

Hydrolytic Enzyme Effects on Total Coliforms in Surfactant-Rich Industrial Wastewater and Soil Extracts: A Control-Adjusted Assessment

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ABSTRACT

Detergent manufacturing wastewater contains surfactants, organic contaminants, and microbial populations that may challenge conventional treatment processes. This study evaluated the response of total coliforms to hydrolytic enzyme treatments in detergent-impacted industrial wastewater and aqueous soil extracts under controlled laboratory conditions. Cellulase, amylase, protease, an enzyme-supplemented detergent formulation, an enzyme-free detergent formulation, and untreated controls were assessed during a 7-day incubation period. Total coliform abundance was determined using the five-tube Most Probable Number (MPN) method, and treatment performance was evaluated using endpoint abundance and residual coliform counts. A substantial decline occurred in the untreated wastewater control, which exhibited an 88.5% reduction in coliform abundance, indicating that natural attenuation represented a major component of the overall response. Among wastewater treatments, cellulase (WW-CEL) and the enzyme-supplemented detergent formulation (WW-D+E) exhibited the lowest endpoint coliform abundances, with endpoint values at or near the analytical detection limit. Relative to the untreated control, WW-CEL was associated with an additional reduction of approximately 11.4% beyond the baseline decline observed under the experimental conditions. In contrast, the enzyme-free detergent treatment produced an endpoint response comparable to the untreated control. Most soil extract treatments exhibited baseline coliform abundances at or below the analytical detection limit, limiting interpretation of treatment-specific responses. These findings suggest that hydrolytic enzymes may contribute to incremental reductions in coliform abundance in surfactant-rich industrial wastewater. However, the overall response was strongly dominated by natural attenuation, and observed treatment effects were highly dependent on matrix characteristics and baseline microbial load.

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1. Introduction

The detergent manufacturing sector represents one of the largest industrial contributors to aquatic pollution, with global production exceeding 15 million tonnes annually and generating complex effluents that challenge conventional wastewater treatment systems. These wastewaters typically contain high concentrations of surfactants, particularly linear alkylbenzene sulfonates (LAS), residual organic matter, and enzymatic additives, forming chemically heterogeneous matrices that complicate microbial control and treatment stability [1]. In addition to chemical pollutants, such effluents frequently carry fecal indicator organisms such as total coliforms and *Escherichia coli*, which are widely used as regulatory benchmarks for assessing microbiological water quality and treatment performance [2].

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Recent evidence from advanced wastewater treatment and bioreactor systems indicates that modern industrial effluents are increasingly characterized by multi-contaminant stress environments, where surfactants, pharmaceuticals, and emerging organic compounds simultaneously reshape microbial community structure and process stability, often reducing the predictability of conventional treatment performance [3]. In parallel, studies on surfactant-acclimated microbial consortia have demonstrated that long-term exposure to linear alkylbenzene sulfonates induces functional adaptation, but also results in altered degradation kinetics and community-level tolerance thresholds [4, 5]. These observations suggest that wastewater systems exposed to complex industrial chemistries cannot be fully interpreted using simplified or single-stressor assumptions.

Conventional disinfection strategies, including chlorination, ultraviolet (UV) irradiation, and ozonation, remain widely implemented for pathogen control. However, their effectiveness is frequently compromised in surfactant-rich wastewater matrices. Elevated LAS concentrations reported in detergent manufacturing effluents have been reported to reduce disinfectant efficiency through competitive adsorption, protection of microbial aggregates, and interference with oxidative reactions [4, 6]. Moreover, conventional chemical disinfection may generate secondary pollutants such as halogenated byproducts, while UV-based systems exhibit reduced efficiency in turbid or high-solids environments and may fail against resistant microbial forms [5, 7, 8]. Collectively, these limitations have contributed to increasing interest in integrated or bioaugmented treatment strategies capable of addressing both chemical complexity and microbial persistence under industrial conditions.

The structural integrity of bacterial pathogens, particularly fecal coliforms, is governed by the hierarchical architecture of the cell envelope, comprising peptidoglycan networks and associated carbohydrate-rich structures that provide mechanical stability and environmental protection [9]. Enzymatic targeting of these structures offers a mechanistically distinct approach to microbial control. Hydrolytic enzymes such as cellulases, proteases, and amylases can degrade extracellular and structural polymeric components associated with microbial cells and biofilms, potentially weakening microbial protective barriers and enhancing susceptibility to external stressors [10-13]. In this context, enzyme-based biocatalytic approaches have been increasingly explored not only for pollutant transformation but also for targeted biological disruption in complex environmental systems, including wastewater and sludge matrices [3, 14].

Recent studies on enzymatic and biological treatment systems further support this direction, demonstrating that enzyme-assisted or bioaugmented processes can influence microbial viability and biofilm-associated tolerance mechanisms in engineered environments, particularly under conditions of high organic and particulate loading [15-19]. For example, hydrolytic enzymes have been reported to disrupt microbial biofilms and extracellular polymeric substances, thereby enhancing susceptibility to chemical or environmental stressors, although their performance remains strongly dependent on system composition and matrix structure [17-19]. Similarly, bioaugmentation strategies in wastewater and sludge systems have highlighted the importance of microbial adaptation and system-specific metabolic pathways in determining overall treatment efficiency [20, 21].

Despite these advances, most existing studies have focused on simplified laboratory systems or model substrates that do not fully capture the complexity of industrial effluents. In particular, enzyme performance in the presence of surfactants such as LAS, which themselves influence microbial physiology and interfacial interactions, remains insufficiently characterized. Additionally, matrix-dependent effects including adsorption to particulate matter, sludge-associated inhibition, and microbial community restructuring have been identified as key determinants of treatment variability in real wastewater systems [7, 8, 22].

However, quantitative evidence describing the response of coliform populations to hydrolytic enzymes under surfactant-rich industrial wastewater conditions, while simultaneously accounting for matrix-dependent effects and natural attenuation processes, remains limited. In this context, the present study investigates the response of total coliform populations to hydrolytic enzyme treatments in two environmentally relevant matrices: (I) detergent manufacturing wastewater characterized by high surfactant and organic loading, and (II) aqueous soil extract representing a lower-load environmental system. This design is motivated by evidence that contaminant behavior, microbial persistence, and treatment efficiency differ substantially between wastewater and solid-associated environmental matrices due to adsorption, bioavailability, and community-level constraints [7, 8, 21].

To our knowledge, few studies have simultaneously evaluated hydrolytic enzyme treatments in surfactant-rich industrial wastewater while explicitly accounting for natural attenuation processes and comparing responses across contrasting environmental matrices. This combination of enzyme treatment, matrix comparison, and control-adjusted interpretation constitutes the primary contribution of the present study. We hypothesize that hydrolytic enzymes may contribute to measurable reductions in coliform abundance in detergent-impacted wastewater; however, the magnitude and interpretability of these effects are expected to be strongly dependent on matrix composition, microbial load, and physicochemical constraints rather than enzymatic activity alone.

