Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Process Optimization and Kinetic Evaluation of Toluene Biodegradation Using Response Surface Methodology

Gaurav Verma
Dr. Ambedkar Institute of Technology for Divyangjan
Vinay Dwivedi
Amity University
Manish Singh Rajput
msr@aith.ac.in

Dr. Ambedkar Institute of Technology for Divyangjan

Research Article

Keywords: Toluene, Batch degradation, 16S rRNA sequencing, CCD-RSM, HPLC, Characterization techniques

Posted Date: March 31st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9072879/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations:
No competing interests reported.

Table 1 to 5 are available in the Supplementary Files section.

Process Optimization and Kinetic Evaluation of Toluene Biodegradation Using Response Surface Methodology

Gaurav Verma[a], Vinay Dwivedi[b*] and Manish Singh Rajput[a*]

[a]Gaurav Verma: Department of Biotechnology, Dr. Ambedkar Institute of Technology for Divyangjan, Awadhpuri Kanpur,India-208024. ORCID: 0000-0003-1862-9889

[a]Manish Singh Rajput: Department of Biotechnology, Dr. Ambedkar Institute of Technology for Divyangjan, Awadhpuri Kanpur, India-208024. ORCID: 0000-0002-5735-9386

[b]Vinay Dwivedi: Amity Institute of Biotechnology, Amity University, Gwalior, Madhya Pradesh,India-474005. ORCID: 0000-0001-8294-0298

[*]Corresponding author:
[a]Manish Singh Rajput: Department of Biotechnology, Dr. Ambedkar Institute of Technology for Divyangjan, Awadhpuri Kanpur, India-208024. E-mail: msr@aith.ac.in, Mo: +91-9838003287.

[b]Vinay Dwivedi: Amity Institute of Biotechnology, Amity University, Gwalior, Madhya Pradesh,India-474005. E-mail: drvinay@yahoo.com, Mo: +91-9868421746.

Abstract

Toluene is a major pollutant detected in opium processing industries causing various environmental hazards significantly harming aquatic as well as terrestrial organisms. Toluene toxicity directly affects the central nervous system, liver, kidney, reproductive organs, hearing and vision loss. Effective degradation strategy for removal of toluene from the biosphere is crucial. Accordingly, this work aimed to isolate potent microbial strains for the degradation of toluene. For this, soil samples were collected from a yard of Opium and Alkaloid factory. A mineral salt medium, supplemented with 100 ppm of toluene was used to isolate the bacterial cultures from these soil samples. Biochemical tests and 16S rRNA sequencing showed the selected strain as *Bacillus haikouensis***.** Batch degradation study was conducted with 50-400 ppm of toluene and the optimal process parameters were evacuated for the temperature (32-40°C), pH (4.0-9.0) and inoculum size (2-8 ml per 100 ml). Central Composite Design with Response Surface Methodology (CCD-RSM) mathematical model was used to predict degradation which was eventually validated experimentally. HPLC was used to validate toluene degradation whereas GC-MS was carried out for treated samples to identify degraded products. Under various experimental conditions, toluene degradation achieved was between 77-82% at 100 ppm initial concentration of toluene. A maximum degradation of 83% was achieved in conjunction to the predicted degradation of 89% by CCD RSM. HPLC chromatogram confirmed degradation of toluene to different lighter

metabolites. GC-MS showed the presence of derivatives of benzene and benzoic acid, toluene (residual), and benzaldehyde.

Keywords: *Toluene, Batch degradation, 16S rRNA sequencing, CCD-RSM, HPLC, Characterization techniques*

Introduction

Increase in discharge of Industrial effluents from various industries has led to risk to living organisms and increased environmental pollution (Wright et al. 2023). Various industries released enormous quantities of aromatic compounds as pollutants into the environment (Kumar et al. 2005). One such chemical is toluene and its derivatives, butanol, methyl isocyanide, 2-methylpentane, and N,N-dimethyl benzamine as major constituents which are frequently found in chemical and pharmaceutical effluents (Verma et al. 2024). Agency for Toxic Substances and Disease Registry' (ATSDR) and U.S. Environment Protection Agency (USEPA) listed the toxicological effect of toluene (ATSDR, 2000, U.S. EPA, 1991). The opium-processing chemical, paints and biopharmaceutical industries, utilizes toluene as a solvent in paints, coatings, cleaning agents, dyes, pharmaceuticals, synthesis of organic chemicals and cosmetic nail products, (Wijekoon et al. 2012). In pharmaceutical industries it is used to produce alkaloid-based therapeutic compounds such as morphine, codeine, and thebaine, generating complex wastewater streams containing high concentrations of organic and inorganic residues (Singh et al. 2018). The Scientific Committee on Occupational Exposure Limits (SCOEL) suggested that an 8 hr exposure to 50 ppm or 15 min short term exposure to 100 ppm of toluene can raise skin notation to indicate that the dermal absorption of liquid toluene which can substantially contribute to the body burden (SCOEL, 2001). Toluene is a colorless, toxic, flammable, sweet, pungent odor released from different chemical and pharmaceutical industries (Mangiapia et al. 2022). Toluene, a monoaromatic hydrocarbon, is widely used as a solvent, a chemical precursor and is classified as a priority pollutant due to its solubility, volatility, and environmental persistence (USEPA, 2016). Conventional treatment approaches such as adsorption, incineration, and chemical oxidation have been found to efficiently remove aromatic hydrocarbons often generating secondary pollutants with high operational costs (Wu et al. 2022). The biological treatment method is considered to be not only cost-efficient but eco-friendly. In the current study, we opted for an ex-situ bioremediation approach for degrading toluene using isolated bacterial strains. Aerobic microbial degradation of toluene has been well-documented, with bacteria such as *Pseudomonas putida, Rhodococcus spp*., and *Mycobacterium spp.,* suggesting their potentials of utilizing toluene as a sole carbon source (Mazzeo et al. 2010). Importantly, understanding substrate biodegradation kinetics using pure culture has direct implications for improving process efficiency, reactor design, and pollutant removal rates in industrial wastewaters (Li et al. 2012). Biodegradation was carried out using a pure bacterial culture. Pure cultures provide better control over experimental conditions, reproducibility of results, and accurate determination of kinetic parameters. The use of a

single, well-characterized strain allows detailed investigation of metabolic pathways and substrate-specific degradation efficiency without interference from interspecies interactions (Cheng et al. 2016). In this study, an ex-situ bioremediation approach was used for the treatment of toluene, as it is one of the major pollutants in many industrial effluents using toluene as a component of processing in the plant. Biodegradation strategy was opted for the study as it is cost-effective and has higher degradation efficiency. This method also allows better control and optimization of process conditions, making it a sustainable treatment strategy.

The current study focused on degradation of toluene using isolated bacterial strain and to enhance the degradation process by optimizing the process parameters. Optimization of multiple parameters are performed in a stepwise manner by conducting a statistically designed experiment followed by determination of coefficient estimate, analysis of the response and finally checking the adequacy of the model as reported by (Singh et al. 2017).

Materials and Methods

Soil Sampling, isolation and characterization of strain

Soil samples were collected from then yard of the Government Opium & Alkaloid Works (GOAW) in the city of Ghazipur, Uttar Pradesh, India and characterized as described by (Prashanthi et al. 2021 and Verma et al. 2024).

