

Bioremediation of distillery effluent using a novel laccase-producing *Bacillus velezensis* HIM03: Process optimization, metabolite characterization, and phytotoxicity studies

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Abstract

Sustainable treatment of distillery industry effluent (DIE) remains challenging due to its strong coloration, complex pollutant profile, and toxicity. However, it is critical to ensure safe disposal/reuse, safeguard soil/water resources, mitigate environmental pollution, and protect public health. This study, for the first time, demonstrates the bioremediation of DIE using a novel ligninolytic enzyme-producing bacterial strain, *Bacillus velezensis* HIM03, attained significant reductions in pollution parameters within 144 h: COD (84.03%), BOD (87.00%), TDS (74.99%), phosphate (69.27%), sulfate (63.17%), nitrate (71.01%), phenol (74.03%), and heavy metals including Zn (85.93%), Cu (78.57%), Mn (75.26%), Fe (66.00%), and Pb (72.73%). The bacterium *Bacillus velezensis* HIM03 (GenBank Accession Number: OP659023) exhibited laccase production with a peak activity of 6.18 IU/mL/min at 96 h under optimal conditions [pH: 8, temperature: 35 °C, glucose and peptone: C and N substrate (0.5%), inoculum size: 10 mL, shaking speed: 120 rpm], indicating its strong oxidative potential for degradation of recalcitrant coloring contaminants (RCCs). FT-IR and GC-MS examination confirmed the efficient degradation and structural transformation of RCCs into simpler, non-toxic metabolites, validating true detoxification rather than mere pollutant removal. Crucially, phytotoxicity assays showed an 82% seed germination in *Phaseolus aureus* L. (mung bean) when irrigated with treated DIE, demonstrating significant reduction in residual toxicity and improved environmental compatibility. These findings validate the role of novel *Bacillus velezensis* HIM03 in achieving regulatory-relevant pollutant removal, showcasing its strong potential for integration into economical and scalable bioremediation strategies for DIE, in line with the UN SDGs on clean water, health, and environmental sustainability.

Keywords: Distillery Industry; Biodegradation; Biodegradation; Biodegradation; Ligninolytic Enzyme; FT-IR, GC-MS; Ecotoxicity

1. Introduction

Distilleries are the major agro-industries which ferment and distil grains, sugarcane, fruits, and molasses to produce a variety of useful products. Among these, alcoholic beverages are the most traded commodities globally, contributing to the economy, employment generation, and export earnings. From USD 656.50 billion in 2025, the global drinking alcohol market is projected to reach USD 788.82 billion by 2030 with a compound annual growth rate (CAGR)

of 3.74% (<https://www.mordorintelligence.com/industry-reports/spirits-market>). Alcohol distilleries in India, one of the world's largest producers, are expected to be worth USD 200.0 billion by 2025 and USD 300.0 billion by 2035, growing at a projected CAGR of 7.2%. (<https://www.futuremarketinsights.com/reports/indian-alcohol-market>). However, distilleries contribute to worldwide environmental pollution since ethanol production consumes a huge amount of fresh water and generates a large quantity of high-strength effluent, referred to as "spent wash". The effluent composition and volume may vary across enterprises due to differences in raw materials, process conditions, distillation technology, plant capacity, and recycling and treatment practices. Typically, distillery industry effluent (DIE) has an acidic pH (3.5-5), significant dark-brown pigmentation from melanoidin production, and extraordinarily high organic loading, with biochemical oxygen demand (BOD) values often surpassing 24,000 mg/L⁻¹ and chemical oxygen demand (COD) that exceeds 90,000 mg/L⁻¹ [1, 2]. Total dissolved solids (TDS) in DIE can reach 20,000-60,000 mg/L, indicating excessive suspended solids, residual sugars, and fermentation by-products [1,3]. DIE also contains various potentially toxic chemicals and recalcitrant coloring contaminants (RCCs) such as caramel, melanoidins, alkaline degradation products, polyphenols, lignin, furfurals, phenolics, long-chain fatty acids, ammonia, sulfates, endocrine-disrupting chemicals, mutagens, carcinogens, organometallic pollutants, and heavy metals (HMs), all of which pose a significant environmental risk [1-3].

The discharge of DIE into the environment either untreated or with insufficient treatment, pollutes water and land resources. The release of DIE into surface waters causes fish kills and extensive harm to aquatic ecosystems by interfering with photosynthesis and rapidly depleting dissolved oxygen [4]. Local farmers use DIE to ferti-irrigate soil to recycle nutrients and organic matter. However, this practice often causes salinization, acidification, and the accumulation of phenolic compounds and HMs, which negatively impact soil health, seed germination, and soil microbial populations [5-9]. The leaching of DIE contaminants into

groundwater causes excessive COD, TDS, and intense coloration, making drinking water unsafe for human consumption [10, 11]. Prolonged exposure to DIE can cause phytotoxicity in plants, decreased agricultural productivity, oxidative stress, impaired enzyme activity, and metabolic disturbance in aquatic and terrestrial flora and wildlife [1, 2, 5]. The toxic effects of DIE in living beings have been well-documented in literature [2, 3, 5], necessitating adequate treatment to prevent and manage pollution.

Recently, DIE treatment and management have gained significant attention in the context of environmental sustainability, regulatory compliance, circular economy, and resource recovery. Strict discharge regulations have pushed distilleries to adopt integrated effluent management strategies emphasizing detoxification, reuse, and sustainability. Adequately detoxified DIE has considerable potential for reuse in irrigation, bioenergy production, and nutrient recycling, thereby reducing freshwater demand and fostering sustainable industrial practices [3]. Nevertheless, recalcitrant organics, residual toxicity, and phytotoxicity often limit DIE reuse potential, emphasizing the need for treatment approaches that ensure ecological safety in addition to pollutant reduction. Consequently, the integration of advanced chemical characterization and biotoxicity assessment have become crucial for accurately determining the environmental compatibility of treated effluents.

Over time, several technologies have been developed to treat and detoxify DIE to control pollution. Conventional DIE treatment uses biological processes such as anaerobic digestion and aerobic polishing, which reduce BOD and produce biogas. However, these methods require huge reactors, long hydraulic retention periods, and may not be able to handle shock loads, colour removal, or refractory pollutants [12, 13]. Physicochemical approaches, including adsorption, chemical oxidation, coagulation and flocculation, and membrane filtration can quickly remove colour, suspended particles, and some refractory organics from DIE. Nevertheless, they produce secondary sludge, incur substantial chemical and energy

expenditures, and fail to mineralize complex chemicals [14, 15, 16]. Emerging treatment techniques like reverse osmosis, supercritical water technology, advanced oxidation methods (ozonation, Fenton's reagent, photocatalysis), and membranes reinforced with nanomaterials appear to degrade DIE's recalcitrant and refractory pollutants, reduce toxicity and sludge generation [17, 18]. However, many of these treatment methods are still in the pilot stage, have scalability issues, and need complex operation or expensive catalysts. Additionally, hybrid or combined systems like aerobic membrane bioreactors or anaerobic treatment with wet oxidation have been developed over time to adequately treat DIE, but they are more expensive and complex [19, 20]. Therefore, environmentally friendly, cost-effective, chemical- and energy-efficient DIE treatment and detoxification methods are needed to meet environmental compliance.

Bioremediation is widely recognized as an eco-friendly and sustainable alternative to conventional physico-chemical treatment methods, which often cause secondary pollution *via* generating chemical sludge. This strategy exploits the metabolic versatility of microorganisms, particularly bacteria that can produce extra-/intra-cellular catabolic enzymes to degrade and detoxify recalcitrant pollutants in DIE [1, 8, 21]. Among these, oxidative enzymes such as laccase, manganese peroxidase, and lignin peroxidase degrade complex pollutants into less toxic compounds for microbial mineralization, thereby improving overall treatment efficiency [1, 8, 21, 22]. The identification and application of robust, high-performance catabolic enzyme-producing microbial strains are therefore critical for advancing bioremediation as a viable and scalable solution for DIE treatment. Enzymatic bioremediation is well-suited for small- and medium-scale distilleries due to its cost-effective application through bioaugmentation or biostimulation. This remediation approach can be readily incorporated into existing effluent treatment plants with minimal infrastructural modifications. Among diverse catabolic enzymes, bacterial laccases, the multicopper oxidases with broad substrate specificity have

emerged as important biocatalysts. Laccases catalyze the oxidation of a wide range of recalcitrant pollutants, including phenolics, melanoidins, dyes, lignin-derived compounds, and other hazardous organics commonly present in DIE [23-28].

Bacterial laccases are of special interest for DIE treatment due to their greater scalability in production and processing compared with fungal laccases [26-28]. Conversely, the application of fungal laccases in DIE treatment is limited by narrow pH-temperature optima, low stability in the presence of salts, phenolics, and HMs, and dependence on expensive redox mediators [1, 22]. Moreover, high production and purification costs, slow kinetics, short shelf life, activity loss in continuous systems, and poor performance under variable industrial conditions limit their large-scale application in DIE treatment [1, 22]. Thus, there is a pressing need to identify novel laccase-producing bacterial strains capable of functioning efficiently under diverse and fluctuating physicochemical conditions associated with DIE. Despite increasing interest in bacterial laccases, their application in DIE bioremediation remains limited. Only a few laccase-producing bacterial species, such as *Bacillus albus* [1] and *Pseudomonas putida* [29], have been reported for DIE degradation and detoxification. This highlights a significant research gap and the need to identify bacterial strains with higher laccase activity and adaptability for effective and sustainable DIE treatment.

