute and chronic/subchronic effects of several antimicrobial compounds, including resorcinol, in three groups of aquatic organisms, providing additional ecotoxicological data for resorcinol.

Microbial biodegradation of phenolic compounds is influenced by numerous factors, including the chemical structure of the pollutant, initial substrate concentration, microbial adaptation,

oxygen availability, nutrient conditions, and the formation of intermediate metabolites. In particular, high concentrations of phenolic substrates may limit microbial growth and degradation rates. Accordingly, determining only the final removal percentage is insufficient to characterize the degradation process; a more comprehensive approach involves jointly evaluating substrate transformation kinetics, relevant metabolic pathways, and the biological systems involved in degradation. Recent literature on phenolic pollutant biodegradation likewise emphasizes the importance of considering degradation pathways, key enzymes, and environmental factors alongside removal performance (Panigrahy et al., 2022; Wang et al., 2024).

Aerobic resorcinol biodegradation has previously been investigated using various microbial cultures and reactor systems. Sharma et al. (2010) examined resorcinol removal in a sequencing batch reactor and showed that increasing biomass concentration and aeration duration improved removal performance. They evaluated several operational parameters, including the initial resorcinol concentration, mixed liquor suspended solids (MLSS) concentration, and aerated fill phase. Dey et al. (2011) investigated the kinetics of resorcinol biodegradation in an aerobic batch system using a mixed microbial culture obtained from a coke-oven wastewater treatment plant. Following adaptation, the culture was reportedly capable of degrading resorcinol at concentrations up to 500 mg/L; however, microbial growth and substrate removal rates were affected by substrate inhibition at higher initial concentrations. Among the inhibition models tested, the Luong model provided the best fit to the experimental data.

The effects of the coexistence of resorcinol and other phenolic substrates on biodegradation kinetics have also been investigated. Dey and Mukherjee (2013) examined interactions between phenol and resorcinol in aerobic batch cultures containing both substrates and found that their simultaneous presence reduced specific microbial growth and substrate removal rates compared with single-substrate conditions. The inhibitory effect of resorcinol on phenol removal was more pronounced. These findings indicate that resorcinol biodegradation depends not only on the ability of a microorganism to utilize the substrate but also on the initial concentration and the presence of other aromatic compounds.

Resorcinol research, however, is not limited to reactor performance and kinetic modeling. More recent approaches have examined the effects of acclimation to resorcinol on microbial communities. Yang et al. (2022) reported changes in bacterial community structure during the adaptation of aerobic activated sludge to resorcinol, differences in the microbial enzyme repertoire revealed by metagenomic analysis, and the identification of Methylotenera, Methylophilus, and Azoarcus among the principal genera associated with resorcinol

degradation. These observations underscore the importance of evaluating resorcinol biodegradation not only in terms of substrate disappearance but also through the cellular and molecular adaptive mechanisms accompanying degradation.

The genus *Pseudomonas* exhibits considerable metabolic capacity for bacterial catabolism of aromatic compounds. In particular, *Pseudomonas putida* is widely used as a model microorganism in environmental and industrial biotechnology because of its ability to utilize diverse aromatic compounds, its tolerance to chemical and physicochemical stresses, and its broad metabolic repertoire (Weimer et al., 2020; de Lorenzo et al., 2024). Genomic analysis of P. *putida* KT2440 identified several central catabolic routes that channel aromatic compounds into central metabolism, including the protocatechuate and catechol branches of the β-ketoadipate pathway and the homogentisate and phenylacetate pathways (Jiménez et al., 2002). This metabolic diversity enables *P. putida* to direct different aromatic substrates into distinct metabolic networks depending on environmental conditions.

Studies of the biochemical basis of resorcinol utilization by *P. putida* date back several decades. Chapman and Ribbons (1976) examined resorcinol-utilizing *P. putida* strains and demonstrated that the organization of resorcinol catabolism may vary among strains. In *P. putida* ORC, resorcinol was shown to be metabolized via hydroxyquinol (hydroxyhydroquinone), followed by ortho-type oxygenative ring cleavage. Subsequent formation of maleylacetate was also reported (Chapman and Ribbons, 1976).

These biochemical observations were further supported by the enzymatic studies of Ohta and Ribbons (1976). The researchers purified resorcinol hydroxylase and orcinol hydroxylase from *P. putida* ORC and showed that both enzymes were FAD-containing flavoproteins catalyzing NAD(P)H/O_2-dependent hydroxylation reactions. They also demonstrated that enzyme synthesis was differentially regulated according to the growth substrate. These classical studies established the importance of hydroxylation in the initial stages of resorcinol degradation by *P. putida* and indicated that resorcinol catabolism may have strain-specific features.

Subsequent genetic investigations of resorcinol catabolism have suggested a broadly similar organization in other bacterial species. Huang et al. (2006) demonstrated that *Corynebacterium glutamicum* could utilize resorcinol as its sole carbon and energy source and characterized gene clusters involved in resorcinol catabolism. Genes associated with resorcinol hydroxylase, hydroxyquinol 1,2-dioxygenase, and maleylacetate reductase activities were experimentally shown to participate in successive transformation steps. These findings support the biological relevance of aromatic ring cleavage following hydroxylation in resorcinol degradation. Nevertheless, the enzymatic and genetic organization of this pathway may differ between

species and strains; therefore, a pathway identified in one organism should not be directly assumed to operate in another strain.

Within the broader context of aromatic compound metabolism, catechol is a central intermediate in the catabolism of numerous compounds. In the catechol branch of the β-ketoadipate pathway, catechol 1,2-dioxygenase is a key enzyme that catalyzes intradiol, or ortho, cleavage of the aromatic ring, converting catechol to cis,cis-muconate (Harwood and Parales, 1996). The presence of cat genes associated with this branch in the *P. putida* KT2440 genome represents an important component of its broad aromatic catabolic capacity (Jiménez et al., 2002).

A study on *P. putida* N6 showed that catechol 1,2-dioxygenase activity can vary substantially depending on the growth substrate. High activity was observed in cells grown on benzoate, catechol, or phenol, whereas cells grown on protocatechuate or 4-hydroxybenzoate predominantly exhibited protocatechuate 3,4-dioxygenase activity. The same study reported that the sequence encoding catechol 1,2-dioxygenase shared 100% sequence identity with catA genes characterized in two other *P. putida* strains (Guzik et al., 2011). Thus, catechol 1,2-dioxygenase may be regulated in a substrate-dependent manner as part of the broader catabolic response to aromatic compounds; however, this does not establish that it is the specific ring-cleavage enzyme responsible for resorcinol catabolism.