The objectives of this study are therefore: (1) to quantify changes in culturable total coliform abundance following exposure to cellulase, amylase, and protease; (2) to evaluate enzyme-associated responses relative to untreated controls using endpoint-based statistical analysis; (3) to assess matrix-dependent differences in wastewater and soil extract systems; and (4) to develop a conservative interpretative framework for enzyme-assisted treatment in surfactant-rich industrial effluents.

2. Materials and methods

2.1. Study site and sample collection

2.1.1. Industrial wastewater

Composite wastewater samples were collected from the effluent discharge point of Active Co. detergent manufacturing facility

(Eshtehard Industrial Zone, 35°42'N, 50°22'E, Alborz Province, Iran). The facility produces household and industrial cleaning products containing amylase, cellulase, and protease. The effluent comprises process rinse streams, equipment washdown water, and incidental spillage, all discharged through a common collection channel.

Triplicate composite samples were independently collected at three time points (0, 15, and 30 min) to capture temporal variability in discharge composition. Each 500 mL sample was collected in sterile borosilicate glass bottles and transported at 4 °C to the Environmental Engineering Laboratory, University of Tehran, within 3 h of collection, complying with APHA holding-time protocols [23].

2.1.2. Soil aqueous extract

Soil samples were collected at six spatially distributed points (0-30 cm depth) using a stainless-steel corer and combined into a single composite per replicate (triplicate sets collected at 0, 15, and 30 min). Aqueous extracts were prepared by mixing 100 g of air-dried soil (2 mm sieved) with 500 mL of sterile deionised water, shaking for 60 min at 150 rpm, and filtering through a Whatman No. 42 filter paper [21]. Extracts were processed identically to wastewater samples within 6 h of preparation.

2.2. Physicochemical characterisation of matrices

Baseline physicochemical parameters were measured at sample receipt (T_0) using calibrated instruments: pH and dissolved oxygen (DO) by portable multi-parameter meter (HQ40d, Hach); temperature by integrated thermistor; turbidity by turbidimeter (2100Q, Hach); COD in wastewater by closed reflux titrimetric method (APHA 5220-C); anionic surfactant LAS by MBAS colorimetric method (APHA 5540-C); electrical conductivity in soil extract by conductivity meter (HQ40d, Hach). These treatment-specific baseline values are therefore reported with the coliform enumeration results rather than in Table 1.

Table 1. Physicochemical characteristics at baseline (T_0).

Parameter	Industrial wastewater	Soil extract	Method
PH	7.2 ± 0.3	6.8 ± 0.4	PH meter (HQ40d)
DO (mg/L)	2.4 ± 0.6	5.1 ± 0.8	LDO probe (HQ40d)
Temperature (°C)	22.5 ± 1.2	20.8 ± 0.9	Integrated thermistor
Turbidity (NTU)	148 ± 22	38 ± 11	Turbidimeter (2100Q)
COD (mg/L)	1,240 ± 180	—	Closed reflux (APHA 5220-C)
LAS (mg/L)	48 ± 9	—	MBAS (APHA 5540-C)
Conductivity (µS/cm)	—	620 ± 45	Conductivity meter

• Values are mean ± SD (n = 3). LAS = linear alkylbenzene sulfonates. Treatment-specific baseline coliform concentrations were determined separately after treatment allocation and are reported with the coliform enumeration results rather than as physicochemical matrix characteristics.

Experimental rationale: The measured pH (7.2 wastewater, 6.8 soil extract) falls within the optimal range for the three enzyme preparations (pH 6.5-7.5 for amylase/cellulase; pH 7.0-8.5 for alkaline protease). However, wastewater DO (2.4 mg/L) was below the threshold for optimal aerobic metabolism (> 4 mg/L); thus, 150 rpm orbital shaking maintained adequate aeration throughout incubation.

2.3. Preparation of enzyme stock solutions

Three commercial enzyme preparations were used: α-amylase (EC 3.2.1.1; ≥ 100 U/mL, Sigma-Aldrich), endo-cellulase (EC 3.2.1.4; ≥ 100 U/mL, Sigma-Aldrich), and alkaline protease (EC 3.4.21.62; ≥ 2.4 AU/g, Novozymes). Enzyme activity was confirmed prior to use by standard spectrophotometric assays (DNS method for amylase/cellulase; azocasein method for protease; [14]).

Each enzyme was dissolved in sterile 50 mM phosphate buffer (pH 7.0) to prepare 100 U/mL stock solutions. Working solutions (10 U/mL final concentration) were prepared fresh daily by volumetric dilution into the respective matrix and stored on ice until use.

Justification of enzyme concentration: A final enzyme concentration of 10 U/mL was selected as a standardised proof-of-concept exposure level for evaluating the potential contribution of hydrolytic enzymes to coliform suppression in detergent-impacted matrices. The selected concentration falls within the activity range commonly employed in laboratory-scale investigations of enzymatic antimicrobial, antibiofilm, and biocatalytic processes [14-19]. No formal dose-response optimisation was conducted prior to experimentation; therefore, the selected concentration should not be interpreted as an optimal operational dose. Rather, it was intended to provide a consistent basis for comparing the relative performance of amylase, cellulase, protease, and detergent-associated treatments under identical experimental conditions.

Twelve treatment groups were established (six wastewater, six soil extract), each prepared in triplicate (n = 3), yielding 36 incubation vessels. Experimental groups are summarised in Table 2.

Each 500 mL vessel received 450 mL of matrix. For single-enzyme treatments, 50 mL of a 100 U/mL stock solution was added (final concentration: 10 U/mL). For detergent treatments (D+E and D–E), 1 g/L commercial liquid detergent (Active Co.) was added to the enzyme-containing formulation (D+E), which included amylase, cellulase, and protease; the enzyme-free formulation (D–E)

contained only surfactants and adjuvants. This design isolates enzymatic effects from surfactant-mediated antimicrobial activity, enabling direct comparison of individual enzyme contributions. Untreated controls received no additives. The overall experimental design and workflow are illustrated in Fig. 1.

Table 2. Experimental treatment groups.

