

Kinetics of BTX Removal by *Rhodococcus erythropolis* in a Slurry Bioreactor

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ABSTRACT

Benzene, toluene, and xylene (BTX) contamination arising from oil-drilling operations constitutes a persistent environmental challenge, necessitating the development of robust bioremediation strategies underpinned by kinetic evaluation. This study systematically investigated the kinetics of BTX removal by *Rhodococcus erythropolis* in a slurry-phase bioreactor employing soil sourced from the PPEJ oil-drilling site in Tuban, Indonesia, with particular emphasis on the influence of inoculum concentration on the k_0 and the K_m . Batch experiments were conducted at inoculum loadings of 12,5%, 15%, and 17,5% (v/v), with periodic monitoring of dissolved-phase BTX concentrations and BOD. Analytical methodologies encompassed gas chromatography–mass spectrometry (GC–MS) for the quantification of BTX and intermediate metabolites, BOD assessment, and kinetic modeling to estimate k_0 and K_m values. The results demonstrated that incremental increases in inoculum concentration consistently elevated the k_0 , thereby expediting BTX removal in reactors with higher biomass. However, a concomitant decline in specific activity per unit biomass was observed at the highest inoculum level, attributable to mass-transfer limitations—specifically, restricted diffusion of oxygen and substrate into microbial flocs—and heterogeneity in enzyme induction. The estimated K_m values exhibited variability across inoculum concentrations, indicating that substrate affinity is modulated by reactor physical parameters and microbial enzymatic adaptation. These findings underscore the necessity of optimizing inoculum concentration in concert with operational parameters such as dissolved oxygen control, agitation intensity, and structured kinetic assays to ensure that enhancements in k_0 are translated into effective per-cell degradation and complete mineralization of BTX contaminants in soils impacted by oil-drilling activities.

KEYWORDS

BTX, *Rhodococcus Erythropolis*, BOD, Michaelis-Menten Constant, Maximum Growth Rate

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A. Introduction

Benzene, toluene, and xylene (BTX) are monoaromatic hydrocarbons extensively utilized in petrochemical manufacturing, solvent production, and as fuel additives. Their widespread application and improper disposal practices have resulted in frequent detection of BTX in polluted soils, groundwater, and industrial effluents. Because of their persistence and toxicity, these compounds are considered priority pollutants: benzene is recognized as a human carcinogen, while toluene and xylene are linked to neurological and developmental disorders. Recent investigations have underscored the environmental and health risks associated with BTX contamination, stressing the importance of sustainable and effective remediation strategies (Chen et al., 2022).

Conventional physicochemical treatments—such as adsorption, chemical oxidation, and air stripping—have been employed to eliminate BTX from contaminated environments. Nevertheless, these methods are often expensive, energy-intensive, and prone to generating secondary pollutants. Microbial biodegradation has emerged as a more environmentally friendly alternative, relying on microorganisms to transform BTX into less harmful products. However, the efficiency of

biodegradation is highly variable, influenced by microbial community composition, environmental conditions, and substrate concentrations, which complicates its application in real-world scenarios ([Nayyeri et al., 2022](#)).

Recent studies have shown that aerobic bacteria, including *Pseudomonas* and *Rhodococcus* species, can degrade BTX via oxygenase-mediated pathways, producing catechol intermediates that feed into central metabolic cycles. Anaerobic degradation has also been documented, though at slower rates, with specialized microbial consortia capable of metabolizing BTX under oxygen-limited conditions ([Al-obaidi et al., 2024](#)). These findings highlight the promise of microbial biodegradation but also point to the necessity of kinetic modeling to better interpret and predict degradation behavior.