Indeed, regulation of aromatic catabolic pathways in *P. putida* may differ according to the substrate utilized. Feist and Hegeman (1969) showed that catechol formed during growth on benzoate was directed through the ortho pathway into the β-ketoadipate pathway via catechol 1,2-oxygenase, whereas catechol or substituted catechol derivatives formed during growth on phenol or cresols could be directed into a meta-cleavage route. They also demonstrated that distinct inducing mechanisms regulated enzymes of the ortho and meta pathways. The comprehensive review of the β-ketoadipate pathway by Harwood and Parales (1996) further emphasized that aromatic catabolism operates through a regulated metabolic network involving intermediates and regulatory systems, rather than merely through the presence of individual enzymes.

Consequently, assessing aromatic pollutant biodegradation solely by endpoint products or removal percentages may provide limited insight into the molecular organization of the process. Initial oxidation reactions and subsequent ring-cleavage steps need not exhibit their maximum activities simultaneously. The ability of the substrate and its intermediates to induce different enzyme systems suggests that the catabolic response may be temporally organized. Classical studies of catabolic regulation by Feist and Hegeman (1969), together with observations of

substrate-dependent resorcinol hydroxylase synthesis by Ohta and Ribbons (1976), demonstrate that aromatic catabolic functions can respond to environmental and metabolic signals.

Genomic, transcriptomic, proteomic, and other omics approaches are increasingly used to elucidate such metabolic responses. Joint investigation of the genes, enzymes, and regulatory networks involved in aromatic compound biotransformation enables a more detailed understanding of the mechanisms underlying the observed degradation phenotype. Vidal-Silva et al. (2025) comprehensively reviewed the growing importance of omics and bioinformatics in identifying microbial diversity, functional genes, and metabolic pathways involved in aromatic hydrocarbon degradation. Likewise, recent studies on *P. putida* emphasize that its extensive metabolic flexibility is relevant to systems biology and metabolic engineering (Weimer et al., 2020; de Lorenzo et al., 2024).

Nevertheless, much of the literature specifically addressing resorcinol has focused on bioreactor performance, substrate removal kinetics, substrate inhibition, enzymatic pathway characterization, or shifts in microbial communities. Sharma et al. (2010) examined operational and reactor parameters; Dey et al. (2011) investigated single-substrate biodegradation kinetics; Dey and Mukherjee (2013) explored dual-substrate inhibition; and Yang et al. (2022) studied activated-sludge adaptation to resorcinol and associated metagenomic changes. Meanwhile, classical investigations of resorcinol metabolism in *P. putida* characterized biochemical and enzymatic features of the pathway but did not integrate modern time-resolved transcriptional analysis with substrate removal kinetics in the same experimental system (Chapman and Ribbons, 1976; Ohta and Ribbons, 1976). Simultaneously assessing bacterial growth, time-dependent changes in resorcinol concentration, and the transcriptional responses of selected aromatic catabolic genes may therefore help clarify the relationships among the phenotypic, chemical, and molecular dimensions of resorcinol biodegradation.

Accordingly, the present study evaluated the utilization of 100 mg/L resorcinol as the sole carbon source by *P. putida* through three complementary data layers. Bacterial growth was monitored by OD_{600} measurements, changes in resorcinol concentration by HPLC, and time-dependent transcriptional responses of selected genes associated with hydroxylation and aromatic ring cleavage by RT-qPCR. The central hypothesis was that substrate removal and bacterial growth during resorcinol utilization by *P. putida* would be accompanied by temporally distinct transcriptional responses of selected genes involved in aromatic catabolism. Thus, the study aimed not only to determine the overall capacity for resorcinol removal but also to characterize temporal relationships among bacterial growth, chemical removal kinetics, and catabolic gene expression.

2. Materials and Methods

2.1. Bacterial strain

The *P. putida* R1 strain used in this study was previously isolated from activated sludge collected from a wastewater treatment plant. The strain was identified by 16S rRNA gene sequencing, and the corresponding sequence was deposited in the NCBI GenBank database under accession number MW248695. Its isolation and molecular characterization have previously been reported by Uyanıker et al. (2025).

2.2. Preliminary optimization and selection of sampling times

Before the final gene expression experiment, preliminary optimization was conducted to characterize the growth and degradation kinetics of *P. putida* R1 in the presence of resorcinol. Initial resorcinol concentrations of 50 and 100 mg/L were evaluated, with inoculated experimental groups and corresponding abiotic controls established for each concentration.

In the preliminary experiments, the active culture was inoculated into Bushnell–Haas (BH) minimal medium at 5% (v/v). Bacterial growth and residual resorcinol concentration were monitored over time by OD_{600} measurements and HPLC, respectively. Samples were initially analyzed at 0, 2, 4, 6, 8, 10, and 12 h and at additional later time points in the subsequent experimental series.

The preliminary results showed that bacterial growth and substrate depletion changed markedly over time, particularly at 100 mg/L resorcinol. In this group, growth became more pronounced at approximately 12–14 h, followed by an accelerated decrease in resorcinol concentration. Based on this kinetic profile, 0, 10, 14, 24, and 32 h were selected as the sampling times for the final gene expression study.

2.3. Preculture and inoculum preparation

Before the experiments, *P. putida* R1 was activated by incubation on Tryptic Soy Agar (TSA) at 30 °C for 12 h. Cells from the fresh culture were suspended in sterile 0.9% (w/v) NaCl, and cell density was standardized spectrophotometrically to OD_{600} = 1.0.

For the preliminary optimization experiments, the inoculum density was recorded as having been standardized to approximately OD_{600} = 1.20–1.28. In contrast, the inoculum prepared for the final experiment was standardized to OD_{600} = 1.0.

2.4. Setup of the final resorcinol biodegradation experiment

The final biodegradation and gene expression experiments were conducted in 500-mL Erlenmeyer flasks with a working volume of 100 mL. All experimental conditions were established with three independent biological replicates.

