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Sustainable Removal of Phenol using *Bacillus Tequilensis* Immobilized in Alginate: Influence of Biomass and Exposure Time

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The impact of phenol on natural environments is a critical environmental issue due to its high toxicity and recalcitrance. Therefore, the search for new alternatives for phenol removal involves adopting sustainable and safe strategies. The objective of this research was to determine phenol removal efficiency at different treatment times and biomass concentrations using *Bacillus tequilensis* immobilized in alginate, isolated from soils of the Lomas del Cerro Campana, Peru—an ecosystem that has never been explored for its microbiological resources in bioremediation. The isolated microbial culture was taxonomically identified through molecular analysis of the 16S rRNA gene, and its tolerance to phenol (100–2000 ppm) was evaluated. For the phenol removal assays, cells were immobilized in 3% sodium alginate at different cell density concentrations (OD_{600} = 0.5, 1.0, and 1.5) and exposed for 3, 5, and 7 days. A multilevel factorial experimental design was applied at 30 °C, 150 rpm, and an initial pH of 7. *Bacillus tequilensis* QCM11 exhibited tolerance up to 1000 ppm of phenol and achieved 28.58 ± 1.99 % phenol removal at an OD_{600} of 0.5 under immobilized conditions. These results confirm its potential as a sustainable biotechnological alternative for optimizing phenol bioremediation applications.

1. Introduction

Phenol is a compound commonly found in petroleum refining, and in the manufacture of textiles, plastics, and pharmaceuticals, resulting in effluent discharges containing up to 10 million tons of phenolic compounds, with concentrations ranging from 0.1 to 1600 mg/L (Panigrahy et al., 2022). However, the main concern lies in its toxicity, as phenol can cause damage to the nervous, renal, and hepatic systems, and at high concentrations, it can even be mutagenic (Mhlongo et al., 2023). Furthermore, it adversely affects aquatic organisms by inducing infertility, mortality, and developmental suppression (Ahmaruzzaman et al., 2024). Therefore, phenolic contamination increases the urgency of implementing effective and environmentally adaptable solutions for its removal. Previous studies have shown that conventional treatment methods used in effluent plants—such as adsorption on activated carbon, advanced oxidation, photodegradation, and Fenton processes—have significant limitations, including high costs, generation of toxic by-products, and high energy consumption (Bibi et al., 2023; Ho et al., 2020). In contrast, the use of microbial or subcellular platforms offers a cleaner, more scalable, and cost-effective process with higher specificity (Chen & Sun, 2023). Nevertheless, high concentrations of phenol can inhibit microbial biodegradation activity. To overcome this challenge, several

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strategies have been proposed, such as the use of microbial consortia, co-substrate addition, and cell immobilization (Sabri et al., 2025). The latter improves the performance, stability, and reusability of microbial cells by providing a higher microbial cell density through physical support (Shahabivand et al., 2022). Cell entrapment using calcium alginate has been widely reported as the most suitable technique due to its ease of use, low cost, and low toxicity (Banerjee & Ghoshal, 2016). However, the efficiency of cell immobilization depends on the biological tolerance of the microorganism; thus, incorporating bacteria adapted to high phenol concentrations could significantly enhance biodegradation performance (Abarian et al., 2019). Autochthonous bacteria are considered desirable candidates because they can withstand variable environmental conditions and complex activities, thereby increasing their likelihood of tolerating contaminants (Shebl et al., 2022). Although phenol biodegradation using immobilized bacteria has been widely reported, the novelty of this study lies in the use of an autochthonous bacterial strain isolated from the soils of Lomas del Cerro Campana, Peru, a local ecosystem not previously explored for its microbiological potential in phenol bioremediation. In this context, the contribution of the present work is twofold: first, it identifies *Bacillus tequilensis* QCM11 as a native phenol-tolerant strain with biotechnological potential; second, it evaluates how immobilized biomass level and exposure time influence its removal performance, providing practical baseline information for future development of locally adapted treatment systems.

2. Method

2.1 Microbial isolation

The methodology described by Quiñones-Cerna et al. (2024) was used. A total of 25 g of soil collected from the Lomas del Cerro Campana (La Libertad, Peru; -11.80901°, -77.0686°) was enriched in 225 mL of Bushnell Haas Minimal Salt Medium (BHM) supplemented with 1 % (v/v) diesel for 7 days at 30 °C. Subsequently, the last two dilutions (10^{-8} and 10^{-9}) were surface-plated on Petri dishes containing nutrient agar (NA). Microbial colonies showing distinct morphologies were subcultured and preserved at 5 °C.