Enrichment of nutrient agar with toluene was done for isolation of toluene degradation bacteria. For this, 0.5 g soil sample was put in 100 mL sterilized distilled water and serially diluted up to 10^{-6} dilutions. A total of eight colonies appeared on the toluene supplemented nutrient agar after 72 hrs. of incubation at 37° C. These appeared eight bacterial colonies were then sub cultured to obtain isolated colonies. Each of these was subsequently subjected to toluene degradation at toluene concentration of 100 ppm per 100 ml of minimal salt media (MSM). The isolate with maximum degradation was subjected to morphological, biochemical and 16S rRNA gene sequencing characterization studies. The selected strain was maintained on nutrient agar slants at 4°C. The selected strain was subjected to morphological characterization and biochemical tests such as Gram staining, Indole test, MR- VP test, Citrate utilization test and others (Margavelu et al. 2012).

Preparation of Minimal Salt Medium (MSM)

MSM used in the study contained following components (g/L): KH_2PO_4, 0.15; $Na_2HPO_4 \cdot 2H_2O$, 0.35; $MgSO_4 \cdot 7H_2O$, 0.02; NH_4Cl, 0.10; $FeSO_4 \cdot 7H_2O$, 0.0001; $CaCl_2 \cdot 2H_2O$, 0.001; glucose, 0.5; tryptone,

3.0; and yeast extract, 3.0. The pH was adjusted to 7 before sterilization. 100 mL of MSM was used for sterilization. 11.7 µl of toluene was filter-sterilized separately using a 0.22 µm PTFE membrane and added to the medium to achieve the desired concentration. For inoculation, 1 % (v/v) actively growing culture (~1×10^4 cells/mL) was introduced in each flask (Zhang et al. 2013).

Batch degradation study

Batch degradation experiment was conducted to study the biodegradation potential of isolated bacterial strain for toluene. 5 different concentrations of toluene 50. 100, 200, 300 and 400 ppm with medium pH of 7, temperature at 35°C and under shaking conditions was done to find out maximum degradation potential of the isolate at different concentrations. The incubation period of the study for toluene removal was of 120 hrs (Aghayani et al. 2019). The total degraded toluene was calculated spectrophotometrically at 269nm, taking pure toluene as reference. Total toluene degradation percentage in this experiment was calculated using below given formulae.

$$\%\ Degradation = \frac{Total\ Initial\ Toluene\ Conc. - Final\ Toulene\ Conc.}{Total\ Initial\ Tolene\ \ Conc.} \times 100 \quad \ldots.1$$

Analytical methods:

UV- Spectrophotometry

UV Spectrometry was employed for initial study of batch degradation. Toluene was scanned from 190-700 nm to obtain maxima and minima. The maxima was observed at 267 nm. The subsequent maxima 267 nm was used to assess the degradation results of toluene with MSM and isolated bacterial strain. The protocol for studying biodegradation is as mentioned above (Farhadian et al. 2010).

High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) studies were conducted using Waters BREEZE QS System (Waters Corporation, USA) having 1525 binary pumps. Waters 2998 PDA detector, was used to detect degraded toluene. 10µl of pure HPLC grade toluene obtained from Thermo Fisher Scientific to obtain a chromatogram with a total run time of 20mins. Waters C18 (25x46 nm) column was used for the study. Methanol: Water, 70:30 % was used as a mobile phase with flow rate set at 1 ml per min. To study the degradation percentage 2 ml of exposed volume of toluene, MSM and bacteria were at 2000

rpm to separate bacterial cells from supernatant. The supernatant was filtered with 22 µm nylon syringe filters before injecting into the detector (Aydin et al. 2021).

Gas Chromatography Mass Spectrometry

Toluene is highly volatile in nature. Gas Chromatography Mass Spectrometry (GCMS) is a technique which is used to study the components profile of the injected sample having a volatile nature. To study degraded components profile, the sample was prepared by filtering it with a 0.22µm syringe filter. 10µl of the filtered sample was then injected at a flow rate of 1 ml per min with a column temperature of 250°C to Shimadzu nexis GC2030. Helium was used as mobile gas. The total run time for GS was 25 mins. To study toluene degraded component profile Shimadzu GCMS-QP2020NX (Shimazu Corporation, Japan) was employed. Shimadzu library was used to BLAST obtained MS chromatogram for identification of degraded compounds (Muccee et al. 2019).

Process optimization

Experimental design and modeling

The aim of the study was to first isolate toluene degrading microbes and then to increase their toluene removal potentials by optimizing process parameters like temperature, pH and toluene concentration initially by manual variations and then validating it with mathematical modelling tools like CCD-RSM. Three independent variables—temperature (A), pH (B), and toluene concentration (C) were selected based on preliminary screening experiments. Each factor was studied at five levels (−α, −1, 0, +1, +α), and a sum of 20 investigational runs were conducted, consisting of six axial points, eight factorial points, and six replicates at the center point to estimate investigational errors. Table 1, represents various ranges of independent parameters chosen for CCD-RSM.

The ranges of these variables were: Temperature: 20–40°C, pH: 6.0–9.0, Toluene concentration: 0.5–3 % (v/v).

Table 1. Range of independent variables chosen for CCD-RSM

All experiments were performed in 250 mL flasks with 100 mL MSM supplemented with toluene. The flasks were incubated on a rotatory shaker at 120 rpm for 72 hrs. under aerobic conditions at 35°C (Pandey et al. 2019). Controls (without inoculum) were maintained to assess abiotic losses. The

response variable (Y) was the percentage of toluene degraded after incubation. Researchers have successfully used CCD-RSM for optimization, improvement and validation of the results obtained using CCD-RSM (Rajput et al. 2020).

Using the response obtained through CCD-RSM, a best fit second-order polynomial quadratic regression equation was generated as mentioned below equation 2.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \ldots.2$$

Where Y is the toluene degradation percentage, and β represents regression coefficients. Statistical analysis, model fitting, and graphical visualization (3-D surface and contour plots) were carried out using Design-Expert 9 (®) software (Stat-Ease Inc., USA). Table 2, below represents values of variable parameters given by CCD-RSM mathematical model for experimental validation of the conditions.

Table 2. Plan of experiment including range and parameters chosen for RSM and wet lab work as per conditions.

Validation of model predictions

The optimal conditions predicted by the RSM model were experimentally validated through triplicate experiments conducted under the predicted parameter settings (Botello-Ojeda et al. 2025). The experimentally observed toluene degradation efficiencies were compared with the model-predicted values to evaluate the accuracy and reliability of the developed regression equation. Furthermore, the biodegradation pathway was corroborated by HPLC analysis, which revealed a substantial reduction in the toluene peak area along with the emergence of intermediate metabolites. These chromatographic changes confirm that toluene removal occurred via biological degradation rather than through simple adsorption mechanisms (Witek-Krowiak et al. 2014).

Statistical analysis

Analysis of Variance (ANOVA) was employed to determine the statistical significance of the developed model as well as the individual process variables. The goodness-of-fit and adequacy of the model was evaluated through several metrics, including the p-value, predicted R^2, coefficient of determination (R^2), adjusted R^2, F-value and Adequate Precision. A p-value below 0.05 was regarded as indicative of statistical significance. All experimental data are presented as the mean ± standard deviation (SD), based on triplicate measurements (Azaman et al. 2010).