Therefore, this study aimed to isolate and characterize a novel laccase-producing bacterium and evaluate its efficiency for bioremediation of DIE. Specifically, the study aimed to (i) identify and characterize a novel laccase-producing bacterium, *Bacillus velezensis* HIM03; (ii) optimize key nutritional and process parameters to maximize DIE decolorization and COD removal under batch conditions; (iii) characterize RCCs and their degradation products using FT-IR and GC-MS/MS analysis; and (iv) evaluate the detoxification potential through comparative phytotoxicity assessment before and after bacterial treatment to gauge environmental safety. To the best of our knowledge, this is the first report demonstrating

effective bioremediation and detoxification of DIE mediated by a novel laccase-producing *Bacillus velezensis* HIM03, highlighting its potential for large-scale distillery wastewater treatment.

2. Materials and methods

2.1 Chemicals and microbiological media

Chemicals (purity $\geq 99\%$) and microbiological media used in the present investigation were bought from Sigma-Aldrich (St. Louis, MO, USA) and HiMedia Laboratories (Mumbai, MH, India), respectively. Bacterial strains were isolated and screened using a particular mineral salt medium (MSM, pH 7.0, g/L) that contained K_2HPO_4 (2.0); Na_2HPO_4 (2.5); $MgSO_4$ (0.01), supplemented with 5% (w/v) of additional C and N sources (glucose and peptone). The DIE was filtered by using a GF/C grade Whatman[®] glass microfiber filter paper (Whatman, England) with a pore size of 1.2 μm . Mung beans (*Phaseolus aureus* L.) seeds, sourced from a nearby vendor in Oachghat, Solan (Himachal Pradesh), India, were used for the DIE phytotoxicity evaluation.

2.2 Description of sampling site and sample collection

All the samples [DIE (biomethanated) and sludge] were collected using a 5 L capacity sterile plastic carboy (Tarson Products Pvt. Ltd., WB, IN) and plastic bags from a release outlet of a major distillery (26.55'N and 80.48'E) in Unnao district, Uttar Pradesh, India. Following the collections, the samples were stored in the lab at 4°C until they underwent different analyses. These included the assessment of physico-chemical parameters of DIE and HMs, bacterial isolation, bioremediation studies, and phytotoxicity assessment.

2.3 Isolation of ligninolytic bacterial strains and growth conditions

To enrich and isolate potential ligninolytic enzyme-producing bacterial strains, a mixture of 5 mL DIE, 1 g sludge, and 5% (50 mg; w/v) synthetic melanoidin [(synthesized as per Santal *et*

al. [30]] was added to 95 mL sterilized MSM broth (pH 7.0) and cultured for 06-days at 35°C and 120 rpm. Furthermore, 50 µL of the enriched bacterial suspension exhibiting visible decolorization was spread onto MSM agar plates (following serial dilution till 10⁻⁶), and these plates were incubated for 24 to 48 h to facilitate colony development. Morphologically different colonies (designated HIM01-10) were chosen and picked-up to purify *via* repetitive streaking for further screening.

2.4 Screening of potential bacterial strains for ligninolytic activity

To ascertain the potential of the isolated strains, HIM01-10, for enhancing DIE treatability, they underwent additional purification and screening, with an emphasis on the synthesis of laccase enzyme following a modified protocol [31]. Each of the chosen isolates was streaked onto the MSM agar plates prepared with CuSO₄ [1 mg/100 mL; w/v (10 mM)] as an inducer and guaiacol (40 µL/100 mL; v/v; purity: ≥ 99%) as a substrate. Colonies exhibiting a brown coloration, a sign of laccase enzyme production, were chosen for further study. The cultures were maintained at 35°C on MSM agar plates added with guaiacol (40 µL/100 mL).

2.5 Characterization and identification of potential bacterial strains

Following preliminary screening, strain HIM03 was chosen and identified using morpho-biochemical procedures defined in "Bergey's Manual of Determinative Bacteriology" [32]. Molecular characterization was based on the 16S rRNA gene sequencing following alkaline lysis to extract the genomic DNA. The PCR products were amplified using universal primers [27 F (5'-GGATGAGCCCGCGGCCTA-3') and 1492R (5'-CGGTGTGTACAAGGCCCGG-3')] at Veriti™ 96-Well Thermal Cycler (Applied Biosystems™ Inc., CA, USA). The PCR conditions consisted of 30 thermo-cycles, which included the first denaturation (3 min; 94°C), the second denaturation (1 min; 94°C) with annealing (1 min; 50°C), the extension (2 min; 72°C), and the final extension (7 min; 72°C). The QIAGEN PCR purification kit (QIAGEN, MD, USA) was used to purify the PCR product (~1.5 kb), which was then sequenced on

Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, MA, USA) with Big Dye Terminator Cycle Sequencing Kit v3.1 (Thermo Fischer Scientific, MA, USA). The resulting 16S rRNA gene sequences (1289 bp) of strain HIM03 were submitted to GenBank following a similarity check *via* the NCBI-BLAST bioinformatic tool (www.ncbi.nlm.nih.gov/BLAST). Mega software (www.megasoftware.net; v7.0) was utilized to generate the ancestral tree of bacterial strain HIM03 utilizing the neighbor-joining approach.

2.6 Optimization of bacterial strains at different process parameters for maximum removal efficiency

Following characterization, the bacterial strain HIM03 was optimized at several process parameters to improve its efficacy in DIE bioremediation. These encompassed nutritional parameters [C sources (sucrose, lactose, maltose, glucose, and starch; 0.5%, w/v) and N sources (peptone, yeast extract, sodium nitrate, ammonium chloride, and urea; 0.5%, w/v)], and environmental parameters (pH, temperature, shaking speed, and inoculum volume). The nutritional factors were optimized by culturing 10 mL of bacterial culture in 90 mL of sterilized DIE-MSM broth composed of DIE (undiluted, 10 mL) + MSM (80 mL, pH 7.0), supplemented with various C and N substrates under shaking conditions (120 rpm) along with abiotic controls at 35°C. The environmental factors were optimized using the same DIE-MSM setup supplemented with the previously identified optimal N and C sources. The pH was optimized from 5 to 9 in 1-unit intervals at 35 °C, whereas temperature optimization was conducted at 25, 30, 35, 40, and 45 °C at the optimized pH. The inoculum volume (2, 4, 6, 8, & 10 %; v/v) and shaking speed (100-140 rpm) were optimized under previously determined optimal nutritional and environmental conditions. COD was finally calculated using the DIE supernatant every 24 h for 06-days after refrigerated centrifugation ($10,000 \times g$; 10 min).

The ranges and specific values of the process parameters selected for optimization were carefully defined based on previous studies on bacterial bioremediation of DIE, bacterial

physiological requirements, and the physicochemical conditions typically encountered in real DIE treatment systems, ensuring their relevance and applicability for potential field-scale bioremediation [1, 8, 21]. The pH range (6-10) was chosen to represent the acidic to alkaline nature of DIE and to evaluate bacterial tolerance and laccase activity under realistic operational conditions. The temperature range (25-45 °C) encompassed sub-optimal, optimal, and supra-optimal mesophilic conditions pertinent to biological treatment processes. Different C & N sources (0.5%, w/v) were selected to assess the influence of readily assimilable and complex nutrients on microbial growth, biomass development, laccase production, and COD removal. The inoculum size (2-10%, v/v) was optimized to ensure rapid biomass establishment and metabolic activity without inducing nutrient limitation. However, the agitation rates (100-140 rpm) were selected to provide adequate aeration and mixing for efficient aerobic degradation and oxidative enzyme activity. Overall, these parameters were selected to facilitate effective translation of the optimized process into practical DIE treatment systems.

2.7 Bioremediation experiment

The efficiency of isolate HIM03 in bioremediating DIE was assessed by monitoring COD reduction and decolorization as the key pollution parameters. The optimized volume (% w/v) of cultured bacterial inoculum was incubated with full-strength DIE (10 mL (v/v)) prepared in the sterilized MSM broth supplemented with the optimal doses of N and C sources (0.5%, w/v) in 250 mL Erlenmeyer flasks along with abiotic controls (lacking bacterial culture) under optimized environmental conditions [pH, temperature (°C)) and shaking (rpm)]. Bacterial growth in DIE and its decolorization were monitored by measuring absorbance ($\lambda_{\text{max}} = 620 \text{ \& } 475 \text{ nm}$), respectively, using an Evolution 201 UV/Visible spectrophotometer (Thermo Scientific™, Australia). The COD (EPA Method No. 5220B; open reflux method) and decolorization rate (%) were determined using the supernatant after removing biomass from

the bacteria-treated (BT) DIE sample every 24 h intervals for 06-days following refrigerated centrifugation ($10,000 \times g$; 10 min; 4°C) as per the following equations:

$$\text{Decolorization (\%)} = A_i - A_t / A_i \times 100 \quad (1)$$

$$\text{Removal Efficiency (\%)} = C_i - C_t / C_i \times 100 \quad (2)$$

[Where A_i = initial absorbance at 0 h; A_t = final absorbance after reaction; C_i = initial concentration of contaminants (mg/L^{-1}) in the untreated (UT)-DIE; C_t = final concentration of contaminants (mg/L^{-1}) in bacterially treated (BT)-DIE after bioremediation]

2.8 Enzymatic studies

The activity of extracellular laccase enzyme was determined as outlined by Bourbonnais *et al.*, [33]. Briefly, the supernatant (crude enzyme extract, see section 2.7) of BT-DIE treatment was withdrawn every 24 h over 6-days during bioremediation studies. The crude enzyme extract (0.5 mL) was added to a mixture of acetate buffer (2.5 mL; 10 mM, pH 5) and guaiacol (2 mM; 4 mL) and incubated at 25°C for 2 h and then measured spectrophotometrically at $\lambda_{\text{max}} = 420$ nm. The enzyme activity was quantified and denoted as unit/mL/min of substrate oxidized per min/mL using the standard formula:

$$\text{UL}^{-1} \text{ min}^{-1} = \Delta Abs \times V/v \times \varepsilon \times T \quad (3)$$

[where ΔAbs = absorbance changes in per minute at specific wavelength; ε = extinction coefficient of each substrate at specific wavelength ($\text{mol}^{-1} \text{ cm}^{-1}$); V = final volume of the reaction mixture in mL; v = enzyme volume (mL), and T = reaction time in (min)]

2.9 Analytical methods

2.9.1 Physicochemical characterization of distillery effluent

The pollution strength of both UT-DIE and BT-DIE was determined in triplicate (mean $\pm$ SE; $n=3$), measuring several physicochemical characteristics (TDS, TSS, pH, BOD, COD, phenol, sulfate, phosphate, nitrate, total nitrogen, and HMs), as detailed in "Standard Methods for

Examination of Water and Wastewater" (APHA-AWWA-WPCF [34]). The acid digestion method [EPA Method No. 3005A; heating of 10 mL of a mixture of HNO₃ and HClO₄ (in a 6:1 proportion) at 85°C] was employed to detect and quantify HMs in DIE at the Thermo Scientific™ iCAP™ 7200 ICP-OES system (Thermo Fisher Scientific Inc., MA, USA).