2.2 Microbial selection and identification

Sterile BHM medium supplemented with 1 % phenol was inoculated with 10 % of the isolated bacterial suspension (OD_{600} = 1) previously subcultured. The cultures were incubated at 30 °C and 150 rpm for 10 days, and optical density (OD_{600}) was monitored on days 0, 5, and 10 using a UV–Vis spectrophotometer (UVILINE 9400, Germany). Subcultures exhibiting OD values above 0.8 and/or the highest growth rates were selected.
For taxonomic identification, total DNA was extracted using the Quick-DNA™ kit, and the 16S rRNA gene was amplified by PCR with primers 27F and 1942R. The amplified product was sequenced by Macrogen (Chile), and the sequence identity was determined by comparison with the EzBioCloud database. A phylogenetic tree was constructed based on sequence alignments to infer evolutionary relationships (Cueva-Almendras et al., 2022).

2.3 Preparation of the phenol solution and bacterial inoculum tolerance

A stock phenol solution (Spectrum, USA) of 10,000 ppm was prepared by dissolving 5 g of phenol in 500 mL of 0.02 N NaOH, according to Baldera-Saavedra et al. (2024). The selected bacterial culture was grown in nutrient broth at pH 7 and 35 °C for 24 h. Cells were then centrifuged and resuspended in sterile saline solution, adjusting the OD_{600} to 0.5–0.6. Phenol tolerance was evaluated in nutrient broth containing phenol concentrations ranging from 100 to 2000 ppm, inoculated with 200 µL of the bacterial suspension. Cultures were incubated at 35 °C for 48 h, and growth was assessed by turbidity measurements (Cruz et al., 2021).

2.4 Cell immobilization

Three bacterial suspensions of the selected strain with OD_{600} values of 0.5, 1.0, and 1.5 were prepared and individually mixed 1:1 with 3 % sodium alginate. The mixtures were dropped by gravity into 0.2 M $CaCl_2$ to form beads via ionic gelation. The beads were cross-linked for 30 min and washed with sterile distilled water before use (Anisha & Prema, 2008).

2.5 Experimental design

A multilevel factorial experimental design was applied with two factors: initial cell biomass (OD_{600} = 0.5, 1.0, and 1.5) and exposure time (3, 5, and 7 days), resulting in 27 treatments performed in triplicate. Each flask contained 20 mL of nutrient broth adjusted to pH 7 and supplemented with phenol at an initial concentration of 1000 ppm, according to the tolerance test. One gram of immobilized cells (wet weight) in alginate beads was added to each flask. Treatments were incubated at 30 °C and 150 rpm in an orbital shaker (BIOBASE, BJPX-200N, China). The best-performing treatment was further replicated to determine the specific growth rate (µ) during phenol removal under similar conditions, using Equation (1).

$$\mu = \frac{lnX2 - lnX1}{t2 - t1} \quad (1)$$

where X_2 is the final cell density, X_1 is the initial cell density, t_2 is the final time, and t_1 is the initial time.

2.6 Analytical methods

Cell biomass was quantified by measuring optical density at 600 nm using a UV/Vis spectrophotometer, while residual phenol concentration was determined by UHPLC equipped with a DAD UV/Vis detector (Thermo Fisher Scientific, Dionex Ultimate 3000) (Patel et al., 2022).

2.7 Statistical analysis

All experiments were performed in triplicate as independent assays, and results were expressed as mean ± standard deviation. Data on residual phenol concentration and cell biomass were analyzed using a multilevel factorial ANOVA in Design Expert v23.1 to evaluate the main effects of initial biomass and exposure time, as well as their interaction.

3. Results and discussion

A microbial isolate coded as QCM11 was selected from 12 isolated cultures, as it exhibited the highest growth (OD_{600} = 1.2) in 1 % phenol at 150 rpm and 30 °C, and was therefore used for the phenol removal assays. Regarding its macroscopic characteristics, QCM11 formed medium-sized colonies with irregular shapes, undulated margins, opaque cream coloration, rough texture, slight elevations, and a mucoid consistency (Figure 1A). Microscopically, the isolate showed a bacillary form, was Gram-positive, and spore-forming. These general characteristics are consistent with those described for the *Bacillus* genus by Gayathri et al. (2025).
Molecular characterization based on 16S rRNA gene sequencing of the QCM11 isolate revealed a 99.93% similarity with *Bacillus tequilensis* KCTC 13622, as reported by Gatson et al. (2006). This identification was further supported by the phylogenetic tree, which showed a close evolutionary distance (0.0007914) to *Bacillus tequilensis*, while more distant relationships were observed with *Bacillus subtilis* and *Bacillus cereus*, showing evolutionary distances of 0.00158 and 0.0609, respectively (Figure 1B). The nucleotide sequence had a total length of 1337 bp and was deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under the accession number PX447481 (https://www.ncbi.nlm.nih.gov/nuccore/PX447481).