Results and Discussion

Identification of toluene degrading microbes:

Standard biochemical procedures, including Gram straining, sugar consumption, were used to biochemically characterize the best isolate obtained from other isolates. A biochemical test is a way to identify microorganisms since it aids in the differentiation of bacteria. These tests indicate different characteristics of microorganisms with respect to subjected bio-chemical tests helping in their identification up to species level. The isolated strain was found to gram +ve, rod shaped showing motility bacteria. The biochemical tests such catalase, oxidase, indole, citrate utilization and urease were positive for the selected isolate, whereas methyl red and voges–proskauer test was found to be negative. Sugar utilization test was also performed to which glucose, sucrose and mannitol utilization was found to be positive whereas the isolate was unable to utilise lactose as a carbohydrate source for growth. All the tests were performed under aseptic conditions. Varshini et al., 2017 highlighted environmental risks posed by oil spills and toluene contamination emphasizing bioremediation as an eco-friendly alternative to conventional cleanup methods. The findings suggest that efficient toluene-degrading bacteria can be scaled up for practical environmental cleanup applications.

The microbe was selected for further, 16S rRNA sequencing. The sequence was subjected to phylogenetic analysis and subsequently was found as *Bacillus haikouensis.* Fig. 1. represents phylogenetic tree for isolated bacterial culture and table 3 shows similarity score, percent identity and the obtained e value verifying identified bacterial strain.

Fig. 1 Phylogenetic tree for isolated bacterial strains a. *Bacillus haikouensis*

Table 3. The blast results revealed that NR_148273.1 (*Bacillus haikouensis)* matches best with our first sequence and NR_042415.1. the similarity score, percent identity, e value and are detailed in the table below.

Biodegradation of toluene

Batch degradation of toluene using *Bacillus haikouensis*

250 mL erlenmeyer flasks were used for studying the batch biodegradation experiments with a working volume of 100 mL to evaluate toluene elimination using *Bacillus haikouensis* and to determine micro-kinetic parameters governing the elimination process. To investigate biomass growth dynamics in presence of toluene as the sole carbon source, substrate concentration in MSM was taken as 50, 100,

200, 300 and 400 ppm. All experiments were performed in duplicates, and the mean values were used for subsequent analysis.

The obtained biomass growth profiles at lower toluene concentrations (<100 ppm), the growth pattern followed a typical microbial degradation trend characterized by distinct lag, exponential, and stationary phases, indicating efficient substrate utilization and cellular adaptation. In contrast, at higher toluene concentrations (>100 ppm), a noticeable decline in growth was observed, attributable to inhibitory and toxic effects of toluene on microbial metabolism. A pronounced decrease in slope beyond 100 ppm indicates onset of substrate inhibition. A maximum of 77% degradation of toluene was evident at 100 pm of toluene when compared with other concentrations. Consistent with this observation, significant growth inhibition was evident at toluene concentrations exceeding 100 ppm, highlighting the concentration-dependent toxicity of toluene during biodegradation. Liu et al., 2010 evaluated benzene biodegradation using individual and mixed *Bacillus* strains isolated from oil sludge. Results highlighted that strain interactions strongly influence biodegradation performance, supporting use of optimized bacterial consortia for benzene remediation. The study led by Liu et al., 2010 supports the identification of *Bacillus spp.* as an evident microbe having aromatic compound degrading potentials likewise toluene.

Parameters Optimization to achieve better degrading efficiency

After studying the batch removal efficiency of *Bacillus haikouensis.* The study was directed to optimization of different parameters to optimize best degradation efficiency of *Bacillus haikouensis* for toluene.

Temperature is one of the important parameters affecting the growth of bacteria. At 100 ppm of toluene, with temperature change of one degree from 31 to 39°C at a constant pH of 7, 35°C maximum degradation of 79.8%% was achieved after an incubation of 72 hrs. The study led by Alagappan et al. 2004, examined the effect of temperature and dissolved oxygen on growth and degradation of benzene and toluene by *Pseudomonas putida* F1. Microbial growth followed established kinetic models, with oxygen availability strongly controlling degradation rates. Similar behavior was observed for *Burkholderia pickettii* PKO1, highlighting the role of temperature in regulating aromatic hydrocarbon biodegradation.

Effect of pH on toluene degradation

pH is another crucial parameter playing a definitive role in bacterial growth. Keeping constant temperature at 35°C and 100 ppm of toluene, pH was varied from 4 to 9 by 1. After incubation of 72 hrs

82% removal was achieved at a pH of 7, i.e 18 ppm of toluene was found to be present from the initial 100 ppm. Any alteration in pH other than 7 resulted in a surge of residual toluene reflecting lesser toluene degradation. Studies led by Men et al. 2011, observed removal of toluene was more at pH 6.5 and they observed a less removal percentage of toluene at all other pH values. Long et al., 2014 also observed similar toluene removal efficiencies increased as the pH of the nutrient mixture in between 5-8. This indicates that most bacteria prefer a weak basic to neutral environment for toluene removal from the medium.

Effect of inoculum volume on toluene degradation

Inoculum concentration also plays a decisive role in biological processes. In current study, varying initial concentrations of *Bacillus haikouensis* from 2 to 8 ml per 100 ml of total volume to study degradation profile of toluene was executed with 35°C as incubation temperature with pH at 7, initial toluene concentration of100 ppm kept under shaking conditions. During the optimization 79% removal of toluene was observed at inoculum concentration of 5 ml per 100 ml. Study conducted by Picone et al. 2013, assessed toluene biodegradation in unsaturated soils under conditions relevant to vapor intrusion using microcosms and soil columns. Biodegradation rates were found to be much higher in unsaturated systems than in liquid batches, indicating importance of soil–air–water interfaces and substrate availability. Study also suggested higher degradation when inoculum size was 5 ml giving support to our study.

Substrate inhibition model for kinetic parameter evaluation

It is well known that toxic substrates either present initially or even produced metabolically influence the growth of both pure as well as mixed cultures in a limiting way. Using the degradation rate data obtained and observed experimentally was fitted to different appropriate kinetic models to estimate kinetic parameters. Toluene kinetic data from batch degradation studies were suitably fitted with Haldane model (Haldane, 1930), Levenspiel model (Levenspiel, 1980) and Edwards model (Edwards, 1970).

Haldane model : $\mu = \frac{\mu_{max} S}{K_S + S + \frac{S^2}{K_I}}$ ….4

Levenspiel model : $\mu = \mu_{max} \left(1 - \frac{S}{K_I}\right)\left(\frac{S}{K_S + S}\right)$ ….5

Edwards model : $\mu = \mu_{max} \left[\exp\left(\frac{-S}{K_I}\right) - \exp\left(\frac{-S}{K_S}\right)\right]$ ….6

Fig.2. Experimental and model predicted profile of specific growth rate for toluene degradation

Here, μ represents specific growth rate, h^{-1}; S represents initial substrate concentration, mg l^{-1}; μ_{max} represents maximum specific growth rate, h^{-1}; K_S represents half saturation constant, mg l^{-1} and K_I represents substrate inhibition constant, mg l^{-1}.

The parameters μ_{max}, K_S and K_I from above three models were studied by the method of least squares. Fig. 2 represents a comparison of model predicted and experimental values for toluene biodegradation. It was observed that in the Levenspiel model, standard deviation for the model predictions of the specific growth rate was very low, indicating an increased degree of fit with R^2 value of 0.97. However, the experimentally observed limiting concentration was found to deviate from the model's predicted limiting concentration. Such variations in model predictions of the limiting concentrations have also been reported in the literature. Rajamanickam et al. 2017 studied toluene biodegradation using mixed microbial consortium for toluene removal. Maximum removal efficiency of 84% was achieved at 100 mg L^{-1}, with *Pseudomonas*, *Bacillus*, and *Escherichia coli* identified as dominant degraders. The effects of pH and inoculum concentration were analyzed, and kinetic data were well described by substrate inhibition models.