2.9.2 Fourier transform-infrared (FT-IR) spectroscopic analysis

FT-IR spectroscopy was employed to examine the various functional groups contained in the toxic recalcitrant pollutants in the UT and BT-DIE by recording the absorption spectrum of the KBr pellet. The standard procedure to prepare the KBr pellet is outlined in Saxena *et al.* [35]. The absorption spectrum was recorded in the mid-IR range between 4000-400 cm⁻¹ and 4 cm⁻¹ resolution under normal air conditions against a pure KBr background spectrum at Thermo Scientific™ Nicolet™ 6700 FT-IR spectrometer (Thermo Fisher Scientific Inc., Massachusetts, USA). OMNIC™ software (v7.4) was used to process the spectral data and the absorption peaks in the FT-IR spectrum were consigned following baseline values detailed in Lambert [36].

2.9.3 Gas chromatography-mass spectrometry (GC-MS/MS) analysis

The UT and BT-DIE samples (50 mL each) were subjected to liquid-liquid extraction (LLE) using 50 mL of high-purity (≥ 99.9%) GC & HPLC grade dichloromethane (DCM) following the protocol by Saxena *et al.* [35], with slight modification. The sample extracts were dried using nitrogen flushing in GC vials and then derivatized with dioxane, pyridine, BSTFA (*N*, *O*-Bis (trimethylsilyl) trifluoroacetamide) & TMSCl (chlorotrimethylsilane). Analytical measurements were performed at Thermo Scientific™ TRACE™ 1300 TSQ™ Duo Triple Quadrupole GC-MS/MS platform manufactured by Thermo Fischer Scientific, USA. The GC-MS/MS instrument was equipped with Thermo Scientific™ TriPlus autosampler, DB-5 MS capillary column [phenyl (5%) + methylpolysiloxane (95 %); (30 m length, 0.18 mm I.D, 0.18 μm film thickness)] & TSQ Quantum XLS™ Triple Stage Quadrupole Mass Spectrometer

from Agilent Technologies, USA. Further, the filtered sample (2 μ L) was put into a GC column using an injection inlet, and the sample was analyzed to scan by a 3.0 min delay of the solvent with a mass range of m/z 50-600. Using helium (purity ≥ 99.99 %; flow rate: 1 mL/min⁻¹) as mobile phase, the mass spectra (MS) were recorded using standard energy (70 eV). The temperature of the column was raised from 60°C to 290°C over 20 min with an increase rate of 10°C per minute. The interpretation of MS data was done using the NIST library (v1.0.0.12), developed by the NIST, U.S., by comparing the MS of recalcitrant pollutants/degradation products with their retention times (RTs) for their identification.

2.10 Phytotoxicity Assessment

The ecotoxicity of the UT and BT-DIE was evaluated using mung bean, *Phaseolus aureus* L., a terrestrial model organism, as per the guidelines of OECD Environmental Safety (<http://www.oecd.org/chemicalsafety/testing/33653757.pdf>). To do so, 50 healthy *Phaseolus aureus* L. seeds were cleansed with tap water, sterilized using a 2.0% HgCl₂ solution to get rid of fungal contamination, and then washed three times with distilled water to get rid of any remaining sterilizing agent residue. Seeds were then positioned onto the Whatman Grade 52 filter-paper discs (England, UK) in petri dishes and flooded with the various volumes (25, 50, 75, & 100%) of the DIE (10 mL; v/v) alongside a control (tap water) to define its effect on the germination/growth of the seeds/seedlings. Moreover, seeds were given a 12 h dark/light cycle for 06-days at room temperature to evaluate and compute the dose-dependent impacts of DIE on seed germination/seedling growth parameters. All data were expressed in mean $\pm$ SE ($n=3$) and statistical comparisons were performed at a significance level of $p < 0.05$. Parameters such as shoot length (SL), root length (RL), germination percentage (GP), seedling mortality (SLM), germination index (GI), seed vigour index (SVI), and phytotoxicity percentage (PP) were calculated using the following formulas:

$$GP (\%) = \text{Number of Germinated Seeds} / \text{Total Number of Seeds Sown} \times 100 \quad (4)$$

$$\text{SLM (\%)} = \text{Number of Non-Germinated Seeds} / \text{Total Number of Seeds Sown} \times 100 \quad (5)$$

$$\text{GI (\%)} = \frac{\text{Total Seeds Germinated in Treatment} \times \text{Average RL in Treatment}}{\text{Total Seeds Germinated in Control} \times \text{Average RL in Control}} \times 100 \quad (6)$$

$$\text{SVI} = \text{Seedling Length} \times \text{GP} \quad (7)$$

$$\text{PP (\%)} = \frac{\text{SL of Control} - \text{SL of Treatment}}{\text{SL of Control}} \times 100 \quad (8)$$

2.11 Quality control and quality assurance

The quantification of HMs (Ni, Cu, Zn, Mn, Cd, Cr, Pb, and Fe) was calibrated in batch and calibrated using CRM multi-element plasma standard stock solution traceable to NIST (Thermo Fisher Scientific Inc., MA, USA). Three replicates of blanks were included to confirm the method accuracy.

2.12 Statistical analysis

The most recent versions of R Programming (v4.5.1) and RStudio (v2025.09.2-418) were used to statistically compute the data and plot the graphs. All the experiments were performed in triplicate ($n = 3$), and results are presented as mean $\pm$ SE. Independent t -tests were used to compare UT and BT-DIE for each physicochemical parameters and HMs. Effect sizes were determined using Cohen's d with 95% confidence intervals to evaluate the magnitude of the differences in these comparisons. Cohen's d (standardized mean difference) was calculated for each parameter as the difference between UT-DIE and BT-DIE means divided by the pooled standard deviation, with 95% confidence intervals. This was used to complement p -values by quantifying the magnitude (practical significance) of treatment effects and enabling unit-independent comparison of effect strength across multiple water-quality and metal parameters. Two-way ANOVA was used to assess the interaction effects of the treatment type and DIE concentration (25%, 50%, 75%, 100%) on the plant growth metrics such as RL and SL. Tukey's post hoc test ($p \leq 0.05$) was applied to determine pairwise differences among factor levels.

Significant differences ($p < 0.05$) were denoted by different letters. Lowercase letters denote comparisons among concentrations within a treatment, while uppercase letters indicate comparisons among different treatment types and the control.

3. Results & Discussion

3.1 Characteristics of bacterial strains

In the present study, among the 10 isolated bacterial strains (HIM01-HIM10), only HIM03 was able to produce laccase enzyme. This was evidenced by the dark brown coloration of the bacterial colony brought on by the catalytic oxidation of guaiacol, which was used as a phenolic substrate [1]. Morphologically, the colony of strain HIM03 was off-white in color, circular in shape, with a firm texture, shiny surface, flat elevation, curled edges, and an opaque appearance. Strain HIM03 was recognized as a motile, rod-shaped and Gram-positive bacterium as confirmed by Gram staining. Biochemically, strain HIM03 showed positive results for malonate, Voges Proskauer's, nitrate reduction, catalase, sucrose, mannitol, and glucose. However, it showed negative results for citrate utilization, ONPG, arginine, arabinose, and trehalose. Strain HIM03 was identified as *Bacillus velezensis* HIM03 (GenBank Accession Number: OP659023) following 16S rRNA gene sequencing. According to BLAST search, strain HIM03 has 99.92% similarity index with *Bacillus velezensis* SN06 among the available sequences. The phylogenetic tree representing evolutionary linkages is presented in supplementary material Fig. S1. This laccase-producing, *Bacillus velezensis* HIM03, may be used to treat and detoxify DIE since the laccase enzyme targets phenolic compounds, which are the major contaminants in DIE. Our findings are strengthened by preceding investigations highlighting the bioremediation potential of *Bacillus velezensis* against various environmental contaminants and industrial waste [37-39].

3.2 Effects of different process parameters on bioremediation of distillery effluent

Notable differences in COD removal efficiency were observed when bacterial performance was evaluated under different process parameters. Among the additional C sources tested, glucose (0.5% w/v) proved to be the most effective, allowing *Bacillus velezensis* HIM03 to achieve a maximum COD reduction of 77.55% in DIE. In contrast, other C sources, including sucrose, maltose, lactose, and starch, resulted in lower COD removal, ranging from 67.29% down to 34.91% (Fig. 1A). Glucose outperformed other sugars due to its rapid assimilation and central role in glycolysis. This pathway generates readily available energy and reducing equivalents needed for enzymatic activity, such as laccase production. Peptone emerged as the most efficient N supplements, enabling *Bacillus velezensis* HIM03 to achieve a peak COD reduction of 78.45% in DIE. In contrast, other nitrogen sources, such as yeast extract, urea, ammonium chloride, and sodium nitrate, supported lower COD removal, ranging from 68.18% to 53.64% (Fig. 1B). Peptone surpassed other N supplements and sugars due to its rich blend of peptides and simple amino acids. These nutrients supported vigorous bacterial growth and likely enhanced microbial metabolism and enzymatic activity required for degrading recalcitrant organics in DIE.