Rhodococcus erythropolis is increasingly recognized as a promising agent for the remediation of petroleum-polluted soils due to its metabolic versatility and resilience. Beyond its established role in degrading hydrocarbons through oxygenase-mediated pathways and biosurfactant production ([Kuyukina & Ivshina, 2019](#)), recent experimental evidence demonstrates its practical effectiveness. Studies have shown that *R. erythropolis* can efficiently degrade xylene in ex situ slurry bioreactors, achieving high removal efficiency within several weeks, as confirmed by gas chromatography analysis ([Ole & Juliastuti, 2023](#)). This highlights the bacterium's ability to adapt to varying nutrient conditions and maintain strong degradation performance in petroleum-contaminated environments. Collectively, these findings reinforce the role of *R. erythropolis* as a robust candidate for large-scale bioremediation strategies, particularly in sites impacted by crude oil and refined petroleum hydrocarbons.

Kinetic models, particularly first-order and Monod equations, are widely applied to describe BTX biodegradation. First-order kinetics are generally suitable for systems with low pollutant concentrations, whereas Monod models provide a more comprehensive description by incorporating microbial growth dynamics and substrate saturation. Experimental investigations using batch and continuous cultures, combined with analytical techniques such as gas chromatography (GC), enable precise quantification of degradation rates and validation of kinetic models ([Chen et al., 2022](#)).

With industrialization and urban expansion intensifying BTX contamination risks, advancing our understanding of biodegradation kinetics has become both urgent and essential. Robust kinetic insights will enhance the predictability of biodegradation processes and support the design of scalable, eco-friendly wastewater treatment technologies.

B. Research Methodology

1. Contaminated Soil Preparation

The petroleum-contaminated soil utilized in this study was collected from oil drilling sites operated by Pertamina–PetroChina East Java (PPEJ) in Tuban, East Java, Indonesia. Prior to experimentation, the soil was manually cleaned by removing leaves, debris, and other coarse materials, and subsequently transported from the contamination site to the laboratory. A preliminary assessment was conducted to determine the initial hydrocarbon concentration in the sample. The soil was then homogenized with water at a ratio of 20:80 (w/v) to prepare a slurry system. To eliminate indigenous microorganisms naturally present in the soil, the prepared samples were sterilized using an autoclave before further biodegradation experiments.

2. Biodegradation of Petroleum-Contaminated Soil

Biodegradation experiments were performed using *Rhodococcus erythropolis* isolates as the bioremediation agent, applied at concentrations of 12.5%, 15%, and 17.5%. The contaminated soil was mixed with the bacterial inoculum in batch reactors, where pH, temperature, and biochemical

oxygen demand (BOD) were monitored and controlled on a daily basis. The biodegradation process was conducted over a period of seven weeks, with samples collected every 14 days for gas chromatography (GC) analysis and kinetic modeling.

3. Biochemical Oxygen Demand (BOD) Analysis

The biochemical oxygen demand (BOD₅) was determined following the Standard Methods for the Examination of Water and Wastewater (Methods, 2023). Samples were diluted with oxygen-saturated nutrient water, and the initial dissolved oxygen (DO₀) was measured using a DO meter. The bottles were then incubated at 20 ± 1 °C in the dark for five days, after which the final dissolved oxygen (DO₅) was recorded. The BOD₅ value was calculated as the difference between DO₀ and DO₅, adjusted by the dilution factor. This measurement not only quantifies the oxygen demand exerted by microorganisms during organic matter degradation but also serves as an indicator of bioremediation efficiency, environmental impact, and microbial activity within the system. Background oxygen demand was accounted for by comparing the slurry sample with a blank control (aquadest), from which the adjusted BOD₅ value was calculated according to the equation below.

$$\text{BOD}_5 \left(\frac{\text{mg}}{\text{L}} \right) = (\text{DO}_s^5 - \text{DO}_f) + \left[\frac{250 \text{ mL}}{10 \text{ mL}} \times (\text{DO}_f - \text{DO}_s^0) \right] \quad (1)$$

Where:

- DO_s⁰ : initial DO of slurry sample (mg/L)
- DO_s⁵ : DO of slurry sample after 5 days (mg/L)
- DO_f : DO of aquadest (blank) after 5 days (mg/L)
- 250 mL : total volume of incubation bottle
- 10 mL : volume of slurry sample used

This corrected BOD₅ value reflects the actual oxygen demand due to microbial activity in the slurry, excluding oxygen depletion from the blank control.