Sterile Bushnell–Haas Broth (BHB) minimal mineral medium containing 100 mg/L resorcinol as the sole carbon source was used as the degradation medium. The final experiment consisted of the following three conditions:

- Resorcinol biodegradation group: BHB containing 100 mg/L resorcinol + *P. putida* R1;
- Abiotic control: BHB containing 100 mg/L resorcinol without bacterial inoculum;
- Gene expression control group: BHB containing 100 mg/L glucose as the sole carbon source + *P. putida* R1.

This experimental arrangement was also described in the final project report as the resorcinol-containing degradation group, resorcinol control, and glucose-containing gene regulation control.

The standardized *P. putida* R1 cell suspension was inoculated into the experimental media at 5% (v/v). Cultures were incubated at 27 °C and 120 rpm in the dark for 32 h.

2.5. Sampling and monitoring of bacterial growth

Samples were collected at 0, 10, 14, 24, and 32 h of incubation. These time points were selected according to the kinetic profile obtained in the preliminary growth and degradation experiments.

At each sampling time, 1 mL of culture was collected to determine bacterial growth by measuring optical density at 600 nm. An additional 2 mL of culture was collected at the same time points for HPLC and RNA analyses.

2.6. Sample preparation for HPLC and RNA analyses

The 2-mL culture samples collected for HPLC and RT-qPCR analyses were centrifuged at 7,000 rpm for 5 min. The supernatants were separated for determination of resorcinol concentrations by HPLC.

Before RNA extraction, cell pellets were washed with 1 mL of sterile 0.9% NaCl, centrifuged again, and the supernatants removed. Samples allocated for HPLC and gene expression analyses were stored at −20 °C until analysis.

2.7. Determination of resorcinol concentration by HPLC

Resorcinol was analyzed using a PerkinElmer Series 200 HPLC system. Chromatographic separation was performed on an Eclipse XDB-C18 reversed-phase analytical column (5 µm, 4.6 × 150 mm) maintained at 30 °C.

The mobile phase consisted of water containing 0.15% phosphoric acid and methanol (75:25, v/v), delivered isocratically at 0.8 mL/min. The injection volume was 10 µL, UV detection was performed at 280 nm, and the total run time was 10 min. Under these conditions, the retention time of resorcinol was approximately 3.2 min.

For quantitative analysis, an external calibration curve was prepared using eight resorcinol standards ranging from 1 to 500 mg/L.

The percentage of resorcinol removal was calculated from the concentrations measured at the beginning of the experiment and at each sampling time.

$$\text{Removal (\%)} = \frac{C_0 - C_t}{C_0}\ x\ 100$$

Here, C_0 represents the initial resorcinol concentration and C_t the concentration measured at the corresponding sampling time.

The abiotic control without bacteria was used to assess possible resorcinol losses unrelated to biological activity under the experimental conditions.

2.8. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the bacterial cell pellets collected at each sampling time using the Presto^{TM} Mini RNA Bacteria Kit (Geneaid Biotech Ltd.) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop 2000C spectrophotometer.

Before cDNA synthesis, RNA samples were standardized to comparable concentrations and reverse-transcribed using the NZY First-Strand cDNA Synthesis Kit. The concentration and purity of the resulting cDNA were checked using a NanoDrop 2000C spectrophotometer.

2.9. Target gene selection, primer design, and specificity assessment

The phenol hydroxylase and catechol 1,2-dioxygenase genes were selected to examine transcriptional responses associated with aromatic catabolism during resorcinol exposure.

The phenol hydroxylase primers were designed with reference to the *P. putida* phenol hydroxylase gene cluster deposited in NCBI GenBank (accession no. D28864.1). The catechol 1,2-dioxygenase primers were designed on the basis of *P. putida* gene sequences available in GenBank.

Primer sequences were selected using Primer3 and related primer-design software, and the in silico specificity of the selected primer pairs was evaluated using NCBI database resources.

Melting-curve analysis following RT-qPCR revealed a single distinct peak for each target gene, supporting the generation of a predominant amplification product under the experimental conditions.

The expected amplicon lengths for phenol hydroxylase and catechol 1,2-dioxygenase were 147 and 177 bp, respectively. Primer sequences are listed in Table 1.

Table 1. Primer sequences used in this study

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)

Phenol hydroxylase	CCCACACCGACTTTCGATCA	GCAAAACTTCACGAAGGCGT	147
Catechol 1,2-dioxygenase	GTCATTCGACTTCCGCTTGC	CGATTCTAGATCACCCGCCC	177
gyrB	GCAGCCGAGGTCATCATGA	GCGTTCACAACCGACACAC	106
gap-1	AGCAAGGCCTGATGACCAC	CGGGATCATCGACTGGGTG	109

2.10. RT-qPCR conditions

RT-qPCR was performed using a LightCycler 96 instrument (Roche Instruments) and LightCycler 480 SYBR Green I Master in a total reaction volume of 20 µL. Each reaction contained 10 µL SYBR Green I Master, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2.5 µL cDNA, and 5.5 µL PCR-grade water.

The thermal cycling program consisted of:

- initial activation/denaturation at 95 °C for 600 s;
- denaturation at 95 °C for 10 s;
- primer annealing at 61 °C for 15 s;
- extension at 72 °C for 15 s.

The amplification steps were repeated for 45 cycles. Melting-curve analysis was performed after amplification to assess product specificity.

2.11. Calculation of relative gene expression

Relative expression levels of the phenol hydroxylase and catechol 1,2-dioxygenase genes were calculated from Ct values obtained by RT-qPCR.

The *gyrB* and *gap-1* reference genes were used jointly for normalization. Amplification efficiencies and Ct profiles were evaluated during reference gene selection, and the arithmetic mean of the Ct values for both reference genes was used for normalization.

For each sample, ΔCt was calculated by subtracting the arithmetic mean Ct of *gyrB* and *gap-1* from the Ct of the target gene.

Target gene expression in the resorcinol-containing group was compared with that in a control culture collected at the same sampling time, containing 100 mg/L glucose as the sole carbon source and no resorcinol.

ΔΔCt values were calculated as the difference between the normalized ΔCt of the resorcinol group and the ΔCt of the time-matched glucose control. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) and expressed as fold changes relative to the control condition.