Among the tested pH values (pH 6-10), pH 8 emerged as the most favourable, yielding the highest COD reduction efficiency (79.68%) in DIE compared to pH 6, 7, 9 & 10 removed COD in range of 62.87% to 46.89% (Fig. 1C). Enhanced performance at pH 8 can be attributed to increased bacterial cell membrane stability and nutrient uptake, which promote growth and maintain sufficient biomass for consistent enzymatic. This likely enhanced the structural stability and catalytic efficiency of laccase enzyme, enabling effective degradation of recalcitrant organics such as melanoidin, phenolics, and lignin. However, the reduced COD removal at pH 6 likely resulted from the acidic conditions that lowered enzyme production or altered active sites, reducing catalytic efficiency. Additionally, acidic stress usually weakens

cell membranes and slows microbial metabolism, resulting in suboptimal pollutant degradation. Furthermore, 35°C was the most efficient temperature, yielding the highest COD reduction (80.72%) in DIE, whereas the lowest reduction (62.34%) occurred at 25°C (Fig. 1D). At 35°C, optimal enzyme kinetics and membrane fluidity improved bacterial metabolism, nutrient uptake, and waste efflux. During the day, ambient temperatures generally fall between 35-37°C, which closely aligns with the mesophilic range favoured by most *Bacillus* species. This also promotes strong expression of degradative enzymes like laccase, which are crucial for degrading melanoidin and other recalcitrant organics in DIE. However, higher temperatures (40-45°C) may have caused thermal stress, leading to enzyme denaturation or disruption of cellular processes. In contrast, lower temperatures (25-30°C) led to slower bacterial growth and reduced enzyme activity.

A 10% inoculum among the others produced the largest COD reduction (81.58%) in DIE (Fig. 1E). This was most likely due to the higher initial cell density, which enhanced enzymatic activity, particularly laccase production, and accelerated microbial metabolism. This, in turn promoted efficient utilization and faster degradation of complex pollutants such as polyphenols, melanoidin, and organo-metallic compounds. Conversely, lower inoculum levels likely slowed microbial adaptation and reduced metabolic activity, subsequently delaying substrate utilisation. Additionally, agitation at 120 rpm was the most effective among the others, achieving the highest COD removal (82.91%) in DIE (Fig. 1F). This likely ensured uniform mixing, optimal aeration, and efficient interaction between DIE pollutants and bacterial cells. Moreover, this likely maintained stable dissolved oxygen levels, further enhancing the oxidative activity of laccase for degrading complex organics. Oppositely, higher agitation (130-140 rpm) could have mechanically damaged bacterial cells or disrupted enzyme production, while lower agitation (100 rpm) probably limited oxygen availability and reduced microbial activity. Thus, the optimal conditions for reducing COD in UT-DIE using the novel

laccase-producing *Bacillus velezensis* were pH 8, 35 °C, 120 rpm, 10% (10 mL) inoculum volume, and 0.5% w/v glucose and peptone.

3.3 Bioremediation of distillery effluent at optimized conditions

The novel laccase-producing *Bacillus velezensis* HIM03 effectively decolourized (80.28%) and removed COD (84.03%) from UT-DIE in 144 h under previously defined optimal conditions (Fig. 1G). These results validate its outstanding potential as a bioaugmentation agent for DIE treatment. Bacterial growth increased over time, accompanied by a parallel rise in decolorization up to 120 h. This indicates that decolorization is directly linked to active bacterial growth and metabolic activity rather than occurring randomly. This likely results from the laccase-producing *Bacillus velezensis* HIM03 actively using RCCs such as melanoidin, polyphenols, and organo-metallic pollutants as nutrient sources [1, 21, 40]. Biodegradation mechanism likely reduced DIE toxicity and its color by breaking conjugated structures and oxidizing aromatic rings. The performance of *Bacillus velezensis* HIM03 offers a novel and extremely effective bacterial contender that hasn't hitherto been described for DIE bioremediation. Further, the ability of *Bacillus velezensis* HIM03 to function in both aerobic and mesophilic environments suggests its suitability for real-time wastewater treatment systems. It could also be applied in facultative lagoons or aerobic bioreactors, offering a cost-effective and eco-friendly alternative to chemical treatments. This bacterial bioremediation system offers a sustainable alternative that avoids secondary pollution from physicochemical treatment methods like ozonation or coagulation-flocculation. Our findings closely align with those of previous studies [1, 6, 21, 40].

3.4 Enzymatic Profile

The bioremediation of UT-DIE by the novel laccase-producing *Bacillus velezensis* HIM03 under optimal conditions exhibited no detectable extracellular laccase activity at 12 h (Fig. 1H). This suggests a slow initial degradation phase due to minimal enzyme production.

However, the increased laccase activity after 24 h is likely due to phenolic and heterocyclic contaminants in UT-DIE acting as substrates for bacteria. A peak laccase activity of 6.18 IU/mL/min at 96 h corresponded with the highest decolorization and COD reduction in BT-DIE. This suggests active microbial metabolism of both phenolic and non-phenolic substances, including recalcitrant melanoidin, which may induce laccase gene expression (Fig. 1H). After 144 h, laccase activity in BT-DIE steadily declined, indicating pollutant biodegradation and biologically regulated enzyme production. No laccase activity was found in the control flasks without bacterial culture. Our findings align with previous studies confirming bacterial laccase's potential in remediating melanoidin and industrial effluents, including DIE [1, 8, 31].

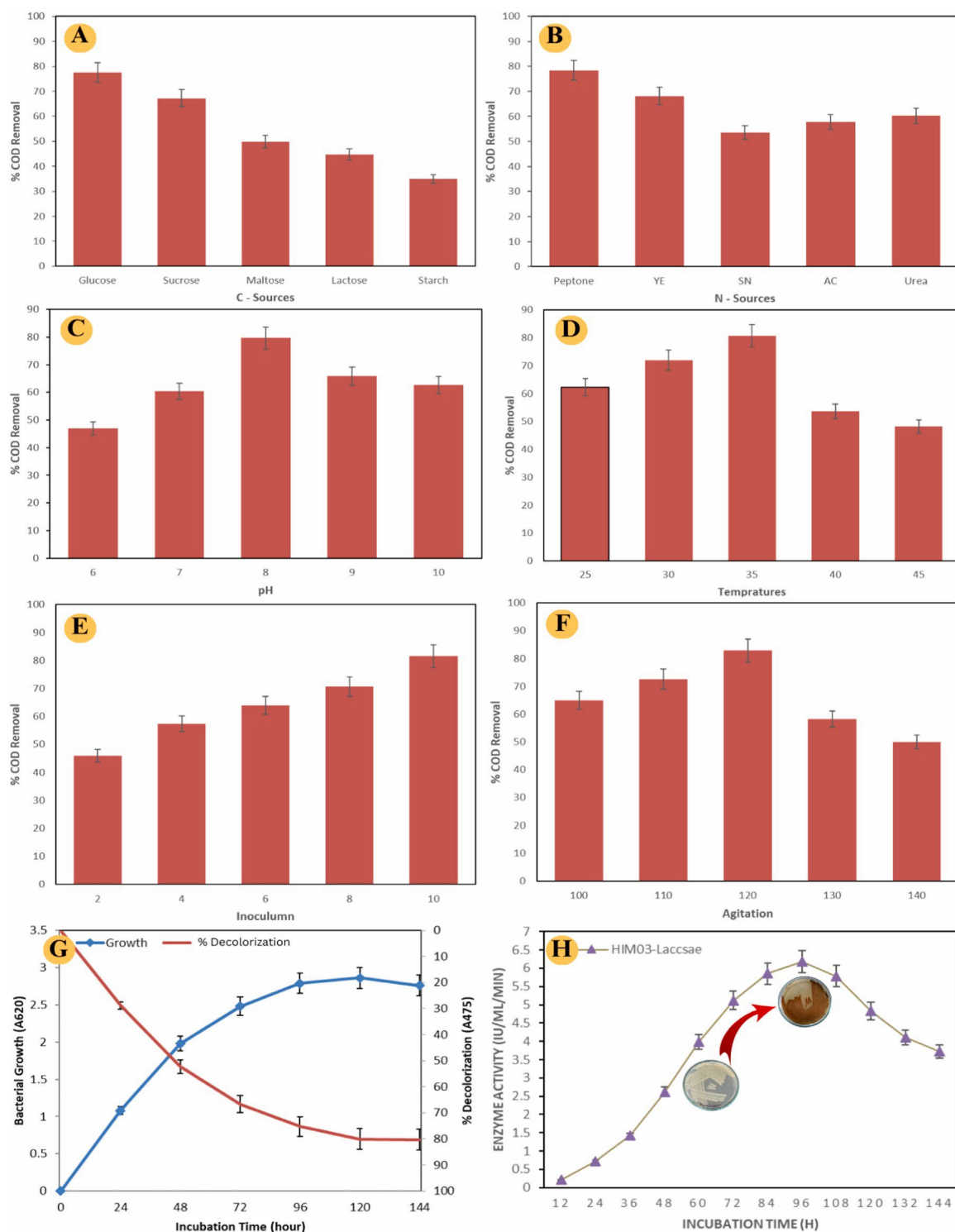


Fig. 1. Effect of (A) C sources (0.5%, w/v), (B) N sources (0.5%, w/v), (C) pH, (D) temperature (°C), (E) inoculum concentration (mL), (F) agitation/shaking speed (rpm) on COD removal from DIE, (G) DIE decolorization, and (H) activities of laccase during treatment with the novel *Bacillus velezensis* HIM03. The standard error (SE) derived from at least three independent experiments carried out under optimal situations (pH 8, 35 °C, 120 rpm, 10 mL inoculum volume, and 0.5%, w/v glucose and peptone) is shown by the error bars (mean $\pm$ SE, $n = 3$).