Optimizing Toluene degradation using CCD- RSM

Biodegradation efficiency of *Bacillus haikouensis* for toluene was evaluated using 20 experimental runs generated using Central Composite Design (CCD). The experimental results showed that toluene removal efficiency varied from 25.2% to 93.3% under different operating conditions, achieved by systematically varying temperature, pH, and toluene concentration. These findings indicate that toluene biodegradation is highly sensitive to physicochemical parameters, with interactive and synergistic effects among the studied variables. Comparable observations have been reported by this study isolated a toluene-degrading bacterium, *Bacillus cereus* ATHH39, from oil-contaminated soil and optimized its degradation performance using response surface methodology. pH, temperature, and toluene concentration were identified as key factors influencing growth and degradation efficiency. Under optimized conditions, the strain achieved about 64% toluene removal, demonstrating its potential for bioremediation of toluene-contaminated wastewater (Heydarnezhad et al. 2018 and Zhang et al. 2021), who demonstrated that moderate temperatures and near-neutral pH conditions significantly enhance microbial degradation of aromatic hydrocarbons.

At inoculum concentrations below 1%, a noticeable decline in toluene degradation efficiency was observed, primarily due to an insufficient population of metabolically active microorganisms. Under such conditions, the limited biomass reduces enzyme availability, thereby constraining substrate uptake rates and slowing oxidative metabolic pathways.

Conversely, inoculum concentrations exceeding 3% occasionally resulted in reduced degradation performance. This behavior can be explained by increased oxygen demand associated with higher biomass densities, leading to dissolved oxygen depletion and oxygen mass-transfer limitations. Additionally, intensified competition for essential nutrients and accumulation of metabolic by-products may induce self-inhibitory effects, further suppressing microbial activity.

Therefore, an intermediate inoculum concentration was identified as optimal, as it ensures sufficient enzymatic and metabolic capacity for toluene biodegradation while maintaining adequate dissolved oxygen levels necessary for sustained aerobic oxidation processes. Desai et al. 2008 compared ANN-GA and RSM approaches for optimizing fermentation media for scleroglucan production. Both methods improved yield significantly, but ANN-GA showed better accuracy and lower prediction error in modeling the non-linear system. Overall, ANN-GA outperformed RSM in predicting and optimizing biopolymer yield.

The response expressed as percentage of toluene degradation was calculated from Eq.3,

$$\% \text{ Degradation} = (C_0 - C_t)/C_0 \times 100 \quad \ldots. 3$$

where C_0 is the initial optical density (OD) and Ct is the OD after time t (min).

Model fitting and ANOVA interpretation

Central Composite Design (CCD) was employed to optimize the biodegradation of toluene by systematically evaluating the effects of temperature (A), pH (B), and inoculum concentration (C) as independent variables. The CCD matrix defined the experimental runs, and the corresponding responses—expressed as toluene degradation (g L^{-1}). Prior to applying the Response Surface Methodology (RSM) model, the experimental error was estimated to ensure data reliability and to validate the suitability of the design for optimization analysis.

The quadratic polynomial model generated through RSM effectively captured the relationship between the selected process variables and the biodegradation response, indicating the presence of both linear and interaction effects. Analysis of variance (ANOVA) confirmed the statistical significance of the model ($p < 0.05$), with a high F-value of 41.62, suggesting that the observed variation in toluene

degradation was primarily attributed to the modeled factors rather than random experimental noise (probability of only 0.01%). The high coefficient of determination ($R^2 = 0.968$) demonstrated that 96.8% of the variability in toluene degradation could be explained by the model.

Furthermore, the adjusted R^2 (0.953) and predicted R^2 (0.929) values were in close agreement (difference < 0.2), confirming the robustness and predictive capability of the developed model. The Adequate Precision value of 22.14 substantially exceeding the minimum desirable threshold of 4 indicated an excellent signal-to-noise ratio, thereby validating the reliability and sensitivity of the response surface model for navigating the design space.

Among the individual model terms, temperature (A), pH (B), and inoculum concentration (C) exhibited statistically significant linear effects ($p < 0.05$), highlighting their dominant roles in governing microbial activity and toluene biodegradation kinetics. In addition, the interaction terms AC and BC significantly influenced degradation performance, reflecting synergistic effects of temperature–inoculum and pH–inoculum interactions on microbial metabolism, enzyme functionality, and oxygen utilization. Similar statistical trends and interaction effects have been reported in RSM-based optimization studies focusing on the biodegradation of aromatic pollutants. Rajput et al. 2020, optimized biodegradation of pyridine raffinate using a laccase-producing *Gamma proteobacterium* through statistical and neural network modeling. Kaur et al. 2025 demonstrate how alternative electron acceptors influence BTEX biodegradation under anoxic conditions using *Bacillus infantis* and *Microbacterium esteraromaticum*. (Rajput et al. 2020 and Kaur et al. 2025).

The Predicted R^2 value (0.9868) exhibited excellent agreement with the adjusted R^2 value (0.9954), as the difference between these metrics was well below the acceptable limit of 0.2, confirming strong predictive reliability of the model. Adequate Precision, which measures the signal-to-noise ratio, is considered satisfactory when greater than 4. In present study, the obtained value of 65.554 indicates an exceptionally strong signal, demonstrating that the developed model is highly reliable and well suited for effective exploration and optimization of the design. Table 4. shows Analysis of Variance for best fit quadratic polynomial model for Toluene removal.

Table 4. Analysis of Variance (ANNOVA) for best fit quadratic polynomial model for Toluene removal

Final Equation in Terms of Actual Factors

Degradation = -120.54704 + 4.72441 Temperature+0.180647 Conc. +41.62781 pH-0.002775 Temperature*okaConc.+0.045000 Temperature * pH+0.004950 Conc. * pH-0.071539 Temperature^2-0.000607 Conc.^{2}-3.47662 pH^2 …4

To make predictions about the responses for given levels of each factor equation in terms of actual factors can be used. Here for each factor, the levels should be specified in the original units. This equation should not be used for determining relative impact of each factor because the coefficients are scaled to accommodate units of each factor. Also, the intercept is not at the center of the design space. Using equation 4, response for given levels of each factor can be predicted in terms of actual factors where levels must be specified in the original units for each factor. Interaction between the predicted and actual response has been shown in fig. 3. In addition, a contour plot and interaction graph displaying relationship between two parameters keeping the remaining as constant is also presented in fig. 4.

Fig.3. Relation between actual response and predicted response

The obtained Model F-value of 462.31 signifies the model is significant. There is only a 0.01% chance having an F-value this large which may be due to noise. P-values less than 0.05 signifies the model is significant. In the present case A, B, C, AB, BC, A^2, B^2, C^2 are significant model terms. Values higher than 0.1000 signifies model terms are not significant. The Lack of Fit F-value of 1.84 signifies not significant relative to the pure error. A higher value of 25.98% Lack of Fit F-value largely could occur because of noise. A non-significant lack of fit is good as we want the model to fit.

Explanation of Response Surface and Contour Plots (Fig. 4. a–c)

Figure 4(a) depicts the combined effects of temperature (factor A) and initial toluene concentration (factor B) on biodegradation efficiency, with pH held constant at its midpoint. The response surface reveals a gradual enhancement in degradation efficiency as temperature increases, reaching an optimal range near 34-36°C, after which a slight decline is noted. This decrease suggests the onset of thermal stress and partial enzyme deactivation at higher temperatures. Such a pattern aligns with typical mesophilic biodegradation dynamics, wherein moderate temperatures promote enzymatic activity and microbial proliferation, whereas elevated temperatures diminish metabolic performance (Khoshpasand et al. 2023). Conversely, an increase in toluene concentration negatively impacted degradation efficiency, especially beyond approximately 200 mg L^{-1}, indicative of substrate toxicity and inhibitory effects on microbial metabolism. The elliptical contour lines further substantiate a statistically

significant interaction between temperature and substrate concentration, demonstrating that optimal biodegradation is achieved under moderate temperature conditions and relatively low toluene concentrations.