2.12. Integrated analysis of phenotypic, chemical, and molecular data

Three independent data layers were evaluated together to characterize the temporal dynamics of resorcinol degradation at different biological levels:

- bacterial growth: OD_{600};
- substrate removal: resorcinol concentration measured by HPLC;
- molecular response: relative gene expression measured by RT-qPCR.

2.13. Experimental replicates and statistical analysis

Bacterial growth, resorcinol removal, and gene expression experiments were performed in three independent biological replicates (n = 3). OD_{600} and HPLC results are presented as the arithmetic mean ± standard deviation (SD) of the biological replicates.

Relative expression of the phenol hydroxylase and catechol 1,2-dioxygenase targets was evaluated using fold-change values obtained by the $2^{-\Delta\Delta Ct}$ method. Descriptive statistics for gene expression were calculated as the arithmetic mean and sample standard deviation of the three replicate values in the results table. For graphical presentation, $\log_{10}$ transformation was applied to the fold-change values, and the means and standard deviations of the $\log_{10}$-transformed replicate values were used.

Data were evaluated using descriptive statistics and time-dependent profiles; no inferential statistical significance tests were applied to between-group comparisons.

3. Results

3.1. Preliminary optimization and selection of sampling times

Before the final gene expression study, preliminary optimization experiments were conducted to determine the growth and substrate removal profiles of *P. putida* R1 in the presence of resorcinol. Initial concentrations of 50 and 100 mg/L were evaluated at different sampling times. Bacterial growth was monitored by OD_{600} measurements, and residual resorcinol concentrations were measured by HPLC.

The preliminary optimization results indicated limited bacterial growth and only a slight decrease in resorcinol concentration during the initial hours of incubation, particularly under the 100 mg/L resorcinol condition. Bacterial growth became more pronounced after approximately 12-14 h, and the decrease in resorcinol concentration accelerated during subsequent incubation.

Based on this kinetic profile, 0, 10, 14, 24, and 32 h were selected as sampling points for the final biodegradation and gene expression experiments.

3.2. Time-dependent bacterial growth and resorcinol removal

Bacterial growth and the time-dependent change in residual resorcinol concentration were evaluated together for *P. putida* R1 grown in Bushnell–Haas minimal medium with an initial resorcinol concentration of 100 mg/L (Figure 1).

The initial OD_{600} value of 0.08 ± 0.02 increased to 0.17 ± 0.02 at 10 h. Over the same period, resorcinol concentration decreased from 100.00 ± 4.09 to 98.34 ± 4.05 mg/L, corresponding to a limited removal of 1.66% relative to the initial concentration during the first 10 h.

Between 10 and 14 h, bacterial growth increased markedly, with OD_{600} reaching 0.49 ± 0.02 at 14 h. At the same time point, resorcinol concentration was 84.60 ± 4.59 mg/L, and cumulative removal increased to 15.40%. These findings indicate that the increase in bacterial growth was accompanied by a decrease in resorcinol concentration.

The highest average decrease in resorcinol concentration among the examined sampling intervals occurred between 14 and 24 h. During this period, OD_{600} increased to 0.62 ± 0.03, while resorcinol concentration declined to 23.95 ± 2.04 mg/L. Thus, resorcinol concentration decreased by 60.65 mg/L between 14 and 24 h, corresponding to an average removal rate of 6.07 mg/L/h.

At 32 h, OD_{600} reached 0.65 ± 0.03, indicating that the increase in growth had slowed relative to earlier periods. At the same time, resorcinol concentration was 5.45 ± 0.59 mg/L. Total resorcinol removal, calculated from the initial and final mean concentrations, was 94.55%.

The joint evaluation of growth and resorcinol concentrations indicated that substrate depletion became more pronounced after 10 h and that the highest average removal rate among the measured intervals occurred between 14 and 24 h. Differences in the temporal profiles of bacterial growth and substrate depletion showed that these parameters did not change at identical rates throughout the process.

Data are presented as mean ± standard deviation (SD) of three experimental replicates (n = 3).

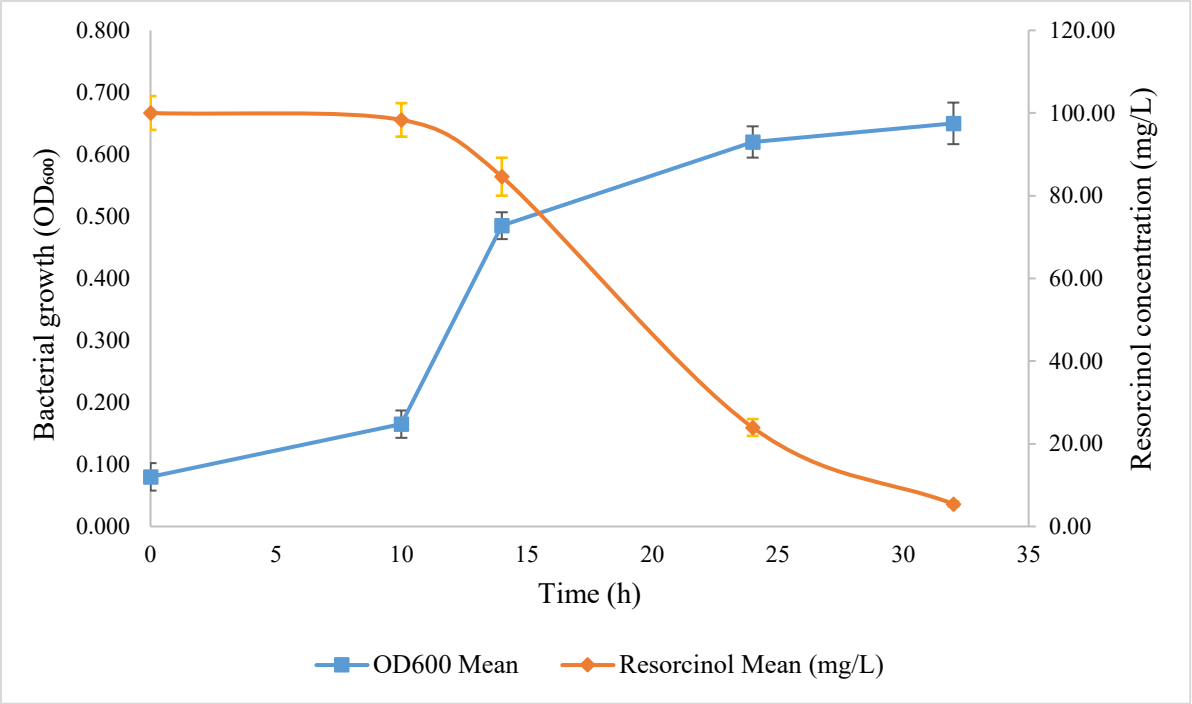

Fig. 1 Time-dependent bacterial growth and resorcinol removal by *P. putida R1*. Bacterial growth was monitored by measuring optical density at 600 nm (OD_{600}; left y-axis), while residual resorcinol concentration was quantified by HPLC (right y-axis) over 32 h of incubation in Bushnell–Haas minimal medium supplemented with 100 mg/L resorcinol as the sole carbon source. Data are presented as mean ± standard deviation (SD) from three independent biological replicates (n = 3).