No.	Treatment group	Matrix	Treatment	Enzyme conc. (U/mL)	Replicates (n)
1	WW-AMY	Wastewater	Amylase	10	3
2	WW-CEL	Wastewater	Cellulase	10	3
3	WW-PRO	Wastewater	Protease	10	3
4	WW-D+E	Wastewater	Commercial detergent + enzymes	10	3
5	WW-D-E	Wastewater	Detergent, surfactant only	—	3
6	WW-CTL	Wastewater	Untreated control	—	3
7	SOIL-AMY	Soil extract	Amylase	10	3
8	SOIL-CEL	Soil extract	Cellulase	10	3
9	SOIL-PRO	Soil extract	Protease	10	3
10	SOIL-D+E	Soil extract	Commercial detergent + enzymes	10	3
11	SOIL-D-E	Soil extract	Detergent, surfactant only	—	3
12	SOIL-CTL	Soil extract	Untreated control	—	3

• All groups were incubated aerobically at 25 ± 1°C for 7 days with orbital shaking at 150 rpm.

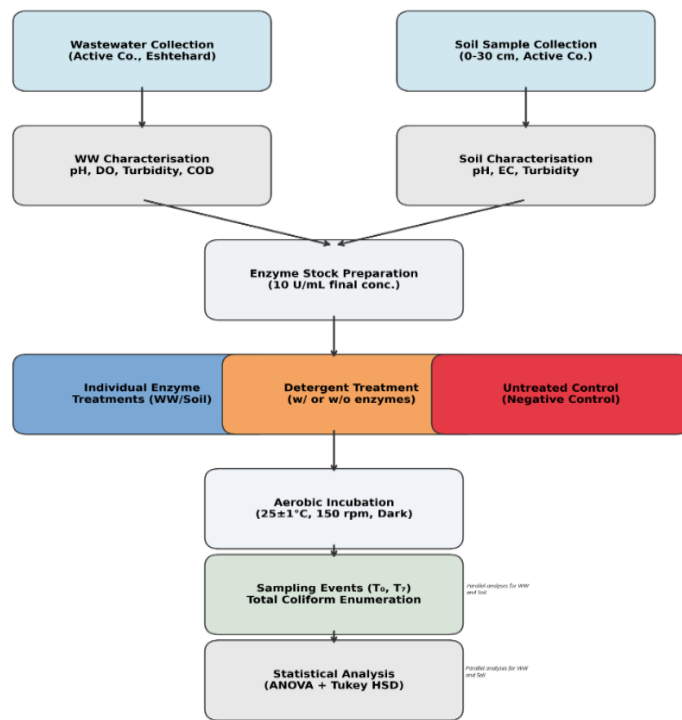


Fig. 1. Experimental design and workflow.

Schematic representation of the study design, including sample collection, enzyme stock preparation, treatment grouping, incubation conditions, and coliform enumeration. Parallel analyses were conducted on industrial wastewater and soil aqueous extracts to ensure a distinct evaluation of enzyme-mediated coliform reduction.

2.4. Incubation conditions

All vessels were incubated aerobically at 25 ± 1 °C under continuous orbital shaking (150 rpm) in a temperature-controlled incubator shaker (Innova 44 R, New Brunswick Scientific) in the dark. Sampling occurred at two time points: immediately upon mixing (T₀) and after 7 days (T₇). At each event, 10 mL was withdrawn aseptically from each vessel for coliform analysis. Physicochemical parameters (pH, DO, temperature) were recorded at both time points.

2.5. Total coliform enumeration by most probable number method

2.5.1. Serial dilution

At T₀ and T₇, 10 mL aliquots were serially diluted in sterile phosphate-buffered saline (PBS; pH 7.0) to yield dilutions from 10⁻¹ to 10⁻⁵. For samples exhibiting high initial coliform abundance (≥ 24 MPN/100 mL), serial dilutions were extended to 10⁻⁶ to ensure quantifiable tube combinations within the standard MPN range.

2.5.2. MPN assay

Total coliforms were quantified using the five-tube, three-dilution MPN method (APHA 9221-B). Five replicate tubes (10 mL Lauryl Tryptose broth each, with inverted Durham tubes) were inoculated with 1 mL of diluted sample and incubated at $35 \pm 0.5^\circ\text{C}$ for 48 ± 3 h. Gas production in the Durham tube or turbidity was recorded as presumptive positive. Presumptive positives were confirmed by subculture into Brilliant Green Lactose Bile broth ($35 \pm 0.5^\circ\text{C}$, 48 ± 3 h); only gas-producing tubes were counted.

MPN determination: MPN indices were determined using standard MPN tables with 95% confidence intervals from tabulated values. This approach avoids uncertainties inherent in mathematical approximations. Results below the detection limit (< 0.03 MPN/100 mL) reflect zero positive tubes across all dilutions. All analyses were conducted in the ISO/IEC 17025-accredited Environmental Engineering Laboratory, University of Tehran.

2.5.3. Quality assurance and quality control

QA/QC procedures included: (I) analytical blanks (PBS only) processed with each sample batch; (II) positive control strain (*E. coli* ATCC 25922, $\geq 10^6$ CFU/mL) in each MPN run; (III) sterilisation of all glassware and media by autoclaving; (IV) coliform enumeration within 6 h of sample withdrawal (APHA holding-time compliance); and (V) blinded analysis of all tube scoring by independent laboratory personnel to eliminate observer bias.

2.6. Calculation of coliform reduction efficiency

Coliform reduction efficiency was calculated as Eq. 1.

$$R (\%) = \left[\frac{C_0 - C_7}{C_0} \right] \times 100 \quad (1)$$

where C_0 and C_7 represent total coliform counts (MPN/100 mL) measured at T_0 and T_7 , respectively. To account for unequal baseline coliform concentrations among treatment groups, a log-reduction endpoint was also calculated for each replicate as follows: $\text{Log reduction} = \log_{10}^{C_0} - \log_{10}^{C_7}$, where C_0 and C_7 represent detection-limit-adjusted MPN values at T_0 and T_7 , respectively. Values below the detection limit were substituted with 0.03 MPN/100 mL prior to log transformation. This log-reduction metric was calculated as a supplementary measure to account for baseline variability among treatment groups, whereas primary statistical comparisons were performed using $\log_{10}$ -transformed T_7 coliform counts. Percentage reduction was retained solely as a descriptive measure of apparent removal efficiency.

2.7. Statistical analysis

All MPN values were $\log_{10}$ -transformed before analysis. Normality was assessed using the Shapiro-Wilk test ($\alpha = 0.05$), and homogeneity of variances was evaluated using Levene's test ($\alpha = 0.05$).

Because baseline (T_0) coliform concentrations differed among treatment groups, percentage reduction values were used only as descriptive indicators and were not used as the primary basis for statistical inference. Primary between-treatment comparisons were instead performed using $\log_{10}$ -transformed T_7 coliform counts, with absolute remaining counts (MPN/100 mL) reported as supporting endpoint data. This approach directly evaluates residual coliform abundance after treatment while avoiding the instability of percentage reduction estimates caused by unequal baseline values and proximity to the analytical detection limit.