Figure 4(b) illustrates interaction between toluene concentration (factor B) and pH (factor C) at a constant temperature. The response surface indicates that biodegradation efficiency reaches its peak within a near-neutral to slightly alkaline pH range (approximately 6.5 to 7.5), conditions that are conducive to enzyme stability, membrane integrity, and cellular metabolic activity. Deviations toward acidic (pH < 5.5) or strongly alkaline (pH > 8) environments result in a significant reduction in degradation efficiency, likely attributable to altered membrane permeability and diminished enzymatic function. Choudhury et al. 2021 evaluated benzene degradation in constructed wetlands, highlighting combined effects of dissolved oxygen, particulate organic matter, and pollutant concentration. Moderate oxygen levels and high organic matter enhanced degradation, while high benzene levels, Cr(VI), and ethanol significantly inhibited microbial activity. The findings provide guidance for optimizing constructed wetland design for effective BTEX-contaminated urban runoff treatment (Choudhury et al. 2021). Furthermore, increasing toluene concentration correlates with decreased biodegradation performance, confirming substrate inhibition at elevated concentrations. The pronounced curvature and enclosed contour patterns underscore a strong interaction between pH and substrate concentration, suggesting that maintaining optimal pH levels can partially mitigate inhibitory effects at moderate toluene concentrations.

Figure 4(c) depicts the combined effects of temperature (factor A) and pH (factor C) on toluene biodegradation, with substrate concentration held constant. An optimal region is clearly defined at moderate temperatures (approximately 32–35 °C) and near-neutral to slightly alkaline pH values (approximately 7.0–7.5), where maximum degradation efficiency is observed. Outside this range, efficiency declines due to reduced enzymatic activity under suboptimal pH conditions and thermal denaturation at elevated temperatures. These findings align with previously reported optimal conditions for aerobic degradation of aromatic hydrocarbons by mesophilic bacteria (Nishad et al., 2025). The elliptical contour patterns further indicate a significant synergistic interaction between temperature and pH, emphasizing the necessity of simultaneous regulation of both parameters to maximize biodegradation efficiency.

Beyond physicochemical parameters, inoculum concentration was found to significantly influence process performance. At a fixed pH of 8.0, increasing inoculum concentration from 0.5% to 2.0% enhanced toluene degradation efficiency up to 89%, beyond which the response plateaued. Excessively

high inoculum densities likely induced limitations in oxygen mass transfer and intensified nutrient competition, thereby constraining metabolic conversion efficiency (Bezerra et al. 2008). Additionally, the interaction between pH and inoculum concentration revealed that near-neutral to slightly alkaline pH conditions favored toluene utilization, whereas extreme pH values suppressed microbial activity due to compromised enzyme stability and cellular function.

Collectively, the response surface and contour analyses demonstrate that toluene biodegradation is governed by complex interactive effects among temperature, pH, substrate concentration, and inoculum density. The observed curved response surfaces and elliptical contour lines validate the suitability of the quadratic central composite design (CCD) model and underscore the importance of multi-parameter optimization. Optimal biodegradation was achieved under conditions of moderate temperature, near-neutral to slightly alkaline pH, intermediate substrate concentration, and controlled inoculum levels, consistent with the kinetics of aerobic microbial degradation and toxicity constraints.

Fig. 4 Three-dimensional response surface and contour plots illustrating the interactive effects of temperature, toluene concentration, and pH on toluene biodegradation efficiency.

Fig. 5. Illustrates the outcomes of numerical optimization conducted via the desirability function methodology aimed at maximizing degradation percentage. The RSM model identified the optimal operational parameters as a temperature of 35.78 °C, a concentration of 214.02 mg/L, and a pH of 7.18. Under these specified conditions, the model forecasts a peak degradation efficiency of 85.37%. The red indicators on each factor's bar denote the optimal solution's position within the experimental range, confirming that the predicted optimum resides well within the design space rather than at its extremities. This observation substantiates the robustness and reliability of the constructed quadratic model. Furthermore, the desirability value of 1.000 signifies complete fulfillment of the optimization objective, reflecting an ideal equilibrium among all process variables to achieve maximal degradation. Collectively, the desirability analysis corroborates efficacy of the RSM-based optimization approach and affirms that the selected operational conditions are both statistically and practically optimal for the degradation process.

Fig. 5. Optimization Results / Desirability Plot (1.00) from RSM

Determination of optimum conditions

Numerical optimization performed using Design-Expert® software established the optimal operational parameters for maximizing toluene biodegradation as a temperature of 35.78 °C, pH 7.18, and concentration of 214.02. Under these optimized conditions, the RSM model predicted a maximum toluene degradation efficiency of 85.37%, closely aligning with the experimentally observed value of 81.1%. This strong concordance between predicted and empirical results validates the adequacy, precision, and predictive reliability of the developed regression model.

The identified optimal parameter ranges align with prior research documenting enhanced biodegradation of aromatic hydrocarbons by Bacillus and Pseudomonas species under moderate temperature and near-neutral pH conditions (Choudhury et al. 2021). The minimal deviation between predicted and experimental values (less than 1%) further attests to the robustness of the RSM–Central Composite Design (CCD) approach in capturing complex interactions among process variables, underscoring its appropriateness for optimizing multivariate biological systems (Wang et al. 2026).

External validation by HPLC for Toluene Degradation

High-Performance Liquid Chromatography (HPLC) analysis was conducted to externally validate the toluene degradation process. Initially, standard toluene was analyzed to establish a baseline chromatographic profile. Pure toluene exhibited a retention peak at 12.27 minutes, whereas biodegradation mediated by *Bacillus haikouensis* resulted in multiple peaks at 2.364, 2.535, 3.175, 3.325, and 11.730 minutes. These findings indicate substantial removal of toluene, suggesting its breakdown into simpler and lighter molecular constituents. Fig. 6 presents a comparative analysis of the HPLC chromatograms of standard toluene and the post-biodegradation sample. Mao et al. 2009 demonstrated petroleum hydrocarbon bioremediation in diesel-contaminated soils showed substantial TPH reduction. Advanced GC×GC analyses revealed formation of heavier, more polar metabolites during degradation. Nutrient addition improved removal rates increasing ecotoxicity due to microbial community shifts (Mao et al. 2009).

Fig. 6. HPLC chromatogram of: a. Pure Toluene, b. *Bacillus haikouensis* treated toluene,

External validation by GCMS analysis

GC-MS was also carried out to validate degradation of toluene using *Bacillus haikouensis.* Fig. 7 (a), represents standard toluene GC-MS chromatogram whereas Fig. 7(b) shows chromatogram of degraded

sample to assess toluene degradation after 72 hrs of incubation. The table 5 below enlists the degraded compounds obtained confirming removal of toluene from the sample.