3.3. Time-dependent expression profile of the phenol hydroxylase target

Relative expression of the phenol hydroxylase gene target in *P. putida* R1 showed marked temporal differences under resorcinol-containing conditions compared with the glucose control (Figure 2).

The relative expression level, initially 1.018 ± 0.039-fold, decreased to 0.052 ± 0.006-fold at 10 h. This value represents lower expression relative to the time-matched glucose control.

By contrast, relative expression of the phenol hydroxylase target increased to 543.072 ± 11.353-fold at 14 h, reaching its highest level among the sampling times investigated.

Although relative expression declined at subsequent time points, it remained above the glucose control. Phenol hydroxylase expression was 112.595 ± 4.815-fold at 24 h and 14.320 ± 0.512-fold at 32 h.

These results demonstrate that the phenol hydroxylase target exhibited a temporally distinct transcriptional profile between resorcinol-containing and glucose-containing conditions. The maximum relative expression observed at 14 h coincided with the sampling period during which bacterial growth increased markedly and the decline in resorcinol concentration accelerated.

3.4. Time-dependent expression profile of the catechol 1,2-dioxygenase target

Relative expression of the catechol 1,2-dioxygenase target followed a different temporal profile from that of the phenol hydroxylase target (Figure 2).

Relative expression increased from an initial level of 1.020 ± 0.160-fold to 9.883 ± 0.227-fold at 10 h. This finding indicates that the catechol 1,2-dioxygenase target exhibited higher expression than the time-matched glucose control during the early sampling period.

At 14 h, relative expression declined to 1.597 ± 0.214-fold, approaching the control level.

Expression subsequently increased again, reaching its maximum of 26.630 ± 2.007-fold at 24 h. At 32 h, relative expression decreased to 21.706 ± 1.178-fold compared with the maximum observed at 24 h.

These results indicate that the catechol 1,2-dioxygenase target exhibited a temporal profile characterized by higher relative expression at 10 and 24 h and lower expression at 14 h. Its maximum relative expression at 24 h differed from the 14 h maximum observed for the phenol hydroxylase target.

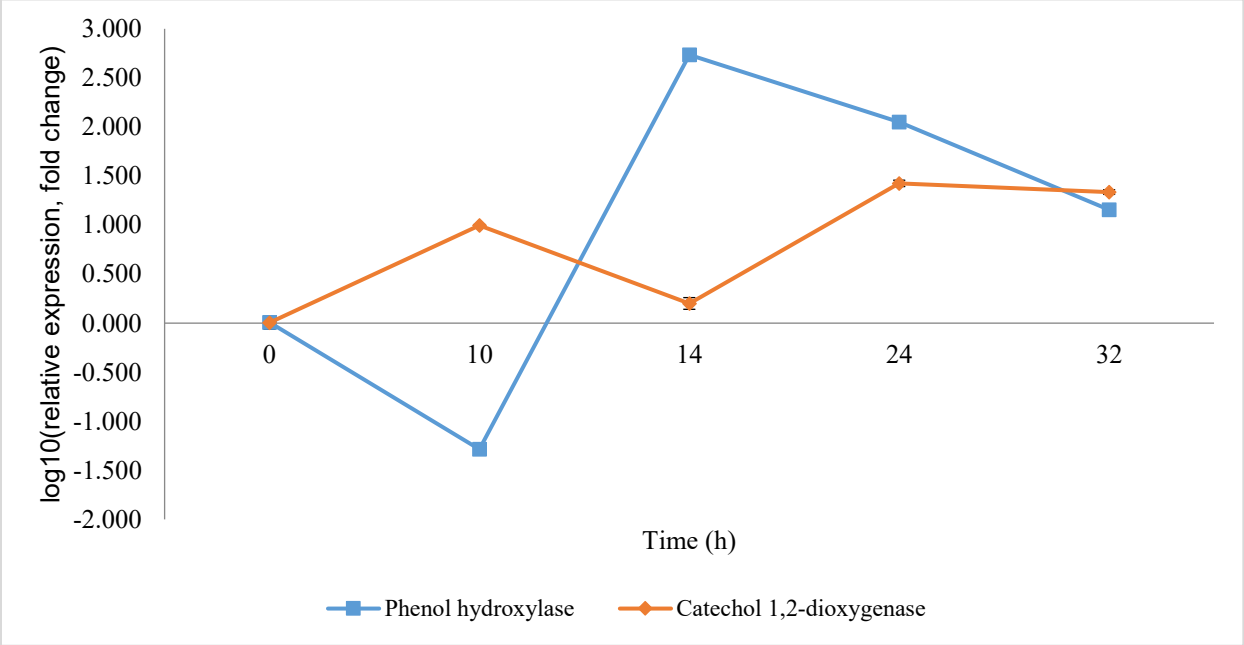

Fig. 2 Time-dependent relative expression profiles of phenol hydroxylase and catechol 1,2-dioxygenase in *Pseudomonas putida* R1 during resorcinol biodegradation. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with *gyrB* and *gap-1* as reference genes and time-matched glucose-grown cultures serving as calibrators. The arithmetic mean Ct values of the two reference genes were used for normalization. Both expression profiles are presented as $\log_{10}$-transformed fold-change values on a common y-axis. Data are presented as mean ±

standard deviation (SD) of $\log_{10}$-transformed replicate values (n = 3). The horizontal reference line at $\log_{10}$(fold change) = 0 corresponds to the control expression level (fold change = 1).