4. BTX Concentration Analysis

The concentrations of benzene, toluene, and xylene (BTX) were measured using the EPA 8270 standard method for semivolatile organic compounds by GC/MS. Soil samples were extracted with n-hexane following EPA procedures, and the extracts were subsequently analyzed with Gas Chromatography–Mass Spectrometry (GC-MS). The analyses were performed at the Environmental Studies Center Laboratory, University of Surabaya, utilizing the HP-6890 GC system with model HP 19095P-QO4. In this study, BTX₀ refers to the initial concentration of benzene, toluene, and xylene (BTX) at the beginning of the experiment, while BTX_n denotes the concentration of BTX measured on the n-th day of sampling. These values were used to calculate the percentage of biodegradation according to Equation (2).

$$\% \text{ biodegradation} = \left[\frac{(\text{BTX}_0 - \text{BTX}_n)}{\text{BTX}_0} \right] \times 100\% \quad (2)$$

C. Results and Discussion

1. BTX Biodegradation

At the initial stage, the concentrations of BTX in the contaminated soil were determined to provide baseline data for comparison. These values represent the pollutant load prior to treatment and serve as a reference point for evaluating the effectiveness of the biodegradation process. The detailed initial concentrations of benzene, toluene, and xylene are presented in Table 1, which forms the basis for comparison with subsequent measurements obtained during the incubation period.

Table 1. Soil Characteristic at the PPEJ Oil Drilling Site, Bojonegoro

Parameter	Characteristic
Benzene	26,44 ppm
Toluene	121 ppm
Xylene	129 ppm
Colour	Light brown
pH	9,1
Temperature	28°C

To investigate the biodegradation potential of *Rhodococcus erythropolis*, contaminated soil samples were treated with this bacterium. Its metabolic versatility enables the degradation of BTX compounds, and the reduction in pollutant concentrations was monitored throughout the incubation period (8 weeks). The detailed results of BTX reduction achieved by *Rhodococcus erythropolis* are summarized in Table 2.

Table 2. Effect of *Rhodococcus erythropolis* Concentration on BTX Biodegradation in Contaminated Soil

BTX concentration (µg/g)											
12.5% <i>Rhodococcus erythropolis</i>				15.0% <i>Rhodococcus erythropolis</i>				17.5% <i>Rhodococcus erythropolis</i>			
B	T	X	total	B	T	X	total	B	T	X	total
26,44	121	129	276,44	26,44	121	129	276,44	26,44	121	129	276,44
25,35	40,59	35,27	101,21	15,37	31,26	35,71	82,34	12,32	20,04	20,97	53,33
20,06	20,73	25,49	66,28	10,96	25,67	25,52	62,15	10,39	10,23	10,58	31,20
9,44	10,39	10,09	29,92	6,03	10,70	10,93	27,66	5,07	8,10	8,01	21,18
3,09	9,04	5,09	17,21	1,05	9,04	5,10	15,19	0,92	6,15	4,09	11,15

The genetic and enzymatic capacity of *Rhodococcus* explains why increasing cell concentration typically accelerates BTX removal: genomes of promising strains encode numerous mono- and dioxygenases and other oxidoreductases that initiate aromatic ring activation and downstream ring-cleavage pathways (catechol formation and ortho/meta cleavage), so each cell carries substantial catalytic potential; when biomass concentration is raised, the total number of enzymatic sites per reactor volume increases and thus the reactor's volumetric BTX transformation capacity rises. However, this benefit depends on enzyme expression and substrate/oxygen availability: enzyme induction can be substrate-dependent so non-adapted cultures may show low specific activity despite high cell counts, and mass-transfer limitations (oxygen or BTX diffusion into flocs) can reduce per-cell activity. In short, higher cell concentrations increase total transformation capacity but do not necessarily improve efficiency per unit biomass unless induction, substrate supply, and oxygen/diffusion are also optimized ([Krivoruchko et al., 2023](#)).