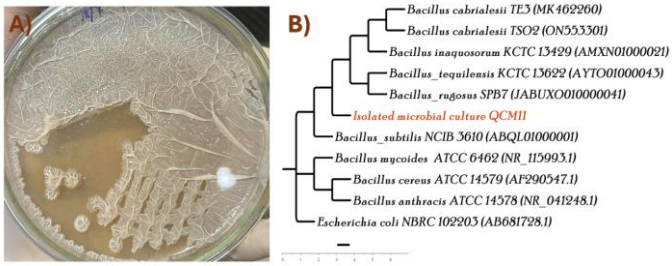

Figure 1: Isolation of the pure culture QCM11 (A) and phylogenetic analysis using the Neighbor-Joining method (B).

In Figure 2A, it is shown that *Bacillus tequilensis* QCM11 was able to grow at phenol concentrations up to 1000 ppm but showed no growth at 1500 and 2000 ppm, which defined the working range for the biodegradation assays. QCM11 exhibited high resistance to phenol toxicity, which is associated with the uncoupling of oxidative phosphorylation, and this tolerance was higher than previously reported in the literature (Anokhina et al., 2025). Moreover, Figure 2B shows that the highest phenol removal percentage was obtained with an initial biomass of OD600 = 0.5 after 7 days (28.58 ± 1.99 %), while the lowest value was observed with OD_{600} = 0.5 after 3 days (4.54 ± 1.36%). These results differ from those reported by Banerjee & Ghoshal (2011), who achieved more than 50% removal of 2000 ppm phenol after 26–36 days using immobilized *Bacillus cereus*. Similarly, Lu et al. (2012) reported that *Bacillus amyloliquefaciens* WJDB-1 immobilized in alginate–chitosan–alginate microcapsules was able to completely degrade 200 ppm of phenol within 36 h. Furthermore, it was observed that the final cell biomass decreased as exposure time increased, possibly due to cellular stress caused by phenol toxicity (Zhang et al., 2023). In addition, mass transfer limitations within the alginate beads explain why higher biomass concentrations do not necessarily lead to improved removal efficiency (Banerjee et al., 2001).
Current results suggest that phenol removal by immobilized *Bacillus tequilensis* QCM11 does not depend exclusively on the amount of inoculated biomass, but rather on the balance between active cell density, physiological adaptation, and substrate diffusion within the alginate matrix. Although higher inoculum levels are

generally expected to enhance biodegradation, our results showed that the lowest biomass condition (OD_{600} = 0.5) produced the highest removal after 7 days. This behavior can be explained by diffusion limitations within the microspheres, reduced oxygen and substrate transfer at higher cell densities, and the accumulation of local stress conditions that limit metabolic efficiency. This interpretation is consistent with the significant interaction observed between biomass and exposure time, as well as with previous reports describing mass transfer limitations in immobilized biodegradation systems (Lin et al., 2020).

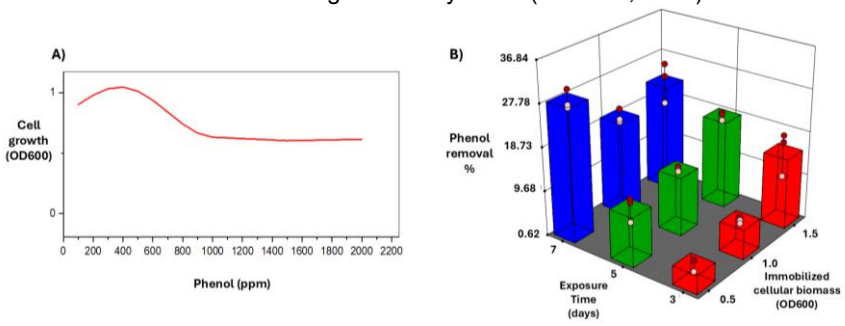

Figure 2: Phenol tolerance assay at different concentrations (A) and effect of exposure time and immobilized QCM11 cells on phenol removal (B).