3.5. Integrated evaluation of bacterial growth, resorcinol removal, and gene expression

Bacterial growth, resorcinol concentration, and the relative expression of selected genes associated with aromatic catabolism were evaluated together at identical sampling times. The findings indicate that bacterial growth, substrate depletion, and transcriptional responses followed different temporal profiles under resorcinol-containing conditions.

During the first 0–10 h, OD_{600} increased from 0.08 to 0.17, whereas resorcinol concentration decreased from 100.00 to 98.34 mg/L. By the end of this interval, relative expression of the phenol hydroxylase target was 0.052-fold compared with the time-matched glucose control, while catechol 1,2-dioxygenase expression reached 9.883-fold.

Between 10 and 14 h, OD_{600} increased from 0.17 to 0.49, and resorcinol concentration decreased from 98.34 to 84.60 mg/L. By the end of this interval, phenol hydroxylase relative expression reached its maximum of 543.072-fold among the sampled time points, whereas catechol 1,2-dioxygenase expression was 1.597-fold.

The highest average rate of decrease in resorcinol concentration was observed between 14 and 24 h. During this interval, resorcinol concentration declined from 84.60 to 23.95 mg/L, corresponding to an average rate of 6.07 mg/L/h. At 24 h, OD_{600} increased to 0.62, and catechol 1,2-dioxygenase expression reached its maximum of 26.630-fold. At the same time point, phenol hydroxylase expression was 112.595-fold.

During the final 24–32 h interval, resorcinol concentration decreased to 5.45 mg/L, while OD_{600} reached 0.65. At the end of this period, relative expression of both targets had declined compared with their respective earlier maxima.

Overall, the maximum relative expression of the phenol hydroxylase and catechol 1,2-dioxygenase targets occurred at 14 and 24 h, respectively. However, the increased expression of the catechol 1,2-dioxygenase target at 10 h indicates that the two targets did not exhibit a simple, definitive sequence of activation. The highest average decrease in resorcinol concentration occurred within the sampling interval between these two expression maxima, indicating a temporal association among growth, substrate depletion, and transcriptional responses.

4. Discussion

4.1. Resorcinol removal capacity and growth kinetics of *P. putida* R1

In this study, *P. putida* R1, isolated from wastewater treatment plant activated sludge, exhibited 94.55% resorcinol removal after 32 h of incubation in Bushnell–Haas minimal medium

containing an initial resorcinol concentration of 100 mg/L, calculated from the initial and final concentrations. An abiotic control group was included in the experimental design to assess potential substrate losses unrelated to biological activity. Over the same period, OD_{600} increased from 0.08 to 0.65, indicating bacterial growth in the resorcinol-containing medium. The removal performance evaluated in the context of the control conditions supports an association between the observed substrate depletion and bacterial treatment. Nevertheless, the decrease in resorcinol measured by HPLC was not interpreted as direct evidence of complete mineralization.

Previous studies on microbial resorcinol biodegradation have shown that removal performance may be influenced by initial substrate concentration, microbial adaptation, and operational conditions. Sharma et al. (2010) investigated resorcinol removal in a sequencing batch reactor and evaluated the effects of biomass concentration and aeration on process performance. Dey et al. (2011) reported that resorcinol biodegradation in aerobic batch cultures depended on substrate concentration and that substrate inhibition at high initial concentrations could affect growth and removal kinetics. Compared with these studies, the present investigation is distinctive in integrating pure-culture removal performance at a single initial resorcinol concentration with bacterial growth and transcriptional responses of selected catabolic genes. However, differences among experimental systems limit direct comparisons of removal efficiencies.

The temporal profiles of bacterial growth and substrate depletion did not fully coincide in the present study. In particular, between 10 and 14 h, OD_{600} increased from 0.17 to 0.49 while resorcinol concentration declined from 98.34 to 84.60 mg/L. In contrast, the highest average substrate depletion rate among the evaluated sampling intervals was calculated as 6.07 mg/L/h between 14 and 24 h. This finding indicates that biomass accumulation and resorcinol removal did not follow identical temporal profiles. The difference may reflect physiological adaptation, changes in substrate utilization, or time-dependent regulation of catabolic processes. However, the present experimental design cannot definitively distinguish among these possible mechanisms.

Recent investigations of biological phenolic pollutant treatment have also emphasized the importance of microbial adaptation and physiological conditions when evaluating removal performance. Yu et al. (2025) investigated the treatment of phenolic wastewater using algal–bacterial granular sludge, while Nunes et al. (2026) evaluated the effects of phenol concentration on aerobic granular sludge characteristics and microbial communities. Although conducted using different microbial communities and experimental systems, these studies

support the importance of jointly considering substrate characteristics and microbial physiology during biological removal of phenolic compounds.

Taken together, the high resorcinol removal and concurrent bacterial growth observed for *P. putida* R1 demonstrate its potential for biological treatment under resorcinol-containing conditions. However, because only one initial concentration was included in the final experiment, definitive conclusions regarding substrate inhibition or concentration-dependent kinetic parameters are limited. Furthermore, a reduction in the parent compound measured by HPLC does not directly demonstrate complete mineralization of resorcinol.

4.2. Transcriptional regulation of aromatic catabolism

The ability of *Pseudomonas* species to metabolize aromatic compounds is a major reason for their importance in environmental biotechnology. Nevertheless, the organization of aromatic catabolic pathways and metabolic capabilities may vary among strains. Through comparative genomic analysis and metabolic modeling, Mueller et al. (2026) demonstrated strain-specific genetic and functional differences in aromatic compound utilization among *P. putida* strains. Similarly, Banerjee et al. (2025) assessed the metabolic capacity of *P. putida* for bioconversion of aromatic carbon sources using a genome-scale modeling approach. These studies support considering strain-specific metabolic properties and genetic organization when investigating aromatic catabolic functions.

In the present study, relative expression of the phenol hydroxylase target was 0.052 ± 0.006-fold at 10 h compared with the time-matched glucose control. By contrast, expression increased to a maximum of 543.072 ± 11.353-fold at 14 h. At subsequent times, relative expression declined to 112.595 ± 4.815-fold at 24 h and 14.320 ± 0.512-fold at 32 h, but remained above the glucose control at both time points. These findings indicate a strong, temporally differentiated transcriptional response of the phenol hydroxylase target under resorcinol-containing conditions.