2. BOD₅ Measurement

The corrected BOD₅ values, obtained by comparing slurry samples with blank controls, reflect the extent of oxygen consumption during incubation and thereby confirm the biodegradation process. Higher BOD₅ values indicate more intensive microbial metabolism, supporting the observed reduction in BTX concentrations. The detailed results of BOD₅ measurements are presented in Table 3, which provides additional evidence for evaluating the efficiency of bioremediation.

Table 3. The result of BOD₅ (S) for bioremediation by *Rhodococcus erythropolis*

Days	BOD ₅ (S) (mg/L)		
	12,50%	15%	17,50%

0	49,45	43,71	43,1
7	32,07	25,54	23,64
14	30,29	23,29	23,35
21	25,59	21,99	21,7
28	18,16	21,72	20,66
35	15,62	18,18	18,18
42	14,91	15,93	16,36
49	13,63	16,53	15,25
56	13,01	15,66	15,13

Rhodococcus erythropolis contributes substantially to BOD reduction via multiple, interconnected processes. Members of this genus can break down a broad spectrum of organic pollutants such as aromatic compounds, plastic monomers, and hydrocarbons, transforming them into cellular biomass and CO₂, which lowers the organic load in wastewater. This degradative ability stems from the genus's diverse metabolic toolkit and oxygen-dependent enzymes (e.g., monooxygenases and dioxygenases) that initiate the breakdown of stubborn substrates, accounting for BOD declines seen in lab and pilot trials. Moreover, population dynamics matter: larger inocula increase the volumetric degradation rate by supplying more active cells per unit volume, but when mass transfer becomes limiting, substrate availability per cell falls; as a result, BOD removal per unit biomass can plateau or even drop unless aeration and mixing are sufficient (Maurya et al., 2023; Wei et al., 2026).

3. Estimation of Reaction Kinetics Based on BOD₅ Data

The biodegradation kinetics of *Rhodococcus erythropolis* in a slurry bioreactor were evaluated using BOD₅ data as a proxy for microbial oxygen consumption. To quantify the reaction rate, the Gates and Marlar method was applied, utilizing Equation (3) to estimate the kinetic parameters (Gates & Marlar, 1968). This approach enabled the calculation of the ultimate biochemical oxygen demand (k₀) and the biodegradation rate constant (K_m), which reflect the extent and speed of organic matter degradation. The resulting values provide a quantitative basis for assessing the efficiency of *Rhodococcus erythropolis* in hydrocarbon bioremediation under varying bacterial concentrations.

$$\frac{1}{t} \ln \frac{S}{S^0} = c \left[\frac{\ln(1+ad)}{t} \right] - b \quad (3)$$

Where

$$a = \frac{Y}{X^0}; \quad b = \frac{k_0}{YK_m} (X^0 + YS^0); \quad c = 1 + \frac{X^0 + YS^0}{YK_m}; \quad d = S^0 - S$$

By plotting $\frac{1}{t} \ln \frac{S}{S^0}$ against $\frac{\ln(1+ad)}{t}$, a straight line is obtained in which c represents the slope and b the intercept. The value of a cannot be determined from a single measurement; therefore, a trial-and-error method may be applied. The parameter a can be established once the best-fitting straight line has been obtained. The kinetic constants, maximum growth rate constant (k₀) and Michaelis–Menten constant (K_m) can then be calculated using the values of a , b , and c derived from the optimal linear plot, as follows:

$$k_0 = \frac{b}{c-1}; \quad K_m = \frac{\frac{1}{a} + S^0}{c-1}$$

From the BOD data presented in Table 3, calculations were performed to obtain the values of the x-axis and y-axis according to Equation (3). These transformed values were then used to construct the linear plot required in the Gates and Marlar method. The processed data are summarized in Table

4, which serves as the basis for determining the slope (c) and intercept (b) of the straight line, and subsequently for calculating the kinetic parameters.