Similar behavior has been reported in other immobilized phenol-degrading systems, where substrate removal was progressive rather than immediate and was associated with microbial growth, acclimation, and transport limitations within the support matrix (Chung et al., 2003). Studies with Ca-alginate-immobilized cells have shown that phenol degradation is governed by coupled growth–degradation kinetics and can be influenced by internal mass transfer effects, while other immobilized systems have also reported lag-phase behavior before efficient phenol removal is established. The ANOVA analysis (Table 1) indicates that the model was significant ($p < 0.0001$), explaining the variability observed in phenol removal efficiency. The factor with the greatest influence was exposure time ($F = 79.90$; $p < 0.0001$), followed by the initial cell biomass ($F = 13.31$; $p = 0.0003$). Additionally, the interaction between both factors (A × B) was also significant ($F = 9.51$; $p = 0.0003$).

Table 1: Analysis of variance (ANOVA) for phenol removal percentage.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1454.63	8	181.83	28.06	< 0.0001	significant
A- Initial cell biomass	172.56	2	86.28	13.31	0.0003	
B- Exposure time	1035.60	2	517.80	79.90	< 0.0001	
AB	246.47	4	61.62	9.51	0.0003	

In Figure 3, it can be observed that the optimal treatment (OD_{600} = 0.5, 7 days) began with an OD_{600} of 0.231 at 6 h, reached 2.098 at 72 h, and decreased to 1.72 at 192 h. Phenol removal was less than 3% after 24 h, increasing progressively to 30.65 % at 192 h. This indicates that degradation intensified during the later stages, when biomass levels were higher and enzymatic systems were fully active. A specific growth rate (μ) of 0.0876 h^{-1} was also observed. In the chromatogram at 0 h (Figure 3B), a dominant peak appeared at a retention time (RT) of 1.86 min, corresponding to phenol. At 192 h (Figure 3C), the phenol peak between RT 1.77–1.86 min showed a marked reduction in area, and a new early peak emerged between RT 1.37–1.50 min, likely corresponding to more polar metabolites or intermediate by-products, possibly related to catechol and cis,cis-muconic acid (Asimakoula et al., 2023).

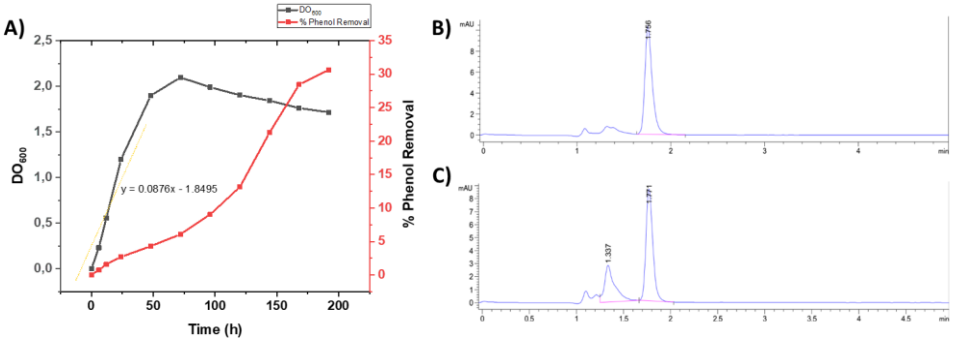

Figure 3: Growth kinetics during phenol removal (A) and chromatograms of phenol removal at 0 h (B) and 192 h (C).

Bacillus tequilensis QCM11 was isolated from the soils of Lomas del Cerro Campana, a Peruvian ecosystem that had not been previously investigated as a source of microorganisms for phenol bioremediation. This is relevant because indigenous strains may exhibit better physiological adaptation to varying environmental conditions and offer advantages for the future design of local and environmentally compatible treatment strategies. From an applied perspective, these findings suggest that Bacillus tequilensis QCM11 immobilized in alginate could be used as a preliminary biotechnological alternative for the treatment of phenol-containing wastewaters under controlled conditions. In practical terms, the best-performing condition identified in this study (OD_{600} = 0.5, 7 d) may serve as a starting operational reference for the design of packed-bed, batch, or polishing bioreactors using immobilized native bacteria. However, before full-scale implementation, additional studies should optimize reactor configuration, bead stability, hydraulic performance, reusability, and phenol removal efficiency in real industrial effluents, where the presence of salts, co-contaminants, and fluctuations in pH and temperature may affect system performance.

4. Conclusions

The results demonstrate that the isolate *Bacillus tequilensis* QCM11 possesses significant potential for phenol removal, achieving 28.58 ± 1.99 % elimination after 7 days with an initial OD_{600} of 0.5. This performance confirms its ability to tolerate concentrations up to 1000 ppm and metabolize toxic compounds, positioning it as a promising biotechnological alternative for the bioremediation of phenol-contaminated environments. These findings highlight the value of unexplored local ecosystems as sources of microorganisms with biotechnological potential and provide a practical basis for the future development of controlled, sustainable, and locally adapted phenol treatment systems.

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