In current analysis, toluene, an aromatic compound, was broken down into simpler aliphatic and aromatic compounds like benzyl alcohol, dihydroxy benzoic acid as intermediate compounds and further into simpler aliphatic fragments. GCMS table 5 also depicts the prominent end products obtained after toluene degradation. Above enlisted metabolites can be recovered and used in industries like chemical, pharmaceutical, food industry and many more. These intermediates have also shown this prominence as antimicrobial agents (Rosell et al. 2003). Imam et al., 2023 demonstrated efficient biodegradation of naphthalene by *Pseudomonas aeruginosa* IIPIS-8, even at high concentrations. Proteomic analysis revealed key enzyme regulation supporting the bacterium's strong metabolic adaptability (Imam et al. 2023).

Fig. 7. GC–MS chromatogram of: a. standard toluene, b. degraded sample

Table 5. The table below enlists the degraded compounds obtained from GC-MS.

Conclusion

In present study, degradation of the environmental pollutant toluene through biological means was performed. Isolation for toluene degrading microbes was carried out from the toluene rich effluent discharging site. From the initial studies, best toluene degrading bacteria was selected and subjected for 16S rRNA sequencing. In batch degradation studies 100 ppm of toluene was found to be the best utilizable concentration of toluene degraded by *Bacillus haikouensis*. Optimization for better degradaing efficiency was performed by keeping 100 ppm constant for optimization of other variables. At 35°C from the range of temperatures 32-39°C by increment of 1°C, 79.8% of toluene degradation was observed. At pH 7, from the pH range of 4.0-9.0 by increment of 1, 82% of degradation was observed. By varying inoculum from the range of 2-8 ml per 100 ml by change of 1ml 79% of degradation was observed. Toluene inhibition model for batch degradation was studied using Haldane, Levenspiel and Edwards model and 100 ppm of toluene was experimentally fitted to the models studied. CCD-RSM (Design-Expert 9 (®) software (Stat-Ease Inc., USA)) mathematical modeling tool was used to design a set of 20 sets of predicted experiments that were experimentally validated and the results obtained were as expected in predicted values. HPLC and GCMS were also conducted to verify toluene degradation. Multiple peaks observed at different retention times in HPLC chromatogram verifying degradation of

toluene. GCMS chromatogram gave 7 different groups of compounds validating degradation of toluene into simpler compounds. Overall, the findings confirm that *Bacillus haikouensis* is a robust and efficient candidate for biological remediation of toluene-contaminated environments. This study underscores the feasibility of employing indigenous microbial resources as eco-friendly and cost-effective alternatives to conventional treatment methods for managing industrial effluent containing aromatic hydrocarbons. The results also provide a strong foundation for future scale-up studies and development of bioreactor-based treatment systems for practical field applications.

Statements and Declarations

Acknowledgements

The author's would like to acknowledge General Manager, Government Opium and Alkaloid Works factory, Ghazipur for allowing sample collection. The author's would also like to thank the Director, Dr. Ambedkar Institute of Technology for Divyangjan, Kanpur for providing all the necessary facilities required for the completion of the work.

Funding

The study was self-sponsored and had received no or any funding from any of the government or private bodies.

Conflict of Interest

All authors have read the manuscript and declare that they have no conflict of interest in the submitted manuscript.

Ethics Approval

This study does not involve any experiments with human participants or animals; therefore, ethical approval was not required.

Reference

1. Wright, T., & Adhikari, A. et al (2023). Utilizing a national wastewater monitoring program to address the US opioid epidemic: a focus on Metro Atlanta, Georgia. *International Journal of*

Environmental Research and Public Health, *20*(7): 5282. https://doi.org/10.3390/ijerph20075282

2. Kumar, A., Kumar, S., & Kumar, S. et al (2005). Biodegradation kinetics of phenol and catechol using Pseudomonas putida MTCC 1194. *Biochemical Engineering Journal*, *22*(2): 151-159. https://doi.org/10.1016/j.bej.2004.09.006
3. Prashanthi, R., Shreevatsa, G. K., Krupalini, S., & Manoj, L. (2021). Isolation, characterization, and molecular identification of soil bacteria showing antibacterial activity against human pathogenic bacteria. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 120. https://doi.org/10.1186/s43141-021-00219-x
4. Verma, G., Dwivedi, V., & Rajput, M. S. et al (2024). Physicochemical study of opium industry effluent and toxicity prediction using in silico method: In silico prediction of opium industry effluent and toxicity. *Indian Journal of Experimental Biology (IJEB)*, *62*(07): 571-577. https://doi.org/10.56042/ijeb.v62i07.12092
5. US Department of Health and Human Services. (1999). Agency for Toxic Substances and Disease Registry-ATSDR. https://doi.org/10.4135/9781412963855.n24
6. Iyer, P. (2017). Guidelines for reproductive and developmental toxicity testing and risk assessment of chemicals. In *Reproductive and Developmental Toxicology* (pp. 179-192). Academic Press. https://doi.org/10.1016/B978-0-12-804239-7.00010-X
7. Wijekoon, C. P., & Facchini, P. J. (2012). Systematic knockdown of morphine pathway enzymes in opium poppy using virus-induced gene silencing. *The Plant Journal*, *69*(6): 1052-1063. https://doi.org/10.1111/j.1365-313X.2011.04855.x
8. Singh, P., Singh, V. K., Singh, R., Borthakur, A., Kumar, A., Tiwary, D., & Mishra, P. K. (2018). Biological degradation of toluene by indigenous bacteria Acinetobacter junii CH005 isolated from petroleum contaminated sites in India. *Energy, Ecology and Environment*, *3*(3): 162-170. https://doi.org/10.1007/s40974-018-0089-8
9. Mangiapia, M. D., Verginelli, I., Baciocchi, R., Bogliolo, M. P., & Berardi, S. (2022). Review of reference values for the assessment of inhalation risks for workers at industrial contaminated sites. *Human and Ecological Risk Assessment: An International Journal*, *28*(5-6): 664-682. https://doi.org/10.1080/10807039.2022.2071206
10. Wu, Z., Liu, G., Ji, Y., Li, P., Yu, X., Qiao, W., ... & Jiang, J. (2022). Electron acceptors determine the BTEX degradation capacity of anaerobic microbiota via regulating the microbial

community. *Environmental Research*, *215*: 114420. https://doi.org/10.1016/j.envres.2022.114420

11. Mazzeo, D. E. C., Levy, C. E., de Angelis, D. F., & Marin-Morales, M. A. (2010). BTEX biodegradation by bacteria from effluents of petroleum refinery. Science of the Total Environment, 408(20): 4334–4340. https://doi.org/10.1016/j.scitotenv.2010.07.004
12. Mukherjee, A. K., & Bordoloi, N. K. (2012). Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by Bacillus subtilis and Pseudomonas aeruginosa strains and a formulated bacterial consortium. *Environmental Science and Pollution Research*, *19*(8), 3380-3388. https://doi.org/10.1007/s11356-012-0862-8
13. Li, L., & Goel, R. (2012). Biodegradation of naphthalene, benzene, toluene, ethyl benzene, and xylene in batch and membrane bioreactors. *Environmental Engineering Science*, *29*(1), 42-51. https://doi.org/10.1089/ees.2010.0362
14. Cheng, Z., Lu, L., Kennes, C., Yu, J., & Chen, J. (2016). Treatment of gaseous toluene in three biofilters inoculated with fungi/bacteria: Microbial analysis, performance and starvation response. Journal of Hazardous Materials, 303: 83–93. https://doi.org/10.1016/j.jhazmat.2015.10.017
15. Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., & Tripathi, C. (2017). Strategies for fermentation medium optimization: an in-depth review. *Frontiers in microbiology*, *7*, 2087. https://doi.org/10.3389/fmicb.2016.02087
16. Margavelu, G., & Ramasamy, D. (2012). Microbial degradation of toluene. *African Journal of Biotechnology*, *11*(96): 16210-16219. 10.5897/AJB12.2251
17. Zhang, L., Zhang, C., Cheng, Z., Yao, Y., & Chen, J. (2013). Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by the bacterium Mycobacterium cosmeticum byf-4. *Chemosphere*, *90*(4): 1340-1347. https://doi.org/10.1016/j.chemosphere.2012.06.043
18. Aghayani, E., Moussavi, G., & Naddafi, K. (2019). Improved peroxidase-mediated biodegradation of toluene vapors in the moving-bed activated sludge diffusion (MASD) process using biosurfactant-generating biomass stimulated with H_2O_2. Journal of Hazardous Materials, 361: 259–266. https://doi.org/10.1016/j.jhazmat.2018.08.076
19. Farhadian, M., Duchez, D., Gaudet, G., & Larroche, C. (2010). Biodegradation of toluene at high initial concentration in an organic–aqueous phase bioprocess with nitrate respiration. *Process Biochemistry*, *45*(11), 1758-1762. https://doi.org/10.1016/j.procbio.2010.01.006