3.5 Characteristics of distillery effluent

The DIE was extremely polluted because almost all the contamination parameters significantly exceeded the discharge limits of CPCB [41] (Fig. 2). However, the studied contamination parameters were considerably reduced with significant differences ($p \leq 0.05$), following treatment with the novel laccase-producing, *Bacillus velezensis* HIM03 within 144 h at the optimal conditions (Fig. 2A & B): COD (84.02%), BOD (87.00%), TDS (74.99%), TSS (70.76%), phosphate (69.27%), sulfate (63.17%), nitrate (71.01%), phenol (74.03%), and HMs including Zn (85.93%), Cu (78.57%), Mn (75.26%), Fe (66.00%), Ni (60.97%), Cd (50.00%), Cr (40.62%), and Pb (72.73%). The dark brown color, pH (8.41 ± 0.11) and electrical conductivity (EC: 3.26 ± 0.01) were changed/or significantly reduced to light brown color, 7.43 ± 0.08 , and 2.12 ± 0.02 , respectively. This reduction in DIE contamination parameters was mainly attributed to HM-biotransformation *via* cellular accumulation or interaction with exopolysaccharides, together with degradation of RCCs, including melanoidins [21]. This novel laccase-producing *Bacillus velezensis* HIM03 demonstrated superior performance and strong competitiveness compared to previous bacterial strains reported for the treatment and detoxification of DIE (Table 1).

Table 1. Bacterial strains reported for the remediation of DIE.

Sr. No.	Bacterial Strain	Type of Biocatalyst	Target Contaminants	Remarks	Reference
01.	<i>Bacillus albus</i> VKDS9	Laccase, LiP, MnP	COD (35.98%)	Future scale-up studies are not recommended	Kumar et al., [1]
02	<i>Pseudomonas aeruginosa</i> SRRBL1	Enzyme characterization was not performed in this study	COD (61.32%)	Further investigation into augmented phytoremediation of DIE is recommended	Ratna and Kumar [6]
03	<i>Staphylococcus saprophyticus</i> + <i>Alcaligenaceae</i> sp.	Enzyme characterization was not performed in this study	COD (81.85%)	Future scale-up studies are not recommended	Chowdhary et al., [4]
04.	<i>Bacillus velezensis</i> HIM03	Laccase	COD (84.03%)	Future scale-up studies are suggested	This study

Further, the bacterial bioremediation of DIE was statistically significant ($p < 0.05$) and substantially affected all the contamination parameters between the treatment (BT-DIE) and control group ([UT-DIE) underscored by the independent t -test (Fig. 2A & B) and Cohen's d effect sizes (Fig. 2C & D). The high effectiveness of the bioremediation process was confirmed by the extremely large negative Cohen's d effect sizes of water quality parameters like COD ($d = -3305$), BOD ($d = -1671$), TDS ($d = -1659$), phenol ($d = -485$), and TSS ($d = -459$), which were reinforced by a dramatic decrease in contaminants and solid loads. Large to moderate impact sizes were also demonstrated by other variables, like phosphate ($d = -294$), sulfate ($d = -135$), and nitrate ($d = -78.0$), indicating efficient nutrient removal. Even variables that normally exhibit little variation, such as pH ($d = -5.57$), EC ($d = -34.2$), and temperature ($d = -2.30$), were statistically significant. However, they had comparatively minor effects, likely indicating a shift towards optimal environmental conditions. The accuracy and consistency of the treatment were demonstrated by the narrow confidence intervals for most of the variables. Statistical analysis revealed strong treatment effects, demonstrating that the observed improvements are statistically, ecologically, and practically significant.

Additionally, the low p -values and narrow confidence intervals further confirmed the exceptionally large effect sizes observed for HMs such as Fe ($d = -55.4$), Mn ($d = -43.2$), and Zn ($d = -22.8$), which reflect strong, precise, and reliable detoxification performance. Statistically significant ($p < 0.05$) reduction in Cu ($d = -13.3$) and Ni ($d = -12.1$) compared to Cd ($d = -4.62$) and Pb ($d = -3.68$) with relatively smaller effect sizes showed the efficacy of the newly developed bioremediation process in detoxifying HM-laden DIE. These effect sizes indicate that the biological process is environmentally relevant, not merely statistically significant. Moreover, the observed differences, as indicated by distinct statistical groupings (lowercase letters: a and b), highlight the pronounced metal-sequestration and biotransformation potential of *Bacillus velezensis* HIM03 (Fig. 2B). Nevertheless, Cr did not

exhibit any statistically significant change, as indicated by a higher p -value (0.0975) and an effect size (d) of -1.76. This suggests a less robust or more varied response to the newly developed bioremediation process for this metal. Together, these results demonstrate the evident potential of the developed bioremediation process to substantially improve the physicochemical quality of DIE for pollution prevention and control. Statistical analysis strongly validates that *Bacillus velezensis* HIM03 induced highly significant and practically meaningful reductions in physico-chemical parameters in UT-DIE. This validates the efficiency, reproducibility, and ecological relevance of the developed bioremediation process.

3.6 Characterization of recalcitrant coloring contaminants and their degradation products

The FT-IR and GC-MS/MS procedures were used to describe recalcitrant pollutants and their degradation products after DIE bioremediation using the novel laccase-producing *Bacillus velezensis* HIM03. Typically, a FT-IR spectrum of UT & BT-DIE displayed many intense peaks at different wave numbers (Fig. 3), like 3405 cm^{-1} , a absorption band signified -OH stretching in phenolic and alcoholic functional groups; 1652 cm^{-1} indicated carbonyl (C=O) stretching coupled with NH_2 deformation in primary amides; 1565 cm^{-1} confirmed N-H bending in secondary amides; 1407 cm^{-1} designated C-N stretching in amides; 1127 cm^{-1} specified C-NH_2 deformation in primary amines; 830.7 cm^{-1} represented aromatic C-H bending of benzene derivatives; 626.9 cm^{-1} demonstrated C-OH bending vibrations, and 547.5 cm^{-1} identified NO_2 in aromatic nitro compounds. A moderately weak band at 2962 cm^{-1} directed to aliphatic C-H stretching in methyl and methylene groups was also noted. Furthermore, a faint peak observed at 1238 cm^{-1} may be due to C-O-C stretching in esters. As a result, UT-DIE appears to contain phenols, alcohol, surfactants, amines, carboxylic acids, benzene, and aromatic skeleton, according to FT-IR study data (Fig. 3).

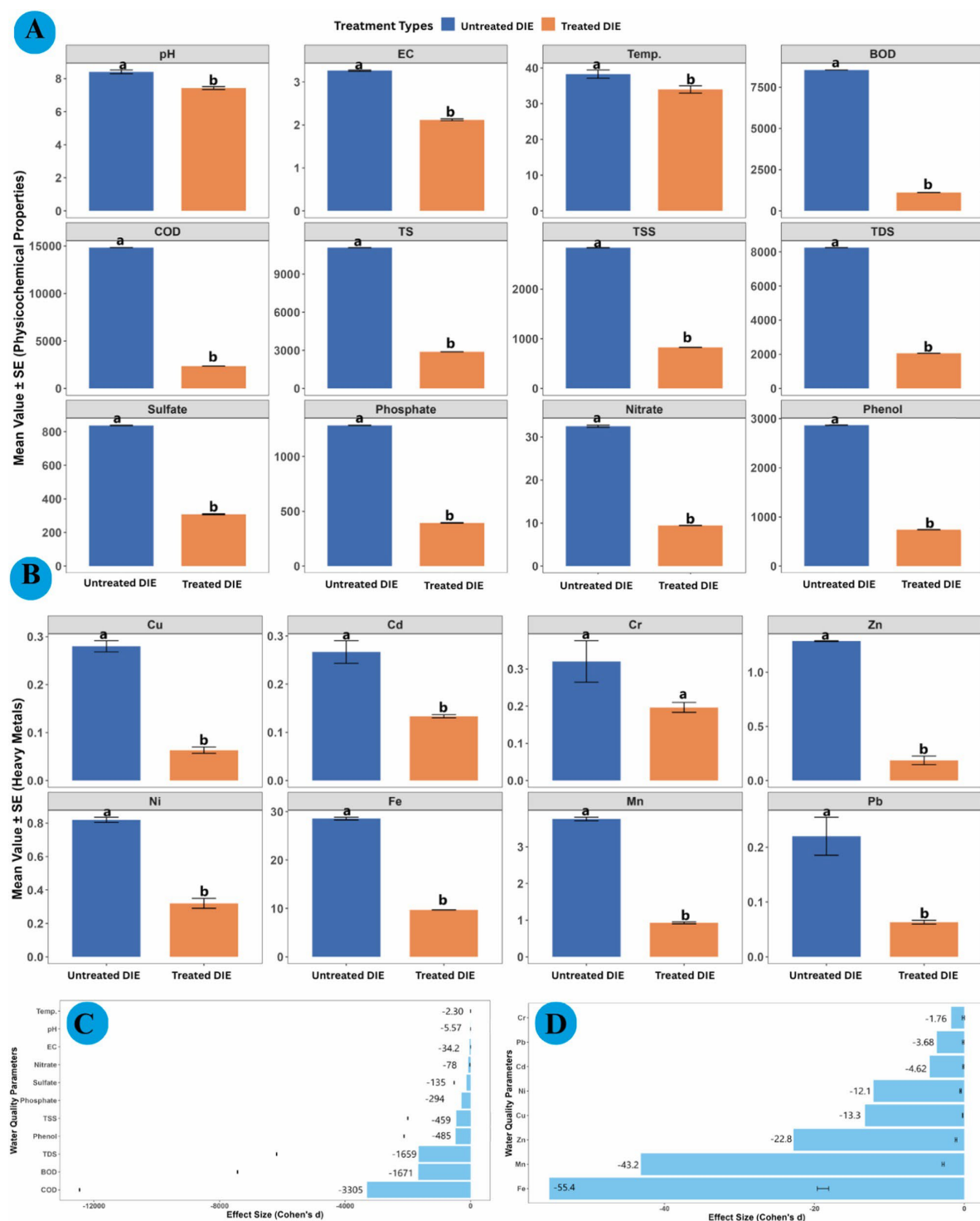


Fig. 2. Comparison of water quality parameters between untreated (UT) and bacterially-treated (BT)-DIE. (A) physicochemical parameters [all were measured in mg/L except pH (dimensionless quantity), EC (mS/cm), Temperature (°C)], (B) HM concentrations (mg/L), (C) Cohen's *d* effect sizes for physicochemical parameters, and (D) Cohen's *d* effect sizes for heavy metals. Independent *t*-tests were performed to determine significant differences between treatment groups ($p \leq 0.05$), and effect sizes (Cohen's *d*) with 95% confidence intervals were calculated to assess the magnitude of differences. In panels C and D, the respective Cohen's *d* values are displayed alongside each parameter for clarity. Different lowercase letters (a & b) above bars indicate statistically significant differences between treatments for each parameter. All the values were mean $\pm$ SE ($n = 3$).