Table 4. Results of BOD data calculations to determine the x-axes and y-axes

Days	12,5% of bacteria		15% of bacteria		17,5% of bacteria	
	$1/t \ln S/S_0$ (y)	$\ln[1+1(S_0-S)]/t$ (x)	$1/t \ln S/S_0$ (y)	$\ln[1+1(S_0-S)]/t$ (x)	$1/t \ln S/S_0$ (y)	$\ln[1+1(S_0-S)]/t$ (x)
7	0,0619	0,0515	0,0768	0,0765	0,0858	0,0822
14	0,0350	0,0280	0,0538	0,0418	0,0438	0,0416
21	0,0314	0,0223	0,0386	0,0292	0,0327	0,0294
28	0,0358	0,0206	0,0294	0,0221	0,0263	0,0229
35	0,0329	0,0175	0,0286	0,0197	0,0247	0,0198
42	0,0285	0,0148	0,0270	0,0175	0,0231	0,0173
49	0,0263	0,0131	0,0224	0,0147	0,0212	0,0153
56	0,0238	0,0116	0,0205	0,0132	0,0187	0,0134

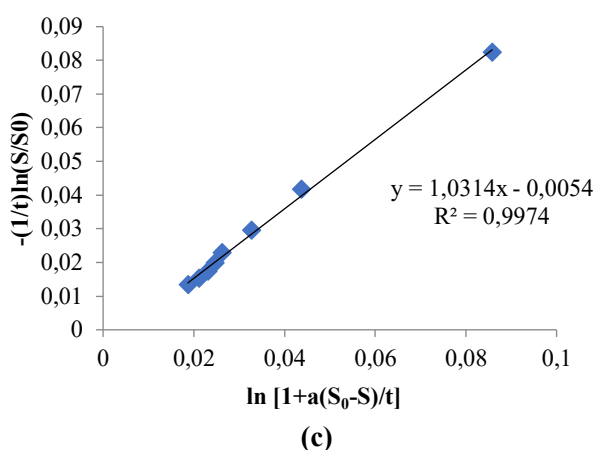
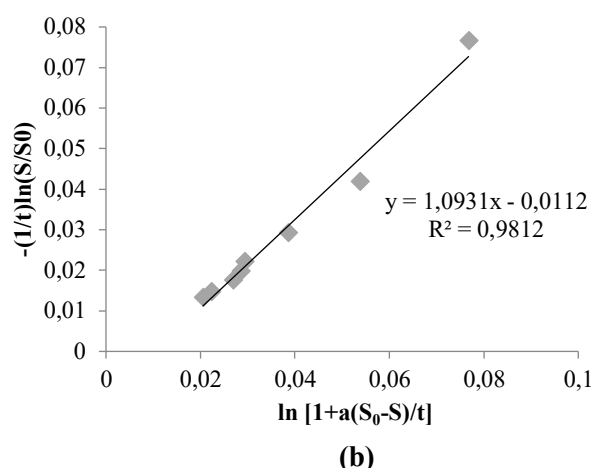
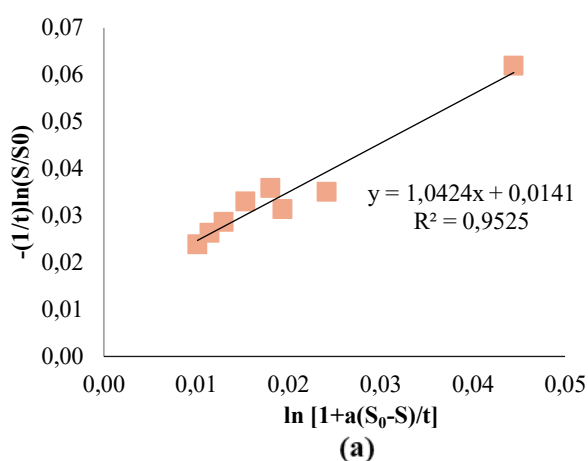


Figure 1. Kinetic constant plots in the slurry bioreactor with *Rhodococcus erythropolis* added at concentrations of (a) 12.5%, (b) 15%, and (c) 17.5

From Figure 1(a), the slope obtained was 1,0424 and the intercept was 0,0141, which were subsequently used to calculate the kinetic constants (k_0 and K_m). Considering the slope and intercept

obtained from the linear plot, the kinetic constants were determined and subsequently summarized in table 5.