Differences in $\log_{10}^{\text{transformed endpoint values}}$ counts among treatment groups were assessed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test ($\alpha = 0.05$). Where ANOVA assumptions were violated, the non-parametric Kruskal-Wallis H-test was applied, followed by Bonferroni-adjusted Mann-Whitney U pairwise comparisons.

Effect sizes were reported as partial eta-squared (η^2). Results from wastewater and soil extract groups were analysed separately because of their fundamentally different physicochemical characteristics, microbial ecology, and sorptive behaviour [22]. All analyses were performed using SPSS Statistics v26.0 (IBM Corp.), and figures were generated using GraphPad Prism v9.0. Data are presented as mean $\pm$ SD ($n = 3$), and statistical significance was defined as $p < 0.05$ (two-tailed).

The observed variation in treatment-group T_0 coliform concentrations, most notably the higher initial count recorded for WW-CEL (24 MPN/100 mL) relative to WW-CTL (4.6 MPN/100 mL), is consistent with enzymatic disruption of cellular aggregates or flocs immediately upon cellulase addition, potentially releasing previously non-culturable cells into suspension and thereby increasing the apparent enumerable coliform density at T_0 [9, 18]. Because primary statistical inference in this study is based on $\log_{10}$ -transformed T_7 endpoint counts rather than on percentage reduction from baseline, this inter-group T_0 variability does not compromise the validity of between-treatment comparisons; residual coliform abundance at T_7 remains the principal outcome measure, independent of initial concentration differences.

3. Results

3.1. Physicochemical baseline characteristics

Physicochemical parameters at T_0 are summarised in Table 1. Industrial wastewater exhibited near-neutral pH (7.2 ± 0.3), low dissolved oxygen (2.4 ± 0.6 mg/L), and high organic loading (COD $1,240 \pm 180$ mg/L), consistent with enzyme-containing detergent effluent. Anionic surfactant concentration (48 ± 9 mg/L LAS) exceeded typical inhibitory thresholds for biological treatment [1]. Soil aqueous extract showed lower organic loading and turbidity (COD N/D; turbidity 38 ± 11 NTU) and a near-neutral pH (6.8 ± 0.4), reflecting limited accumulation of process chemicals in the surrounding soil.

Overall, these baseline characteristics indicate two distinctly different exposure matrices: a high-strength, surfactant-rich wastewater environment with potential biological stressors, and a comparatively diluted soil aqueous extract with reduced organic and chemical loading.

3.2. Total coliform counts and reduction efficiency

Total coliform counts at T_0 and T_7 , $\log_{10}^{\text{transformed endpoint values}} [\log_{10}^{T_7}]$, reduction efficiencies (R%), and statistical significances are presented in Table 3. In accordance with the revised statistical framework, $\log_{10}^{T_7}$ values were used as the primary endpoint for between-treatment comparisons, while percentage reduction (R%) was retained solely as a descriptive indicator and not used for statistical inference. Visual comparisons of coliform abundance across treatment groups are presented in Fig. 2, while overall suppression patterns are summarised in Fig. 3. Wastewater and soil extract treatments were evaluated separately because of their fundamentally different physicochemical and microbiological characteristics [22].

Table 3. Total coliform abundance at baseline (T_0) and endpoint (T_7), $\log_{10}^{\text{transformed endpoint values}} [\log_{10}^{T_7}]$, reduction efficiency, and statistical significance across wastewater and soil extract treatment groups.

No.	Treatment group	Matrix	T_0 (MPN/100 mL)	T_7 (MPN/100 mL)	$\log_{10}^{T_7}$	Reduction (%)	P-value	Significance
1	WW-AMY	Wastewater	0.09	< 0.03	≤ -1.52	66.7	0.032	*
2	WW-CEL	Wastewater	24.0	< 0.03	≤ -1.52	99.9	< 0.001	***
3	WW-PRO	Wastewater	0.12	< 0.03	≤ -1.52	75.0	0.018	*
4	WW-D+E	Wastewater	24.0	0.19	-0.72	99.2	< 0.001	***
5	WW-D-E	Wastewater	0.24	0.03	-1.52	87.5	0.041	*
6	WW-CTL	Wastewater	4.60	0.53	-0.28	88.5	—	Reference
7	SOIL-CEL	Soil extract	0.06	< 0.03	≤ -1.52	51.6	0.043	*
8	SOIL-D+E	Soil extract	< 0.03	< 0.03	≤ -1.52	N/D	> 0.05	ns
9	SOIL-D-E	Soil extract	< 0.03	< 0.03	≤ -1.52	N/D	> 0.05	ns
10	SOIL-PRO	Soil extract	< 0.03	< 0.03	≤ -1.52	N/D	> 0.05	ns
11	SOIL-AMY	Soil extract	< 0.03	< 0.03	≤ -1.52	N/D	> 0.05	ns
12	SOIL-CTL	Soil extract	2.40	0.09	-1.05	96.2	—	Reference

* Values represent the mean of three independent replicates ($n = 3$). T_0 = immediately after treatment allocation and mixing (Day 0); T_7 = after 7 days of incubation. LOD = analytical detection limit (0.03 MPN/100 mL); N/D = not determinable (both T_0 and T_7 below the detection limit); ns = not significant ($p > 0.05$). $\log_{10}^{T_7}$ values were used as the primary statistical endpoint for between-treatment comparisons, while percentage reduction values are presented solely for descriptive interpretation of apparent removal efficiency. WW-CTL and SOIL-CTL served as untreated reference controls for wastewater and soil extract matrices, respectively.

3.2.1. Wastewater treatment groups

Substantial coliform decline was observed in the untreated wastewater control (WW-CTL), which decreased from 4.60 to 0.53 MPN/100 mL over the 7-day incubation period (88.5% reduction; Table 3). This pronounced background decline highlights the importance of accounting for environmental attenuation when interpreting treatment-associated responses. The observed trend may be consistent with conditions typically associated with industrial wastewater matrices, including nutrient depletion, low dissolved oxygen availability (2.4 ± 0.6 mg/L), and prolonged exposure to surfactant-containing environments [1, 4, 6, 22].

Given this control behaviour, treatment performance is described primarily in terms of residual endpoint abundance [$\log_{10}^{T_7}$] rather than percentage reduction values. WW-CEL and WW-D+E consistently exhibited the lowest residual coliform abundances at T_7 , with endpoint values reaching or approaching the analytical detection limit (Table 3). In comparison, WW-CTL remained at 0.53 MPN/100 mL, whereas WW-D-E reached 0.03 MPN/100 mL.