The maximum phenol hydroxylase expression at 14 h coincided with the period in which bacterial growth became more pronounced and the decline in resorcinol concentration accelerated. In particular, the increase in OD_{600} from 0.17 to 0.49 and the decrease in resorcinol concentration from 98.34 to 84.60 mg/L between 10 and 14 h indicate a temporal association among transcriptional response, growth, and substrate depletion. However, it cannot be concluded that the observed expression change resulted directly from a particular enzymatic step in resorcinol metabolism.

Liu et al. (2026) investigated microbial removal of aromatic compounds at the gene transcription level in a system using phenol as a co-substrate and reported that catabolic gene

expression could be associated with biodegradation. Zou et al. (2025) evaluated biodegradation performance in the phenol-degrading strain *Pseudomonas alloputida* BF04. These studies support jointly investigating microbial metabolic capacity and catabolic functions to understand the biological removal of aromatic pollutants. However, differences in the chemical structures and catabolic transformation pathways of phenol and resorcinol preclude direct extrapolation of these findings to the present study.

Strong induction of catabolic genes during biodegradation of aromatic compounds has been reported previously. Chen et al. (2016) investigated the transcriptional regulation of para-nitrophenol degradation in *Pseudomonas putida* DLL-E4 and identified a 519.1-fold increase in expression of pnpA, encoding para-nitrophenol 4-monooxygenase, by RNA-Seq and a 152.7-fold increase by RT-qPCR validation. These findings show that genes encoding oxidative enzymes involved in aromatic compound metabolism can exhibit transcriptional responses of several hundred-fold in *P. putida* strains. The 543.072-fold relative expression observed for the phenol hydroxylase target at 14 h in the present study likewise supports a strong transcriptional response. However, differences in target gene function, aromatic substrates, reference conditions, and analytical methods mean that the fold-change values cannot be directly compared between studies. Furthermore, a high relative transcript level does not necessarily indicate a proportional increase in the catalytic activity of the target enzyme.

Classical biochemical studies of bacterial resorcinol metabolism established the importance of hydroxylation reactions in its transformation. Ohta and Ribbons (1976) purified and characterized resorcinol hydroxylase and orcinol hydroxylase from *P. putida* ORC. Chapman and Ribbons (1976) reported that resorcinol in the same strain was metabolized through hydroxyquinol, followed by oxygenative ring cleavage. These findings support a role for hydroxylation in the initial steps of resorcinol catabolism.

Nevertheless, the phenol hydroxylase gene target examined here should not be assumed to be identical to the gene encoding the resorcinol hydroxylase enzyme. The primer design based on a reference sequence from a *P. putida* phenol hydroxylase gene cluster, the bioinformatic specificity assessment, and the observation of a single distinct RT-qPCR melting-curve peak support the specificity of amplification.

Moreover, the lower relative expression at 10 h and the higher expression at 14 h were calculated against different time-matched glucose controls. Therefore, the entire change cannot be attributed unequivocally to induction directly caused by resorcinol. Substrate availability, growth phase, and physiological differences associated with carbon source utilization may also influence the observed transcriptional profile.

Overall, these findings indicate that the phenol hydroxylase target in *P. putida* R1 exhibited a strong, time-dependent relative transcriptional response under resorcinol-containing conditions.

4.3. Temporal characteristics of the catechol 1,2-dioxygenase response

Ring-cleavage reactions are among the principal processes that channel aromatic structures into subsequent metabolic steps during bacterial catabolism. The activity and gene expression of the dioxygenases involved may vary with the chemical structure of the aromatic substrate and the metabolic characteristics of the microorganism. Catechol 1,2-dioxygenase is an important enzyme in the catechol branch of the β-ketoadipate pathway, catalyzing intradiol (ortho-type) cleavage of catechol to form cis,cis-muconate (Guzik et al., 2011).

In the present study, relative expression of the catechol 1,2-dioxygenase target followed a different temporal pattern from that of phenol hydroxylase. Expression was 9.883 ± 0.227-fold at 10 h relative to the time-matched glucose control, decreased to 1.597 ± 0.214-fold at 14 h, reached a maximum of 26.630 ± 2.007-fold at 24 h, and declined to 21.706 ± 1.178-fold at 32 h. These results show that the catechol 1,2-dioxygenase target exhibited a variable, rather than continuously increasing, relative transcriptional response under resorcinol-containing conditions.

Notably, catechol 1,2-dioxygenase expression was above the control level at 10 h, whereas phenol hydroxylase expression was below the corresponding control at 0.052-fold. Conversely, at 14 h, phenol hydroxylase expression reached its maximum while catechol 1,2-dioxygenase expression approached the control level. These contrasting profiles indicate that the two gene targets did not exhibit identical temporal transcriptional responses to the experimental conditions.

Previous studies have also demonstrated substrate-dependent differences in catechol 1,2-dioxygenase activity. Guzik et al. (2011) observed high catechol 1,2-dioxygenase activity in *P. putida* N6 grown on phenol, benzoate, or catechol, whereas a different dioxygenase system predominated in cells grown on protocatechuate and 4-hydroxybenzoate. Zhang et al. (2023) evaluated the kinetics and associated metabolic pathway of phenol biodegradation by *Pseudomonas* sp. SAS26. These studies emphasize that enzyme systems involved in aromatic ring cleavage must be interpreted in the context of the substrate used and experimental conditions. However, because they concern phenol and other aromatic substrates, their findings cannot be directly extended to resorcinol catabolism.

This distinction is particularly important for aerobic resorcinol catabolism. Chapman and Ribbons (1976) demonstrated that resorcinol was metabolized through a hydroxyquinol

intermediate in *P. putida* ORC, followed by ortho-type oxygenative ring cleavage and maleylacetate formation. Similarly, genetic and biochemical analyses by Huang et al. (2006) identified genes associated with resorcinol hydroxylase, hydroxyquinol 1,2-dioxygenase, and maleylacetate reductase during transformation of resorcinol through hydroxyquinol in Corynebacterium glutamicum. These studies indicate that substrate-specific enzymes, such as hydroxyquinol 1,2-dioxygenase, may participate in the ring-cleavage stage of resorcinol catabolism.