Table 5. Results of the kinetic calculations using the slope and intercept from Figure 1

	12,5% <i>R. erythropolis</i>	15% <i>R. erythropolis</i>	17,5% <i>R. erythropolis</i>
Slope (c)	1,065	1,039	1,0113
Intercept (b)	0,013	0,010	0,0052
a	0,025	0,039	0,0400
k_0 (day ⁻¹)	0,200	0,256	0,4602
K_m (mg/L)	815,538	1059,001	3551,3274

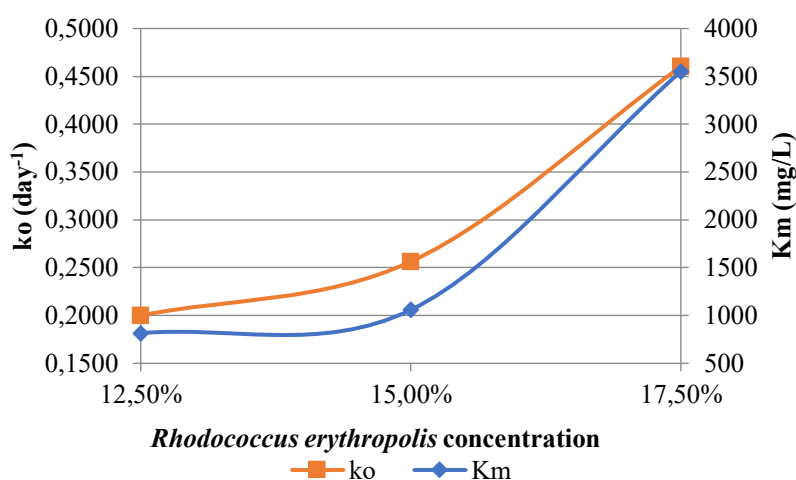


Figure 2. Kinetic constant plots in the slurry

Figure 2 shows that both the Michaelis-Menten constant (K_m) and the maximum specific growth rate (k_0) increase as the concentration of added bacteria rises. This trend reflects the higher biomass density in the bioreactor, leading to increased cell proliferation. Under favorable environmental conditions and sufficient substrate, bacterial growth progresses until the population ultimately enters the decline (death) phase. The observed simultaneous increase in k_0 and K_m with higher *R. erythropolis* concentration can be interpreted as a trade-off between greater catalytic capacity at the reactor scale and lower per-cell substrate accessibility. An increased inoculum raises active biomass and therefore the volumetric degradation rate (reflected in higher k_0), but dense biomass and cell aggregation tend to create internal diffusion limitations and oxygen gradients that effectively reduce substrate availability to individual cells, producing an apparent rise in K_m . This coupling of kinetics and mass transfer has been reported for biodegradation systems and explained mechanistically by models that link external transport to the Monod form of rate laws ([Enouy et al., 2022](#)). Studies on *Rhodococcus* species further indicate that hydrocarbon degradation rates depend both on intrinsic enzymatic potential and on operational factors that influence mass transfer, so the observed rise in K_m likely reflects transport or physiological effects rather than a simple change in enzyme affinity. Consequently, increasing inoculum past an optimal level may boost total removal but lower per-biomass efficiency and require enhanced aeration and mixing to prevent oxygen or substrate limitation ([Malhautier et al., 2022](#)).

Rhodococcus erythropolis degrades BTX via oxygenase-mediated hydroxylation to catechol and methylcatechol intermediates followed by ortho- or meta-ring cleavage that channels products into the TCA cycle for assimilation and mineralization; increasing inoculum raises the reactor's

volumetric catalytic capacity (higher k_0) but can introduce mass-transfer and enzyme-induction limitations that reduce per-cell activity and manifest as an increased apparent K_m (Kuyukina & Ivshina, 2019).