20. Aydin, D. C., Pineres, J. Z., Al-Manji, F., Rijnaarts, H., & Grotenhuis, T. (2021). Direct analysis of aromatic pollutants using a HPLC-FLD/DAD method for monitoring biodegradation processes. *Analytical Methods*, *13*(13), 1635-1642. 10.1039/d1ay00083g
21. Muccee, F., Ejaz, S., & Riaz, N. (2019). Toluene degradation via a unique metabolic route in indigenous bacterial species. *Archives of microbiology*, *201*(10), 1369-1383. https://doi.org/10.1007/s00203-019-01705-0
22. Pandey, A. K., Pandey, K., Pandey, A., Morya, V. K., & Singh, L. K. (2019). Response surface and artificial neural network simulation for process design to produce L-lysine by Corynebacterium glutamicum NCIM 2168. *Indian J Biotechnol*, *18*, 269.
23. Rajput, M. S., Dwivedi, V., & Awasthi, S. K. (2020). Enzymatic degradation of pyridine raffinate using response surface and artificial neural network simulation. *Indian Journal of Experimental Biology (IJEB)*, *58*(08): 584-592. https://doi.org/10.56042/ijeb.v58i08.39418
24. Botello-Ojeda, A. G., Águila-Almanza, E., Hernández-Cocoletzi, H., Chavez-Bravo, E., Bernabé-Loranca, M. B., Zhang, C., ... & Conde-Hernández, L. A. (2025). Essential oils extracted from Mexican giant hyssop (Agastache mexicana subsp. xolocotziana) by a central composite face-centered (CCF) design. *Biomass Conversion and Biorefinery*, *15*(24): 31133-31148. https://doi.org/10.1007/s13399-025-06910-0
25. Witek-Krowiak, A., Chojnacka, K., Podstawczyk, D., Dawiec, A., & Bubała, K. (2014). Application of response surface methodology and artificial neural network methods in modelling and optimization of biosorption process. *Bioresource technology*, *160*: 150-160. https://doi.org/10.1016/j.biortech.2014.01.021
26. Azaman, S. N. A., Ramakrishnan, N. R., Tan, J. S., Rahim, R. A., Abdullah, M. P., & Ariff, A. B. (2010). Optimization of an induction strategy for improving interferon-α2b production in the periplasm of Escherichia coli using response surface methodology. *Biotechnology and applied biochemistry*, *56*(4): 141-150. https://doi.org/10.1042/BA20100104
27. Varshini, D. V., & Sumathy, V. J. H. (2017). Isolation of toluene degrading bacteria from polluted environment. *Eur J Pharm Med Res*, *4*, 341-346. https://doi.org/10.20959/wjpr20179-9314
28. Liu, J. H., Maity, J. P., Jean, J. S., Chen, C. Y., Chen, C. C., & Ho, S. Y. (2010). Biodegradation of benzene by pure and mixed cultures of Bacillus spp. *World Journal of Microbiology and Biotechnology*, *26*(9), 1557-1567. https://doi.org/10.1007/s11274-010-0331-9

29. Alagappan, G., & Cowan, R. M. (2004). Effect of temperature and dissolved oxygen on the growth kinetics of Pseudomonas putida F1 growing on benzene and toluene. *Chemosphere*, *54*(8), 1255-1265. https://doi.org/10.1016/j.chemosphere.2003.09.013
30. Men, J., & Cheng, F. (2011). Biodegradation and growth characteristics of a toluene-degrading strain. *African journal of Biotechnology*, *10*(61), 13299-13306. https://doi.org/10.4314/ajb.v10i61
31. Long, A., Lei, Y., & Zhang, H. (2014). Degradation of toluene by a selective ferrous ion activated persulfate oxidation process. *Industrial & Engineering Chemistry Research*, *53*(3), 1033-1039. https://doi.org/10.1021/ie402633n
32. Picone, S., Grotenhuis, T., van Gaans, P., Valstar, J., Langenhoff, A., & Rijnaarts, H. (2013). Toluene biodegradation rates in unsaturated soil systems versus liquid batches and their relevance to field conditions. *Applied microbiology and biotechnology*, *97*(17), 7887-7898. https://doi.org/10.1007/s00253-012-4480-7
33. Heydarnezhad, F., Hoodaji, M., Shahriarinour, M., Tahmourespour, A., & Shariati, S. (2018). Optimizing toluene degradation by bacterial strain isolated from oil-polluted soils. *Polish Journal of Environmental Studies*, *27*(2). https://doi.org/10.15244/pjoes/75811
34. Zhang, Y., Liu, J., Chen, Y., & Li, J. (2021). Screening and study of the degradation characteristics of efficient toluene degrading bacteria combinations. *Environmental Technology*, *42*(21): 3403-3410. https://doi.org/10.1080/09593330.2020.1732477
35. Rajamanickam, R., Kaliyamoorthi, K., Ramachandran, N., Baskaran, D., & Krishnan, J. (2017). Batch biodegradation of toluene by mixed microbial consortia and its kinetics. *International Biodeterioration & Biodegradation*, *119*: 282-288. https://doi.org/10.1016/j.ibiod.2016.11.014
36. Desai, K. M., Survase, S. A., Saudagar, P. S., Lele, S. S., & Singhal, R. S. (2008). Comparison of artificial neural network (ANN) and response surface methodology (RSM) in fermentation media optimization: case study of fermentative production of scleroglucan. *Biochemical Engineering Journal*, *41*(3): 266-273. https://doi.org/10.1016/j.bej.2008.05.009
37. Kaur, G., Verma, S., Krol, M., & Brar, S. K. (2025). Analysis of benzene, toluene, ethylbenzene, xylene (s) biodegradation under anoxic conditions using response surface methodology. *International Biodeterioration & Biodegradation*, *198*, 105973. https://doi.org/10.1016/j.ibiod.2024.105973