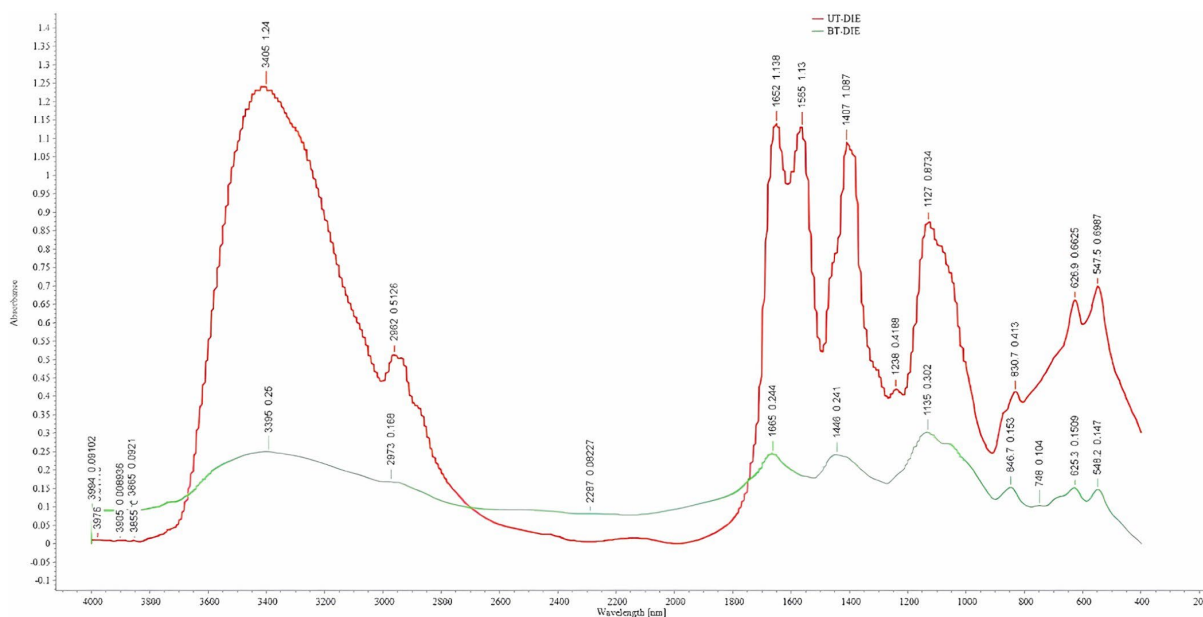


Fig. 3. A FT-IR spectrum of untreated (UT) and bacterially-treated (BT, treatment with the novel *Bacillus velezensis* HIM03)-DIE. The FI-IR spectrum was plotted and superimposed on F. Menges “SpectraGryph” Software (v1.2.16) available at <http://www.effemm2.de/spectragryph/>.

Notable spectral alterations were observed, characterized by the disappearance of multiple peaks associated with UT-DIE and the emergence of new peaks in BT-DIE at various wavenumbers (Fig. 3). A weak absorption at 2973 cm^{-1} corresponded to aliphatic C–H stretching vibrations of CH_3 and CH_2 groups, while a very weak band at 748 cm^{-1} was suggestive of out-of-plane C–H bending of ortho-disubstituted aromatic rings. Additionally, a weak band at 625.3 cm^{-1} may be associated with ester-related skeletal or O–C–O bending vibrations. Newly emerged prominent peaks in BT-DIE included 3395 cm^{-1} , corresponding to O–H stretching; 1665.4 cm^{-1} , attributed to alkenic C=C stretching; 846.7 cm^{-1} , associated with trisubstituted alkene deformations; and 548.2 cm^{-1} , ascribed to aldehydic C–C=O stretching. Moreover, a medium-intensity band at 1446 cm^{-1} suggested carboxylic acid O–H bending, while a dominant peak at 1135 cm^{-1} corresponded to aliphatic ether C–O–C asymmetric stretching. Furthermore, a very faint peak observed at 2287 cm^{-1} may be due to the presence of weak nitrile ($\text{C}\equiv\text{N}$) stretching vibrations. Collectively, these spectral alterations reflect

chemical transformations arising from bacterial metabolism of recalcitrant pollutants present in UT-DIE and are corroborated by prior studies [1, 6, 23].

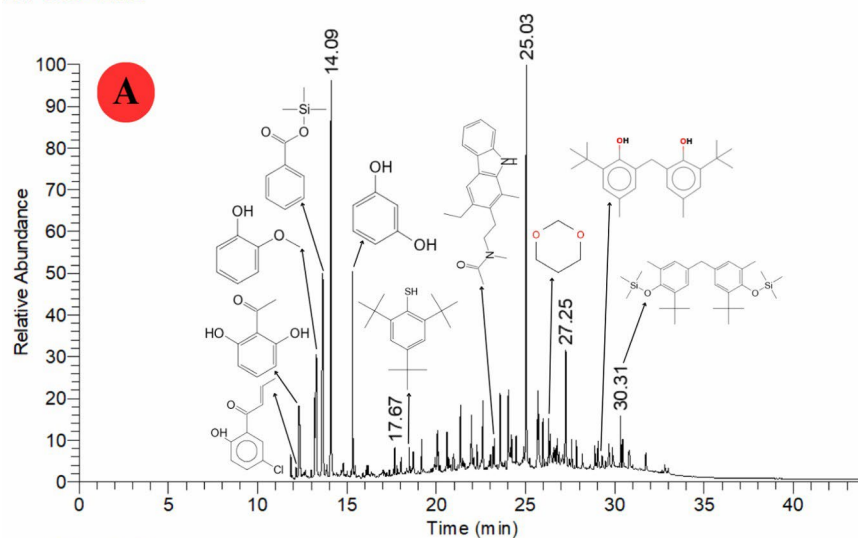
Additionally, a few weak absorption bands were detected in the extreme high-wavenumber region, appearing around 3976, 3905, and 3855 cm^{-1} in UT-DIE and at 3994 and 3865 cm^{-1} in BT-DIE in the FT-IR spectrum (Fig. 3). These peaks are largely the result of atmospheric and instrumental interference (noise) rather than true chemical absorptions and, were therefore excluded from further consideration. These interferences arise mainly from atmospheric water vapour and carbon dioxide, reduced detector sensitivity at high wavenumbers, imperfect background correction, and scan-to-scan instability during liquid effluent analysis, which together generate spurious, non-chemical peaks in the 3700-4000 cm^{-1} region. Further, FT-IR findings were validated by GC-MS/MS analysis. This revealed multiple peaks at various retention times (RTs) corresponding to various toxic organic pollutants in IT-DIE, including surfactants, phenols, endocrine disruptors, and aquatic toxicants (Fig. 4A & Table 2). However, following treatment with the novel laccase-producing *Bacillus velezensis* HIM03, these compounds were substantially reduced or transformed into environmentally safe, non-toxic metabolites in BT-DIE (Fig. 4B & Table 2), indicating effective DIE treatment and detoxification.

3.7 Phytotoxicity of distillery effluent

Following irrigation with UT-DIE (25-100%; v/v), *Phaseolus aureus* L. showed a marked reduction in all physiological indicators linked to seed germination/seedling growth compared to the control (TP: tap water) and thus, revealed potential toxicity of UT-DIE (Table 2). However, the toxic effects are governed by the type of crops to be irrigated and the concentration of UT-DIE. A decrease in all physiological markers linked to seed germination and seedling growth was likely due to high concentrations of toxic organics, HMs, and organo-metallic contaminants, COD, TDS, TSS, and salt load in UT-DIE [1]. This was probably due

to the altered functioning of growth-promoting hormones and enzymes, including cytokinins, gibberellins, auxins, and amylases. In addition, the development of anaerobic conditions and high osmotic pressure together hampered physiological indicators. However, across the concentration gradient (25, 50, 75 and 100% v/v), the seeds' irrigation with BT-DIE consistently promoted/improved seedling growth, with significantly higher SL & RL than UT-DIE (two-way ANOVA with Tukey's post hoc test, $p \leq 0.05$; Table 3, Fig. 5A–B).