Although WW-CEL and WW-D+E showed descriptive reduction efficiencies of 99.9% and 99.2%, respectively, these values reflect both treatment-associated effects and the background decline observed in the untreated control. Accordingly, percentage reduction values were not used as the basis for treatment ranking.

An elevated T_0 value was observed in WW-CEL (24 MPN/100 mL) compared with WW-CTL (4.60 MPN/100 mL). Because treatment allocation preceded T_0 measurement, this difference may reflect variability associated with sample distribution following treatment addition. One possible explanation is the temporary dispersion of microbial aggregates following enzyme addition, which may increase the number of culturable units detected by the MPN assay. Similar enzyme-associated biofilm disruption and aggregate

dispersion phenomena have been reported in previous studies of hydrolytic enzyme systems [15, 17-19]. However, this interpretation remains hypothetical, as aggregate structure was not directly assessed in the present study.

The enzyme-free detergent treatment (WW-D-E) resulted in an 87.5% reduction, which was similar in magnitude to the decline observed in the untreated control (88.5%). Under the conditions examined, exogenous surfactant addition did not result in a clearly distinguishable change in coliform abundance relative to background attenuation based on the measured endpoint counts. However, this observation is limited to the specific concentration and exposure conditions applied in this study and should not be interpreted as evidence of intrinsic inefficacy of LAS. Previous studies have shown that surfactant behaviour is strongly dependent on concentration, microbial adaptation, matrix composition, and biodegradation dynamics [1, 4-6].

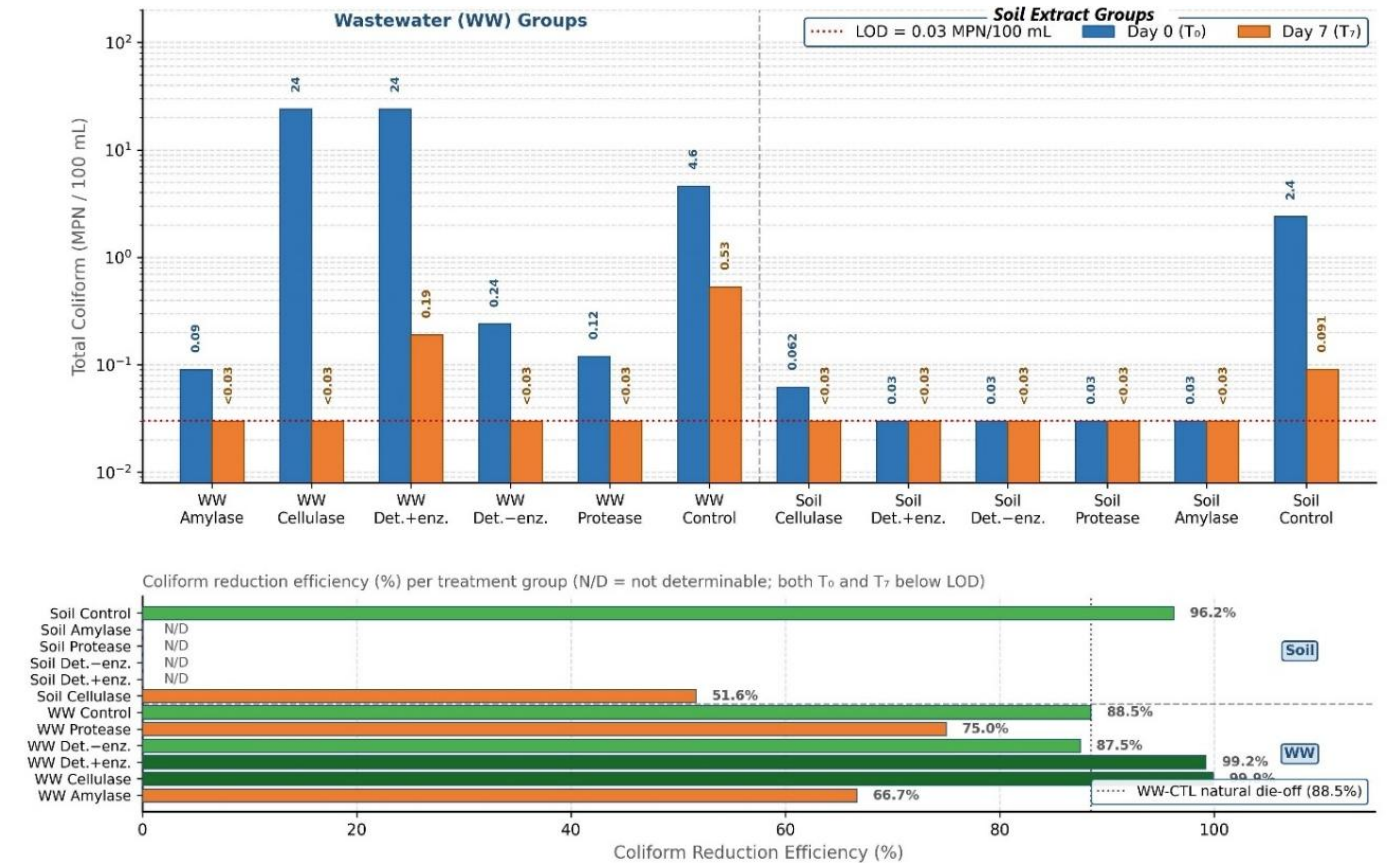


Fig. 2. Total coliform counts at T0 and T7 (Log Scale).

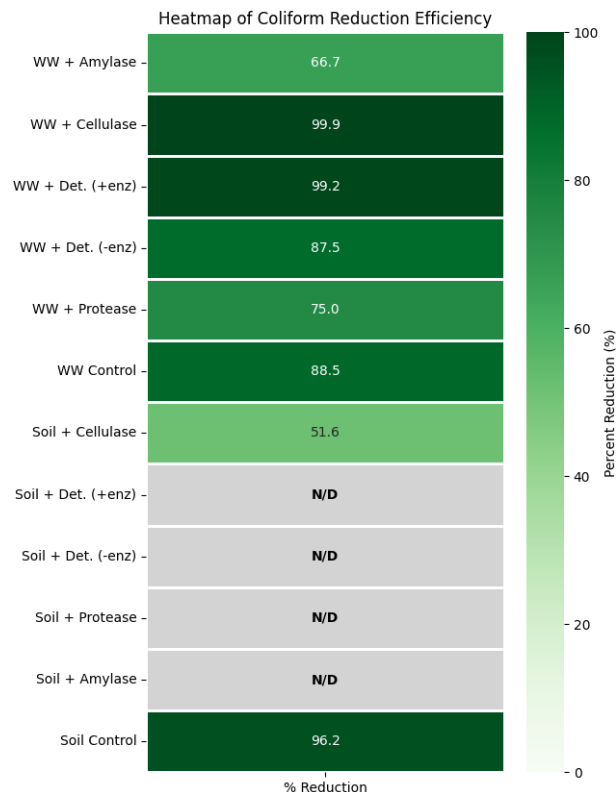


Fig. 3. Heatmap of coliform reduction efficiency.