Accordingly, the increased expression of the catechol 1,2-dioxygenase target observed here was not interpreted as evidence that this enzyme directly catalyzes resorcinol ring cleavage in strain R1. Catechol 1,2-dioxygenase and hydroxyquinol 1,2-dioxygenase are enzymes with different substrate specificities. Because neither the intermediates formed during resorcinol transformation nor the catalytic activity of the target enzyme were directly determined, the observed transcriptional response cannot be assigned to a specific catabolic reaction in R1.

The maximum catechol 1,2-dioxygenase expression at 24 h coincided with the end of the 14–24 h interval, during which the highest average decrease in resorcinol concentration was observed. This temporal association suggests that transcriptional responses related to aromatic catabolism may vary across different stages of substrate depletion.

Overall, the findings demonstrate that the catechol 1,2-dioxygenase target in *P. putida* R1 exhibited a time-dependent relative expression profile under resorcinol-containing conditions that differed from that of phenol hydroxylase. This supports the occurrence of distinct transcriptional responses among selected aromatic catabolic genes during resorcinol removal but does not directly identify the specific resorcinol ring-cleavage mechanism in strain R1.

4.4. Temporal relationships among bacterial growth, resorcinol removal, and gene expression

A central finding of the present study is that bacterial growth, the decline in resorcinol concentration, and the relative expression of two selected aromatic catabolic gene targets followed different dynamics along the same experimental timeline in *P. putida* R1. The period of pronounced bacterial growth, the interval with the highest average decrease in resorcinol concentration, and the times of maximum relative gene expression did not coincide completely. Thus, physiological, chemical, and transcriptional responses may follow distinct temporal patterns during resorcinol removal.

The increase in OD_{600} from 0.17 to 0.49 between 10 and 14 h indicated pronounced bacterial growth. At the end of this interval, relative expression of the phenol hydroxylase target reached its maximum of 543.072-fold. In contrast, the highest average rate of decrease in resorcinol

concentration, 6.07 mg/L/h, was recorded between 14 and 24 h. These observations indicate that bacterial growth, gene expression, and substrate depletion were temporally associated but did not proceed in complete synchrony.

Maximum relative expression of the catechol 1,2-dioxygenase target was observed at 24 h, at the end of the interval with the highest average decrease in resorcinol concentration. However, this target also showed a 9.883-fold relative expression level at 10 h, indicating that the two genes cannot be interpreted as participating solely in a simple, consecutive activation sequence. Consequently, their different peak expression times should be regarded as distinct transcriptional responses of selected aromatic catabolic genes, rather than direct evidence of a sequential series of metabolic reactions.

Previous research on bacterial aromatic compound catabolism has shown that metabolic pathways may differ according to substrate and microbial genetic background. Chapman and Ribbons (1976) demonstrated that resorcinol utilization may involve alternative metabolic organizations in different *P. putida* strains and that the corresponding catabolic enzymes may be differentially regulated. Ohta and Ribbons (1976) characterized the substrate-dependent synthesis and catalytic properties of aromatic hydroxylases in *P. putida* ORC. These observations support the substrate- and strain-specific regulation of enzyme systems involved in aromatic compound transformation. Nevertheless, these biochemical findings cannot be considered direct mechanistic explanations for the transcriptional changes observed in strain R1.

The use of time-matched glucose controls to calculate gene expression must also be considered when interpreting the results. The observed relative expression differences may reflect both the transcriptional response under resorcinol-containing conditions and the differing effects of the two carbon sources on bacterial growth and physiological state. Furthermore, because gene expression was measured at discrete sampling times, the present data do not permit definitive identification of a time lag or direct causal relationship between the maximum transcriptional response and changes in substrate removal.

The original contribution of this study lies in the concurrent evaluation of changes in resorcinol concentration, bacterial growth, and relative expression of two selected aromatic catabolic genes in *P. putida* R1 within the same experimental system and at the same sampling times. This integrated approach demonstrates that phenotypic and transcriptional changes accompanying resorcinol removal may follow different temporal profiles. Rather than confirming a specific resorcinol catabolic pathway, these findings contribute to a time-resolved characterization of biodegradation through chemical, physiological, and molecular indicators.

5. Conclusion

This study investigated the time-dependent relationships among bacterial growth, resorcinol removal, and the transcriptional responses of selected aromatic catabolic genes in *P. putida* R1. Under the investigated conditions, the strain achieved 94.55% resorcinol removal within 32 h, while bacterial growth increased from an initial OD_{600} of 0.08 to 0.65. The highest average resorcinol removal rate (6.07 mg/L/h) was observed between 14 and 24 h, indicating that bacterial growth and substrate removal exhibited distinct temporal patterns.

RT-qPCR analysis revealed different relative expression profiles for the two investigated gene targets. Phenol hydroxylase expression reached its maximum at 14 h (543.072-fold), whereas catechol 1,2-dioxygenase exhibited its highest relative expression at 24 h (26.630-fold), following an earlier increase at 10 h (9.883-fold). These findings demonstrate that the selected aromatic catabolic genes displayed distinct transcriptional responses during resorcinol removal. However, the observed differences in expression timing do not establish a sequential enzymatic activation mechanism or confirm the direct involvement of these enzymes in a specific resorcinol degradation pathway.

The integration of HPLC-based substrate quantification, bacterial growth measurements, and RT-qPCR analysis provided a time-resolved characterization of the physiological, chemical, and transcriptional changes associated with resorcinol removal. Overall, the findings support the potential of *P. putida* R1 for the biological treatment of resorcinol-containing wastewater and highlight the value of combining complementary analytical approaches to investigate microbial aromatic pollutant biodegradation.

Statements and Declarations

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Competing interests

Tayyibe Alpay Uyanıker is an employee of SOCAR Turkey R&D and Innovation Co., which provided financial support for this study. The other authors declare that they have no relevant financial or non-financial interests to disclose.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

Muharrem Oğuz Ersöz and Selin Sarıçayır performed the experiments and analyses. Büşranur Ütsügör contributed to the study design. Tayyibe Alpay Uyanıker contributed to the coordination and management of research activities and provided scientific guidance. Güven Özdemir contributed to the study design, wrote the manuscript, and provided scientific guidance. All authors read and approved the final manuscript.

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