RT: 0.00 - 44.12



RT: 0.00 - 44.09

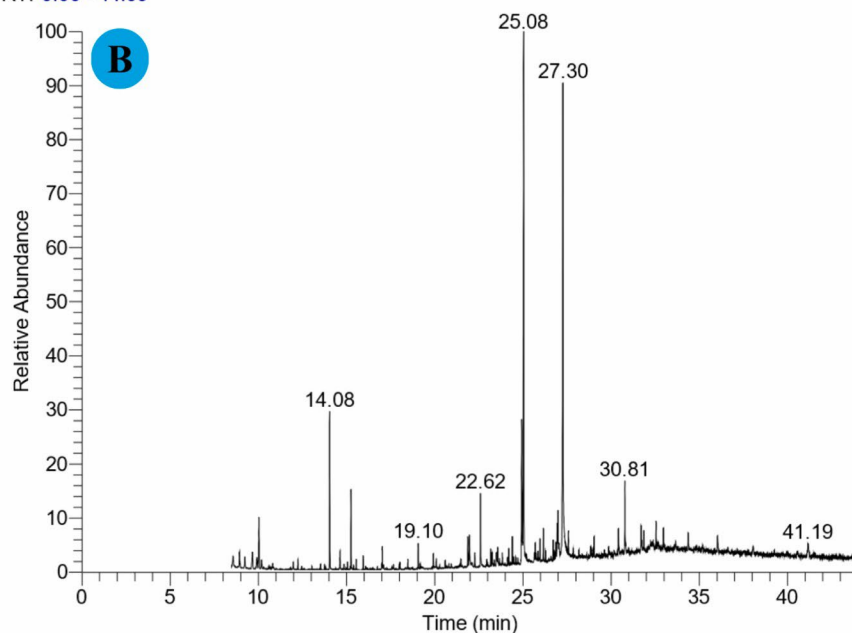


Fig. 4. GC-MS chromatogram of DIE before (A) untreated and after (B) treatment with the novel *Bacillus velezensis* HIM03

Table 2. Organic pollutants and their degradation products identified as TMS derivatives by GC-MS analysis of UT and BT-DIE.

Sr. No.	RT (min)	Compounds Identified	Molecular Formula	Molecular Weight	Molecular Structure	Toxicological Information	Distillery effluent	
							UT-DIE	BT-DIE
01.	12.29	4-Chloro-2-crotonylphenol	$C_{10}H_7ClO_2$	196		Aquatic Toxicant, Endocrine Disruptor	+	-
02.	12.33	2,6-Dihydroxyacetophenone, 2TMS derivative	$C_{14}H_{24}O_3Si_2$	296		Skin, Eyes & Respiratory Irritant	+	-
03.	13.28	Guaiacol, TBDMS derivative	$C_{10}H_{12}O_2Si$	238		Skin & Eyes Irritant; Aquatic Toxicant	+	-
04.	13.64	Benzoic Acid, TMS derivative	$C_{10}H_{14}O_2Si$	194		Skin, Eyes & Respiratory Irritant	+	-
05.	14.09	Glycerol, 3TMS derivative	$C_{12}H_{22}O_5Si_3$	308		Mild Skin and Eyes Irritant	+	-
06.	15.31	1,3-Benzenediol, 2TBDMS derivative	$C_{14}H_{24}O_3Si_2$	338		Skin, Eyes & Respiratory Irritant; Endocrine Disruptor	+	-
07.	17.67	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{34}$	240		Skin and Eyes Irritant; Asphyxiant; Acute Toxicant	+	-
08.	18.50	2,4,6-Tri- <i>t</i> -butylbenzenethiol	$C_{18}H_{24}S$	278		Skin, Eyes & Respiratory Irritant; Acute Toxicant	+	-
09.	19.17	Hexadecane	$C_{16}H_{34}$	226		Skin, Eyes & GI-tract Irritant; Acute Toxicant; Asphyxiant; CNS depressant	+	-
10.	20.06	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{25}H_{54}$	366		Acute Toxicant; Skin and Eyes Irritant	+	-
11.	20.60	Methyl glycocholate, 3TMS derivative	$C_{18}H_{36}NO_5Si_3$	695		Skin, Eyes & Respiratory-tract Irritant	+	-
12.	21.48	tert-Hexadecanethiol	$C_{16}H_{34}S$	258		Skin, Eyes & Respiratory-tract Irritant	+	-
13.	22.09	17-Pentatriacontene	$C_{35}H_{70}$	490		No information available	+	-
14.	22.60	Pentadecanoic acid, TMS derivative	$C_{18}H_{36}O_2Si$	314		Skin, Eyes & Respiratory Irritant; Acute Toxicant	+	-
15.	23.24	Acetamide, N-[2-(3-ethyl-1-methyl-9H-carbazol-2-yl)ethyl]-N-methyl-	$C_{28}H_{34}NO$	308		Carcinogen; Skin & Eyes Irritant; Acute Toxicant	+	-
16.	23.57	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270		Skin, Eyes & Respiratory Irritant; Acute Toxicant	+	-
17.	24.03	L-(+)-Ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{76}O_8$	652		Nausea and Diarrhea (in excess)	+	-
18.	25.03	Tridecanoic acid, TMS derivative	$C_{16}H_{32}O_2Si$	286		Skin, Eyes & Respiratory Irritant	+	-
19.	25.70	Fumaric acid, 2-ethylhexyl tridec-2-yn-1-yl ester	$C_{25}H_{42}O_4$	406		Skin and Eye Irritant; abdominal cramps, diarrhea and nausea	+	-
20.	26.29	1,3-Dioxane, 4-(hexadecyloxy)-2-pentadecyl-	$C_{25}H_{50}O_2$	538		Skin, Eyes & Respiratory Irritant; Acute Toxicant	+	-
21.	27.25	Nonadecanoic acid, TMS derivative	$C_{22}H_{44}O_2Si$	370		Skin and Eye Irritant	+	-
22.	29.29	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	$C_{20}H_{22}O_2$	340		Skin and Eye Irritant; Acute Toxicant; Reproductive and Developmental Toxicant	+	-
23.	30.31	Benzene, 1,1'-methylenebis[3-(1,1-dimethylethyl)-5-methyl-4-[(trimethylsilyl)oxy]-	$C_{29}H_{40}O_2Si_2$	484		Skin Burns and Eye Damage	+	-
24.	10.08	D-(-)-Lactic acid, 2TMS derivative	$C_5H_{10}O_3$	90		Nontoxic	-	+
25.	14.08	Glycerol, 3TMS derivative	$C_{12}H_{22}O_5Si_3$	308		Nontoxic	-	+
26.	14.67	Butanedioic acid, 2TMS derivative	$C_{10}H_{20}O_4Si_2$	262		Nontoxic but Mild Skin, Eye, and Respiratory Irritant	-	+
27.	15.30	1-Hexadecanol	$C_{16}H_{34}O$	242		Nontoxic	-	+
28.	17.07	Heptadecane	$C_{17}H_{36}$	240		Nontoxic	-	+
29.	19.10	1-Octadecanol	$C_{18}H_{38}O$	270		Nontoxic	-	+
30.	22.00	1,4-Benzenedicarboxylic acid, ethyl trimethylsilyl ester	$C_{24}H_{44}O_4$	166		Nontoxic	-	+
31.	22.62	Myristic acid, TBDMS derivative	$C_{20}H_{40}O_2Si$	342		Nontoxic	-	+
32.	24.44	1-Docosene	$C_{22}H_{44}$	308		Nontoxic	-	+
33.	25.08	Palmitic Acid, TMS derivative	$C_{18}H_{36}O_2Si$	328		Nontoxic	-	+
34.	26.19	Heptadecanoic acid, TBDMS derivative	$C_{20}H_{40}O_2Si$	384		Nontoxic	-	+
35.	27.30	Stearic acid, TBDMS derivative	$C_{22}H_{44}O_2Si$	398		Nontoxic	-	+
36.	30.45	Cyclodecasiloxane, eicosamethyl-	$C_{20}H_{40}O_{10}Si_{10}$	741		Nontoxic	-	+
37.	30.81	1-Monopalmitin, 2TMS derivative	$C_{35}H_{70}O_4Si_2$	474		Nontoxic	-	+
38.	32.58	Glycerol monostearate, 2TMS derivative	$C_{27}H_{54}O_4Si_2$	502		Nontoxic	-	+
39.	34.41	Heptasiloxane, hexadecamethyl-	$C_{16}H_{36}O_7Si_7$	533		Nontoxic	-	+
40.	36.06	Octasiloxane, hexadecamethyl-	$C_{16}H_{36}O_8Si_8$	577		Nontoxic	-	+
41.	41.19	α -Sitosterol, TMS derivative	$C_{27}H_{48}OSi$	486		Nontoxic	-	+

Footnote: +: present; -: absent; TMS: trimethyl silyl; RT: retention time; UT: untreated; BT: bacterially treated; DIE: distillery industry effluent

Table 3. Phytotoxicity of DIE before and after bacterial treatment

DIE Type (%)	GP (%)	SLM (%)	GI (%)	SVI	PP (%)
UT-DIE (25%)	68.00	32.00	28.86	391.907	37.61
BT-DIE (25%)	82.00	18.00	72.71	666.933	26.83
UT-DIE (50%)	54.00	46.00	27.00	295.2	45.85
BT-DIE (50%)	65.33	34.66	52.39	468.222	36.59
UT-DIE (75%)	38.00	62.00	15.77	145.667	65.37
BT-DIE (75%)	46.66	53.33	29.49	262.889	50.24
UT-DIE (100%)	32.00	68.00	4.528	33.0667	92.2
BT-DIE (100%)	38.00	62.00	19.71	181.133	57.07
Control (TP)	100.00	0.00	100.00	1036.67	0.00

Footnote: All the values are mean of triplicates ($n = m$) $\pm$ SE; DIE: Distillery industry effluent; UT-DIE: Untreated DIE; BT-DIE: Bacterially treated DIE; TP: Tap water (control); GP: Germination percentage; SLM: Seedling mortality; GI: Germination index; SVI: Seed vigour index; PP: Phytotoxicity percentage

In contrast to the suppressive impacts of UT-DIE, irrigation with different volumes of the BT-DIE also resulted in a significantly higher SL and RL at the same concentrations, as evidenced by different letter groupings (Fig. 5A-B). This statistical separation confirms that bioremediation significantly mitigated the phytotoxic effects of DIE. At lower concentrations (25-50%), BT-DIE values were statistically closer to the control, particularly for SL, indicating partial recovery of normal plant growth. The seedlings irrigated with UT-DIE showed reduced and altered growth of SL (4.27 ± 0.30 , 3.70 ± 0.12 , 2.37 ± 0.34 , and 0.53 ± 0.03 cm). In contrast, SL increased to 5.00 ± 0.50 , 4.33 ± 0.09 , 3.40 ± 0.26 , and 2.93 ± 0.29 cm in the seedlings irrigated with BT-DIE at 25-100% (v/v). The growth-promoting effects of BT-DIE on seedling development were also evident in the RL. Seedlings irrigated with UT-DIE showed RL values of 1.50 ± 0.29 , 1.77 ± 0.19 , 1.47 ± 0.26 , and 0.50 ± 0.12 cm, whereas those treated with BT-DIE exhibited corresponding increases of 3.13 ± 0.13 , 2.83 ± 0.17 , 2.23 ± 0.15 , and 1.83 ± 0.09 cm at 25-100% (v/v). The control (TP without DIE) consistently recorded the

highest RL and SL, indicated by the distinct letter 'A,' confirming optimal growth under non-stressed conditions and establishing a reference standard for plant performance. Statistical groupings (different letters, two-way ANOVA and Turkey post-hoc through pair-wise comparison) further confirm that differences among treatments and concentrations were significant. This emphasizes the strong phytotoxicity of UT-DIE and the substantial mitigation achieved through the developed bioremediation process (Fig. 5A-B). Further, the relatively small SE bars across treatments (UT-DIE & BT-DIE, TP) indicate low variability among replicates and high experimental precision. The clear statistical stratification among treatments and DIE concentrations underscores the robustness of the experimental design. It confirms that the observed improvements in plant growth were not due to random variation but were a direct consequence of bioremediation.