3.2.2. Soil extract treatment groups

In the soil extract matrix, the majority of treatment groups exhibited baseline coliform counts at or below the LOD (0.030 MPN/100 mL; Fig. 2), precluding calculation of meaningful reduction efficiencies (designated N/D for SOIL-D+E, SOIL-D-E, SOIL-PRO, and SOIL-AMY; Table 3).

The fixed 10 U/mL enzyme dosage was standardized to address high-microbial-load wastewater scenarios (worst-case dosing strategy), resulting in a relative excess of enzymatic activity in the lower-load soil matrix, leading to a “floor effect” in which measured coliform abundance approached the analytical detection limit rather than reflecting treatment-driven dynamics. Only SOIL-CEL yielded a quantifiable baseline count ($T_0 = 0.06$ MPN/100 mL), which declined to <0.03 MPN/100 mL at T_7 (51.6% reduction; $p = 0.043$; Table 3 and Fig. 2). The soil control (SOIL-CTL) exhibited 96.2% natural reduction ($T_0 = 2.40$; $T_7 = 0.09$ MPN/100 mL), indicating substantial background attenuation under the applied incubation conditions.

Logarithmic bar plot displaying MPN/100 mL ($\log_{10}$ scale, y-axis) for all 12 treatment groups at both sampling time points (T_0 Day 0, T_7 Day 7, x-axis). Error bars represent $\pm$ SD ($n = 3$). Horizontal red dotted line indicates method detection limit (LOD = 0.030 MPN/100 mL). Percentages above each bar pair denote reduction efficiency (R%) relative to T_0 . Wastewater (WW) treatment groups shown in blue; soil extract (SOIL) groups in green. An asterisk indicates groups with T_0 below LOD values are presented for descriptive comparison; statistical inference is based on $\log_{10}^{T_7}$ endpoint analysis as described in Section 2.7.

Colour-gradient heatmap displaying magnitude of coliform suppression (0-100% reduction scale) across all 12 treatment groups from T_0 to T_7 . Treatment groups (WW and SOIL) listed on the y-axis; reduction percentage (0-100%) on the x-axis. Darker green shading indicates higher reduction efficiency; grey shading indicates N/D (not determinable: both T_0 and T_7 at or below LOD). Numeric labels within each cell display the exact reduction percentage. A horizontal dividing line visually separates wastewater and soil groups. Reduction values are shown for descriptive interpretation only and were not used for statistical comparisons. The temporal patterns of coliform decline in the wastewater treatment groups are illustrated in Fig. 4.

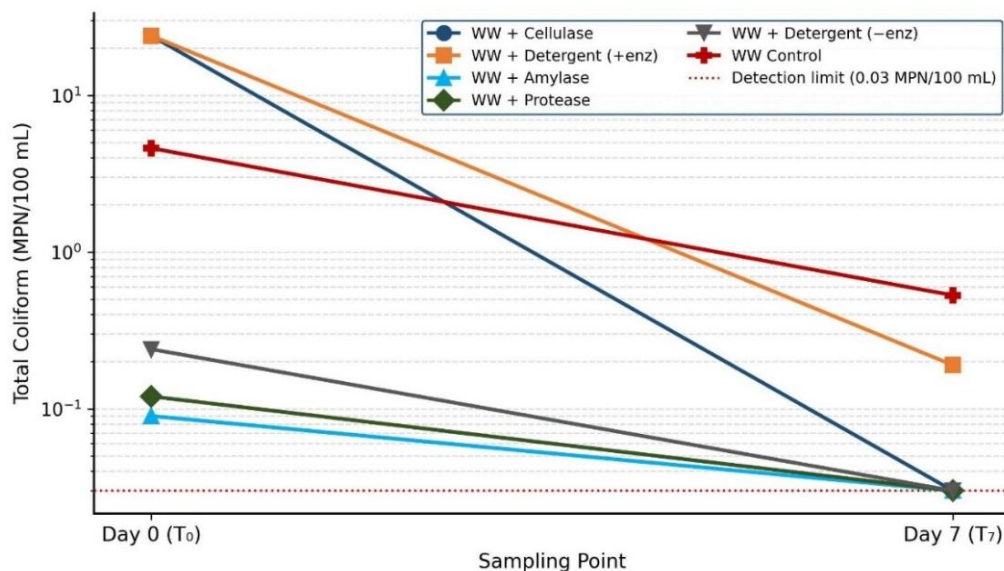


Fig. 4. Temporal decline in coliform counts wastewater treatment groups (log scale).

Semi-logarithmic plot displaying temporal dynamics of total coliform counts ($\log_{10}$ MPN/100 mL on y-axis) versus incubation time in days (0-7 days on x-axis) for six wastewater treatment groups: WW-AMY (amylase), WW-CEL (cellulase), WW-PRO (protease), WW-D+E (enzyme-supplemented detergent), WW-D-E (enzyme-free detergent), and WW-CTL (untreated control). Each treatment is represented by a distinct colour-coded line with error bars ($\pm$ SD, $n = 3$) at T_0 and T_7 . The horizontal red dotted line indicates method detection limit (LOD = 0.030 MPN/100 mL). Slope differences illustrate the rate of coliform decline under each treatment regime. Temporal trends are presented as observational patterns; endpoint statistical comparisons are based on $\log_{10}^{T_7}$ values.

The absence of detectable treatment effects in SOIL-D-E relative to SOIL-CTL may additionally reflect matrix-specific constraints on surfactant bioavailability, including possible adsorption of LAS onto soil particles and the potential for surfactant concentrations to fall below the critical micelle concentration (CMC) under the applied conditions [1, 5]; however, adsorption processes were not directly measured, and this interpretation is offered only as a plausible hypothesis.

4. Discussion

The present study evaluated the response of total coliform populations to hydrolytic enzyme treatments in detergent-impacted industrial wastewater and soil aqueous extracts under controlled laboratory conditions. A central finding was the substantial background attenuation observed in the untreated wastewater control, which exhibited an 88.5% decline in coliform abundance during the 7-day incubation period. This observation indicates that a large proportion of the overall reduction occurred independently of enzyme addition and highlights the importance of distinguishing treatment-associated responses from natural environmental attenuation.

In accordance with the revised statistical framework, treatment performance was interpreted primarily using residual endpoint abundance $\log_{10} T_7$ and absolute remaining coliform counts rather than percentage reduction values alone. Under these conditions, WW-CEL and WW-D+E exhibited the lowest endpoint coliform abundances among the wastewater treatment groups, whereas the enzyme-free detergent treatment (WW-D-E) produced an endpoint response that was comparable to the untreated control.