The kinetic parameters obtained here ($k_0 \approx 0.20\text{--}0.46 \text{ day}^{-1}$ and apparent $K_m \approx 8.2 \times 10^2\text{--}3.6 \times 10^3 \text{ mg} \cdot \text{L}^{-1}$) are consistent with moderate volumetric rates but unusually high apparent substrate affinities reported for *Rhodococcus* in two-phase or poorly mixed systems: reported k_0 values for *Rhodococcus* on hydrophobic aromatics commonly fall in the low–moderate range ($\approx 0.05\text{--}0.6 \text{ day}^{-1}$) while fast-growing planktonic degraders such as *Pseudomonas* often show higher $\mu_{\max}$ under fully dissolved substrate conditions, so the k_0 values here align with *Rhodococcus*'s slower, versatile catabolism but are lower than ideal planktonic benchmarks (Ibrahim et al., 2020). The very large apparent K_m values are best interpreted as transport-limited artifacts sorption to soil/slurry phases, floc formation, and oxygen diffusion gradients inflate observed K_s/K_m compared with intrinsic enzyme affinities measured in well-mixed, planktonic assays an effect documented for *Rhodococcus* adaptation to solvents and for mechanistic derivations linking external mass transfer to Monod-type kinetics (Carvalho & Fatah, 2007).

Slurry bioreactors inoculated with *Rhodococcus erythropolis* are practically attractive for large-scale BTX remediation because they combine enhanced substrate bioavailability (soil suspension and solvent desorption) with controllable aeration, nutrient dosing, and biomass management features that allow the strain's broad oxygenase repertoire and solvent-tolerance genes to be exploited at scale. *Rhodococcus* genomes encode multiple mono- and dioxygenases and biosurfactant pathways that improve uptake and catabolism of hydrophobic aromatics, supporting robust degradation in ex-situ reactors when cells are adapted and oxygen transfer is sufficient. In practice, successful scale-up requires engineering solutions to three recurring constraints observed in slurry systems: (1) oxygen transfer: design impellers, sparging, or staged aeration to meet the high O_2 demand of oxygenase-driven pathways; (2) mass transfer and bioavailability: use agitation, surfactant production (or controlled biosurfactant addition), or two-liquid-phase strategies to reduce sorption and diffusion limits that inflate apparent K_m ; and (3) biomass management: control inoculum density, floc size, and retention time to avoid internal diffusion gradients that lower per-cell activity while preserving volumetric k_0 . Pilot studies and treatability tests are essential to quantify intrinsic Monod parameters under well-mixed planktonic conditions and to translate them into reactor-scale operating points (impeller power, gas flow, solids loading) for techno-economic evaluation (Afordoanyi et al., 2024; Mahdinia et al., 2019).

This study has several limitations that constrain its generalizability and guide future work: experiments were performed on sterilized slurry using a single *Rhodococcus erythropolis* isolate and only three inoculum levels, so ecological interactions, strain variability, and the true inoculum optimum remain unassessed; the very high apparent K_m values likely reflect unquantified mass-transfer constraints (oxygen and substrate diffusion into flocs and sorbed phases) because DO profiles, floc sizing, and mass-transfer coefficients were not measured; inhibition thresholds and intermediate-metabolite fate were not determined; and key engineering metrics for scale-up (impeller power, gas flow, solids handling) were not reported—therefore, subsequent studies should determine intrinsic Monod parameters and K_i in well-mixed bacteria assays, mass-transfer characteristics, evaluate a broader range of inoculum strategies in nonsterile soils (including biosurfactant or two-phase approaches), and validate findings in pilot reactors to inform aeration, mixing, and techno-economic feasibility.

D. Conclusion

Inoculation with *Rhodococcus erythropolis* increases the volumetric rate of BTX decline, so dissolved-phase BTX concentrations decrease more rapidly in treatments with high inoculum than with low biomass. This increase in biomass generally elevates the reactor's volumetric removal capacity (k_0), but its effect on reaction constant (K_m) and specific activity per unit biomass depends on enzyme induction and mass-transfer constraints. Therefore, inoculum optimization should be performed together with controlled DO, agitation, and structured kinetic assays to ensure volumetric gains translate into efficient per-cell degradation.

Daftar Pustaka

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