The production of non-toxic metabolites (Table 1) during the degradation of recalcitrant contaminants in UT-DIE with the novel laccase-producing *Bacillus velezensis* HIM03 likely contributed to a remarkable reduction in the phytotoxicity of BT-DIE by 92.2% at 100%. This also led to improvements in all physiological parameters, reinforcing the effectiveness of the bioremediation process. Moreover, this was further supported by the presence of certain metabolites in BT-DIE that mitigated the phytotoxic effects of UT-DIE (Table 1). These include sitosterol (RT 41.19), which enhances root development and stress tolerance like phytohormones [42]; butanedioic acid (RT 14.67), which promotes plant growth [43]; and lactic acid (RT 10.08), known to alleviate phytotoxicity [44]. As a result, BT-DIE consistently outperformed UT-DIE at concentrations ranging from 25 to 50% (v/v). This indicates that, when appropriately diluted, it could be utilized as a non-toxic liquid fertilizer for irrigation purposes. Conclusively, the statistical analysis strongly validates that bacterial treatment of DIE significantly and reproducibly reduced phytotoxicity, with clear concentration-dependent effects distinguished among UT-DIE, BT-DIE, and control treatments. These results provide

strong quantitative evidence supporting the safe reuse of appropriately diluted biotreated effluent for agricultural applications.

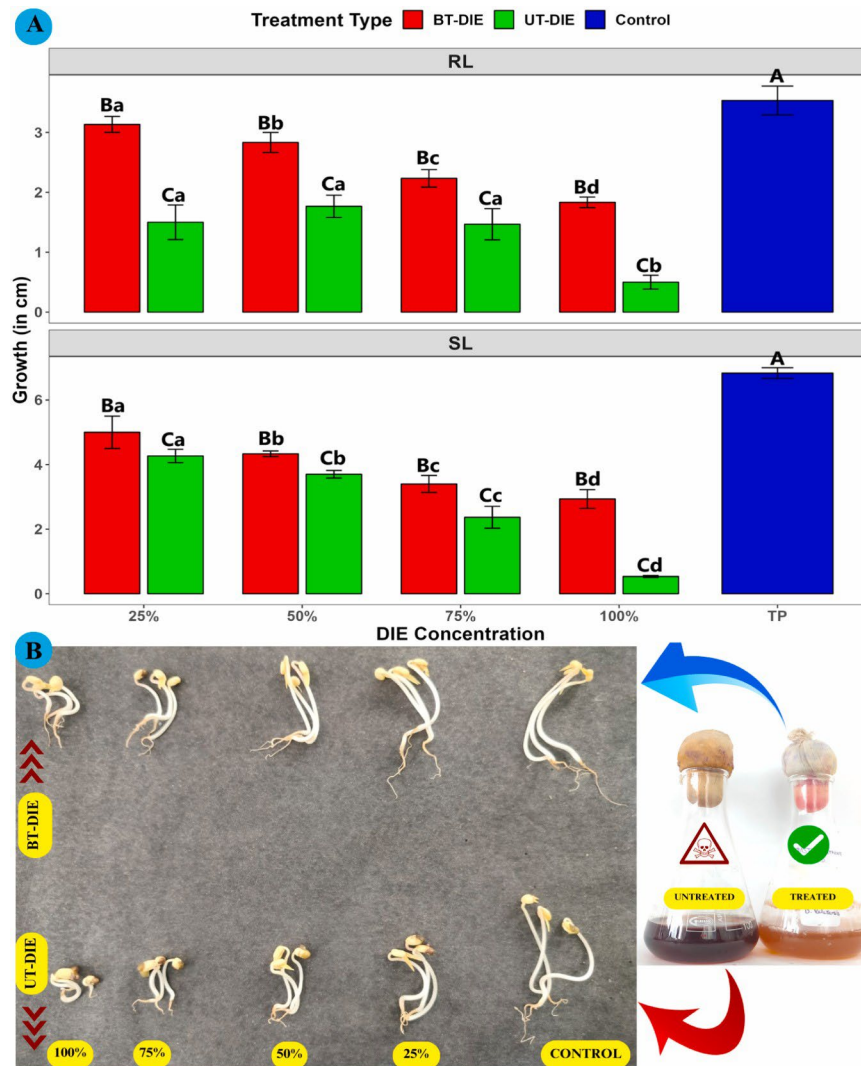


Fig. 5. Effect of varying concentrations of untreated (UT) and bacterially-treated (BT)-DIE on (A) root length (RL) and shoot length (SL), and (B) seedling growth of *Phaseolus aureus* L. Two-way ANOVA followed by Tukey's post hoc test ($p \leq 0.05$) was used to assess main effects of treatment type (BT-DIE vs. UT-DIE), concentration (25%, 50%, 75%, and 100%), and their interaction. Control (TP with 0% DIE) was analyzed separately for comparison with treatment means. Different lowercase letters (a, b, c, d) indicate significant differences among DIE concentrations within each treatment group, while different uppercase letters (A, B, C) indicate significant differences among treatment types (BT-DIE, and UT-DIE) and control (TP). All the values were mean $\pm$ SE ($n = 3$). Bars sharing the same letter are not significantly different, whereas bars with different letters differ significantly ($p < 0.05$).

4. Conclusion

The present study convincingly demonstrates the strong bioremediation and detoxification potential of a novel laccase-producing bacterium, *Bacillus velezensis* HIM03, for treating highly polluted DIE. Under optimal situations (pH 8, 35 °C, 120 rpm, 10% inoculum, 0.5%, w/v glucose and peptone), the strain reduced COD by 84.03% and colour by 80.28% in 144 h, demonstrating its robust metabolic and enzymatic capacity. Peak laccase activity (6.18 IU/mL/min) at 96 h coincided with maximum decolorization and COD removal, confirming laccase's key role in oxidative degradation. Significant reductions were also observed in other contamination parameters, including BOD, TDS, TSS, sulfate, phosphate, nitrate, phenols, and HMs, with most parameters approaching regulatory discharge standards, confirming broad-spectrum detoxification. The formation of non-toxic metabolites in BT-DIE and a significant reduction in phytotoxicity (up to 92.2%), further confirmed effective biodegradation and detoxification of UT-DIE. These findings strongly support the potential reuse of appropriately diluted DIE for agricultural irrigation, aligning with principles of sustainable wastewater management and resource recovery. Together, the results establish *Bacillus velezensis* HIM03 as a highly efficient, eco-friendly, scalable and sustainable bioaugmentation agent for DIE treatment. To the best of our knowledge, this is the first study to integrate biodegradation, detoxification, molecular transformation, and phytotoxicity alleviation of DIE using this specific laccase-producing bacterial strain. The ability of HIM03 to perform efficiently under mesophilic and aerobic conditions highlights its promise as a low-cost and eco-friendly alternative to conventional physicochemical treatments, which are highly polluting and costintensive. The developed bioremediation process shows strong potential for meeting regulatory discharge standards and mitigating ecological and public-health risks associated with DIE. Comprehensive scale-up investigations are warranted to facilitate the successful transition of this process to pilot- and industrial-scale operations. This will require systematic

evaluation of suitable reactor configurations, such as stirred-tank batch reactors, fed-batch systems, and continuous-flow bioreactors. Concurrently, key operational parameters such as hydraulic retention time (HRT), aeration rate, mixing efficiency, and biomass immobilization strategies should be optimized to ensure sustained laccase activity and consistent, efficient pollutant removal under real DIE conditions. Moreover, future research should examine the long-term stability and reusability of the bacterial strain under repeated and prolonged operational cycles. Focused evaluation of sustained enzyme productivity, resilience to variations in effluent composition, and the feasibility of biomass recycling or immobilization-based reuse will be essential to determine robustness, economic viability and operational sustainability at larger scales.

CRedit authorship contribution statement

Himani Chandel: Conceptualization, Methodology, Investigation, Data Curation, Writing - original draft, Formal Analysis, Software, Visualization, Validation. **Rahul Yadav & Mudita Chaturvedi:** Software, Data Curation, Formal Analysis, Visualization, Validation. **Kirti Shyam & Geetansh Sharma:** Formal analysis, Visualization. **Sibiraj Murugesan:** Writing - original draft, Visualization. **Diane Purchase:** Writing - review & editing, Visualization. **Gaurav Saxena:** Conceptualization, Supervision, Resources, Project administration, Investigation, Writing - original draft, review & editing, Visualization

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Declaration of Competing Interest

The authors report no declarations of interest.

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