Although descriptive reduction efficiencies exceeded 99% in WW-CEL and WW-D+E, these values incorporate both treatment-associated effects and the substantial background decline observed in WW-CTL. Consequently, the findings are more appropriately interpreted as evidence of an incremental treatment-associated response superimposed upon a strong natural attenuation process rather than as a direct measure of enzymatic contribution alone. Relative to the untreated control, the descriptive reduction efficiencies observed for WW-CEL and WW-D+E exceeded the control response by approximately 11.4% and 10.7%, respectively. These values are presented only as descriptive indicators and should not be interpreted as formal estimates of enzymatic effect size.

4.1. Natural attenuation and wastewater responses

A prominent finding of the present study was the substantial decline observed in the untreated wastewater control (WW-CTL), which exhibited an 88.5% reduction in coliform abundance over the 7-day incubation period. This result indicates that environmental attenuation constituted a major component of the overall response and therefore required explicit consideration when interpreting treatment-associated effects. The observed decline is consistent with multiple stressors known to influence microbial persistence in industrial wastewater environments, including nutrient depletion during prolonged incubation, low dissolved oxygen availability (2.4 ± 0.6 mg/L), and exposure to surfactant-containing matrices. Similar interactions between surfactant-rich environments and microbial community dynamics have been reported in recent wastewater studies [1, 4, 6, 22].

Because baseline coliform concentrations differed among treatment groups, treatment performance was evaluated primarily using residual endpoint abundance [$\log_{10} T_7$] and absolute remaining counts rather than percentage reduction values alone. Under this framework, WW-CEL and WW-D+E exhibited the lowest residual coliform abundances at the end of the incubation period, with endpoint values reaching or approaching the analytical detection limit. These observations indicate that enzyme-containing treatments were associated with lower endpoint coliform abundance than the untreated control under the experimental conditions evaluated.

The difference between gross reduction and treatment-associated response is particularly important in the present dataset. Although descriptive reductions exceeded 99% in WW-CEL and WW-D+E, a substantial proportion of this decline was also observed in the untreated control. Consequently, the findings are more appropriately interpreted as an incremental response superimposed upon a strong background attenuation process. Relative to WW-CTL, the additional reduction associated with WW-CEL corresponded to approximately 11.4% beyond the control baseline, indicating a measurable but considerably smaller effect than implied by percentage reduction values alone.

An additional observation was the elevated T_0 count measured in WW-CEL relative to WW-CTL. Because treatment allocation occurred before baseline enumeration, this difference may not necessarily represent a true difference in the initial microbial burden of the wastewater. One possible explanation is the partial dispersion of microbial aggregates immediately following enzyme addition, thereby increasing the number of culturable units detected by the MPN assay. Hydrolytic enzymes have previously been reported to disrupt extracellular polymeric substances, biofilm structures, and microbial aggregates, potentially increasing the recovery of individual culturable cells [15, 17–19]. However, aggregate structure was not directly evaluated in the present study, and this explanation should therefore be regarded as a plausible hypothesis rather than a confirmed mechanism.

The response observed in the enzyme-free detergent treatment (WW-D-E) further highlights the importance of matrix context when interpreting surfactant effects. Although WW-D-E exhibited substantial coliform decline, the magnitude of reduction was similar to that observed in the untreated control. Under the concentration, exposure duration, and wastewater conditions examined here, exogenous surfactant addition did not produce a clearly distinguishable endpoint response beyond the background attenuation process. This finding should not be interpreted as evidence that LAS is intrinsically ineffective as an antimicrobial stressor. Previous studies have demonstrated that surfactant behaviour is strongly influenced by concentration, microbial adaptation, biodegradation pathways, and wastewater matrix characteristics [1, 4–6]. Therefore, the absence of a detectable additional effect in the present study is most appropriately interpreted as being specific to the experimental conditions evaluated rather than as a general property of LAS-containing systems.

4.2. Matrix-dependent behaviour: wastewater and soil extract responses

The contrasting outcomes between wastewater and soil extract matrices reflect fundamental differences in microbial abundance, substrate availability, and matrix characteristics. Previous studies have shown that microbial responses in environmental systems are strongly shaped by matrix-specific physicochemical conditions, resource availability, and community structure, often resulting in markedly different treatment outcomes despite identical interventions [12, 21, 22]. In the wastewater matrix, relatively higher baseline coliform abundances were observed ($T_0 = 4.60$ MPN/100 mL for the untreated control; up to 24.0 MPN/100 mL in WW-CEL and WW-D+E), whereas most soil extract treatments exhibited coliform counts at or below the analytical detection limit at baseline. Consequently, interpretation of treatment-associated responses was considerably more constrained in the soil matrix than in wastewater, particularly because statistical comparisons relied on endpoint abundance rather than percentage reduction values.

The predominance of sub-detection-limit counts in soil extract treatments is consistent with a floor effect, whereby measured

coliform abundance approached the analytical detection limit irrespective of treatment. Under these conditions, the ability to distinguish treatment-associated responses from background variability becomes inherently limited. The fixed enzyme dosage (10 U/mL) was originally selected as a standardised proof-of-concept exposure level for detergent wastewater and may therefore have exceeded the effective requirement for the substantially lower microbial loads present in the soil extract matrix. Similar substrate-limited responses have been reported in environmental microbial systems, where treatment effects become increasingly difficult to resolve as target populations approach low-abundance thresholds [12, 22].

In addition to low baseline microbial abundance, matrix-specific interactions may also have influenced treatment performance. Sorption processes are known to alter the environmental availability of biologically active compounds in heterogeneous solid-containing systems. Studies examining sludge and particulate-rich environmental matrices have demonstrated that adsorption phenomena can substantially influence microbial accessibility to contaminants and bioactive agents [7, 8]. Consequently, enzymes and surfactants introduced into the soil extract may have undergone partial adsorption onto soil particles, thereby reducing their effective bioavailability. Although adsorption processes were not directly measured in the present study, such mechanisms provide a plausible explanation for the limited differentiation observed among soil treatments.

The sole quantifiable soil treatment, SOIL-CEL ($T_0 = 0.06$ MPN/100 mL; 51.6% reduction, $p = 0.043$), exhibited a decline to below the analytical detection limit at T_7 . However, given the extremely low baseline abundance and the proximity of both measurements to the detection limit, this observation should be interpreted cautiously and not as definitive evidence of superior cellulase performance in the soil matrix.

The untreated soil control (SOIL-CTL) exhibited a 96.2% reduction over the incubation period ($T_0 = 2.40$ MPN/100 mL; $T_7 = 0.09$ MPN/100 mL), indicating substantial background attenuation under the experimental conditions. This observation suggests that intrinsic matrix properties exerted a dominant influence on endpoint coliform abundance. In addition, adsorption of surfactants onto soil particles may have reduced the freely available fraction of LAS, thereby limiting exposure of microorganisms to surfactant-associated stress. Such matrix-dependent reductions in bioavailability have been widely reported for contaminants and biologically active compounds in complex environmental systems [7, 8, 21]. Consequently, the present data do not support strong conclusions regarding enzyme-specific effects in soil extracts, but rather indicate that treatment-associated responses were difficult to resolve under low-load conditions approaching the analytical detection limit.

4.3. Practical implications for industrial wastewater treatment

The findings of the present study suggest that hydrolytic enzyme addition may influence endpoint coliform abundance in detergent-impacted wastewater under the experimental conditions evaluated. Because substantial background attenuation was observed in the untreated wastewater control (88.5% reduction), interpretation of treatment performance should focus on the incremental response observed beyond this baseline rather than on gross reduction values alone.

Within this context, WW-CEL and WW-D+E exhibited the lowest residual coliform abundances at T_7 , indicating that enzyme-containing treatments may contribute to additional suppression beyond the attenuation observed in untreated wastewater. However, the magnitude of this contribution should be interpreted in relation to the strong background decline observed in the control rather than as an independent measure of treatment efficacy.

These observations support the potential value of hydrolytic enzymes as supplementary components within wastewater treatment strategies rather than as stand-alone replacements for established treatment or disinfection processes. The present study was designed as a proof-of-concept investigation and did not evaluate regulatory compliance, treatment reliability under operational conditions, pathogen inactivation kinetics, or performance at pilot or full scale. Consequently, direct comparisons with conventional disinfection technologies such as chlorination or ultraviolet treatment are beyond the scope of the current dataset.

The comparatively low endpoint abundances observed in WW-CEL and WW-D+E nevertheless suggest that further investigation of enzyme-assisted treatment approaches may be warranted, particularly in detergent-rich industrial effluents where surfactants, organic loading, and microbial stressors coexist. Similar process-specific optimisation strategies have been widely advocated in industrial biocatalysis and environmental biotechnology applications [4, 14].

More broadly, the results emphasise the importance of distinguishing treatment-associated responses from natural attenuation processes when evaluating biological interventions in environmental systems. Adoption of endpoint-based statistical comparisons, together with explicit consideration of control performance, may provide a more conservative and transparent framework for assessing future enzyme-assisted wastewater treatment strategies.

4.4. Limitations and future research directions

The attainment of coliform counts at or below the analytical detection limit ($LOD = 0.03$ MPN/100 mL) in several treatment groups demonstrates the limited resolution of cultivation-based enumeration methods when endpoint abundances approach the detection threshold. While low endpoint counts were observed in WW-CEL and WW-D+E, measurements near the detection limit inherently constrain discrimination among highly suppressed treatment groups. This limitation was particularly evident in the soil extract matrix, where several treatments exhibited baseline and endpoint counts at or below the analytical detection limit.

An additional limitation relates to the use of the Most Probable Number (MPN) method, which quantifies culturable coliforms rather than directly measuring cellular damage, physiological stress, or mechanisms of inactivation. Consequently, the present study

cannot determine whether the observed reductions reflect cell death, loss of culturability, aggregate disruption, or a combination of these processes.

Future investigations could improve mechanistic resolution by integrating cultivation-based methods with complementary molecular and cell-viability approaches, including quantitative PCR, fluorescence-based viability assays, and microscopic assessment of aggregate structure. Such methods would help distinguish between reductions in culturability and broader changes in microbial physiological status.

The experimental design was based on two sampling points (T_0 and T_7), which allowed evaluation of endpoint responses but did not permit detailed assessment of treatment kinetics. Additional intermediate sampling intervals would provide insight into the temporal dynamics of coliform attenuation, potential transient effects, and the possibility of microbial recovery during prolonged incubation. Likewise, longer observation periods would help determine the persistence of treatment-associated responses beyond the initial 7-day incubation period.

A further limitation concerns the use of a single enzyme concentration (10 U/mL), which was intentionally selected as a standardised proof-of-concept exposure level rather than an optimised operational dose. Because no dose-response analysis was performed, the minimum effective concentration and the optimal treatment range remain unknown. Future studies should therefore evaluate multiple enzyme concentrations across matrices with differing microbial abundances and physicochemical characteristics. Such work would facilitate assessment of treatment efficiency, operational feasibility, and matrix-specific performance under environmentally relevant conditions.

Finally, the marked differences in baseline coliform abundance among some wastewater treatment groups highlight the challenges associated with environmental microbial datasets. Although the revised statistical framework relied primarily on $\log_{10}$ -transformed endpoint counts and absolute residual abundance rather than percentage reduction values, future studies would benefit from larger sample sizes, increased replication, and experimental designs specifically developed to minimise baseline heterogeneity among treatment groups.

5. Conclusions

This study investigated the response of total coliform populations to hydrolytic enzyme treatments in detergent-impacted wastewater and soil aqueous extracts under controlled laboratory conditions. A major finding was the substantial background attenuation observed in the untreated wastewater control, indicating that natural environmental decay represented a significant component of the overall reduction observed during incubation.

When treatment performance was evaluated using residual endpoint abundance [$\log_{10} T_7$] and absolute remaining coliform counts, enzyme-containing treatments were associated with lower endpoint coliform abundances than the untreated control in the wastewater matrix. Among the evaluated treatments, cellulase-containing systems exhibited the lowest residual coliform abundance under the experimental conditions investigated.

The findings further indicate that, under the tested conditions, addition of an enzyme-free LAS-containing detergent formulation did not produce a clearly distinguishable response beyond the background attenuation observed in the untreated control. This observation should be interpreted within the context of the specific matrix characteristics, concentration levels, and exposure conditions examined.

Overall, the results suggest that hydrolytic enzymes may contribute to incremental reductions in coliform abundance in detergent-impacted wastewater, while natural attenuation processes remain a dominant component of the overall response. These findings provide preliminary evidence supporting further evaluation of enzyme-assisted approaches for the management of microbiological contamination in surfactant-rich industrial wastewater systems.

Statements & declarations

Author contributions

Siamak Boudaghpour: Investigation, Supervision, Resources, Writing - Original Draft.

Amirali Bahadori: Formal analysis, Project administration, Writing - Review & Editing.

Ehsan Qamari: Conceptualization, Methodology, Resources, Writing - Original Draft.

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Data availability

The data presented in this study are available on request from the corresponding author.

Declarations

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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