38. Khoshpasand, F., Nikpay, A., & Keshavarz, M. (2023). Optimization of the photocatalytic oxidation process in toluene removal from air. *Pollution*, *9*(2), 567-578. https://doi.org/10.22059/poll.2022.347254.1595
39. Choudhury, S. P., & Kalamdhad, A. S. (2021). Optimization of electrokinetic pretreatment for enhanced methane production and toxicity reduction from petroleum refinery sludge. *Journal of Environmental Management*, *298*, 113469. https://doi.org/10.1016/j.jenvman.2021.113469
40. Nishad, V., Kumar, S., & Sastry, S. V. A. R. (2025). Process Parameter Optimization for Cr (VI) Reduction Using Zero-Valent Iron Nanoparticles Via Response Surface Methodology. *Environmental Claims Journal*, 1-25. https://doi.org/10.1080/10406026.2025.2556802
41. Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, *76*(5): 965-977. https://doi.org/10.1016/j.talanta.2008.05.019
42. Wang, J., Zheng, D., Liu, Y., Zhao, Z., Chen, D., & Xiao, Z. (2026). Optimization of Typical Constructed Wetland Substrate Conditions and Their Feasibility for Removing the Co-contamination of Benzene and Cr (VI)/ethanol. *Water, Air, & Soil Pollution*, *237*(1), 27. https://doi.org/10.1007/s11270-025-08700-w
43. Mao, D., Lookman, R., Weghe, H. V. D., Weltens, R., Vanermen, G., Brucker, N. D., & Diels, L. (2009). Combining HPLC-GCXGC, GCXGC/ToF-MS, and selected ecotoxicity assays for detailed monitoring of petroleum hydrocarbon degradation in soil and leaching water. *Environmental Science & Technology*, *43*(20), 7651-7657. https://doi.org/10.1021/es9015603
44. Rosell, M., Lacorte, S., Ginebreda, A., & Barceló, D. (2003). Simultaneous determination of methyl tert.-butyl ether and its degradation products, other gasoline oxygenates and benzene, toluene, ethylbenzene and xylenes in Catalonian groundwater by purge-and-trap-gas chromatography–mass spectrometry. *Journal of Chromatography A*, *995*(1-2), 171-184. https://doi.org/10.1016/S0021-9673(03)00500-4
45. Imam, A., Suman, S. K., Singh, P., Vempatapu, B. P., Tripathi, D., Ray, A., & Kanaujia, P. K. (2023). Proteomic response of Pseudomonas aeruginosa IIPIS-8 during rapid and efficient degradation of naphthalene. *Environmental Research*, *216*, 114511. https://doi.org/10.1016/j.envres.2022.114511

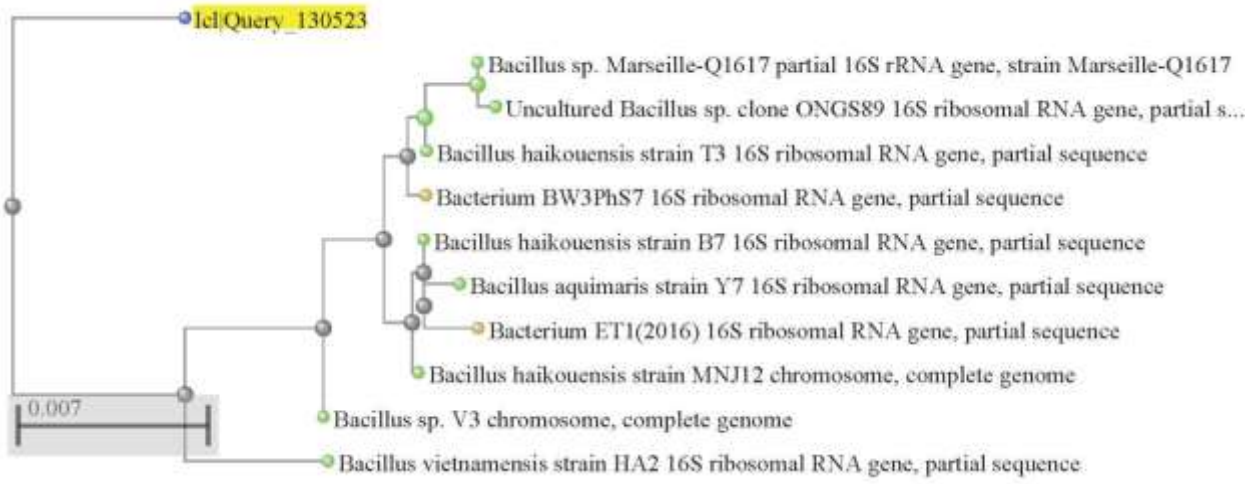


Fig. 1. Phylogenetic tree for isolated bacterial strains a. *Bacillus haikouensis*

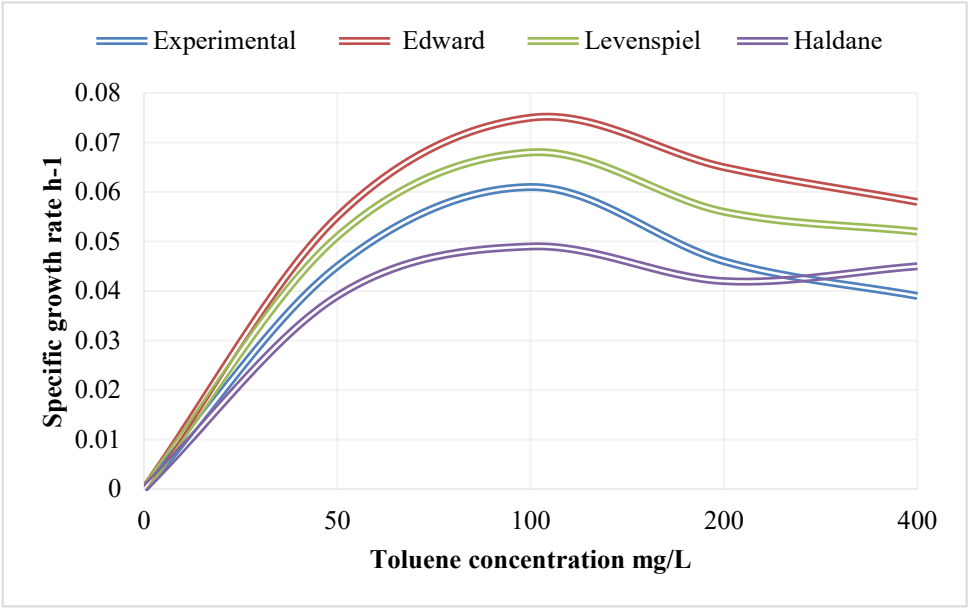


Fig.2. Experimental and model predicted profile of specific growth rate for toluene degradation

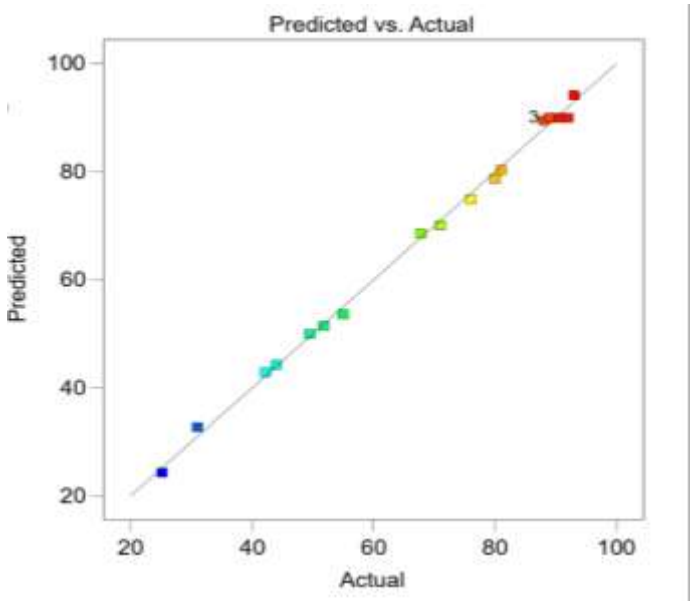


Fig.3. Relation between actual response and predicted response

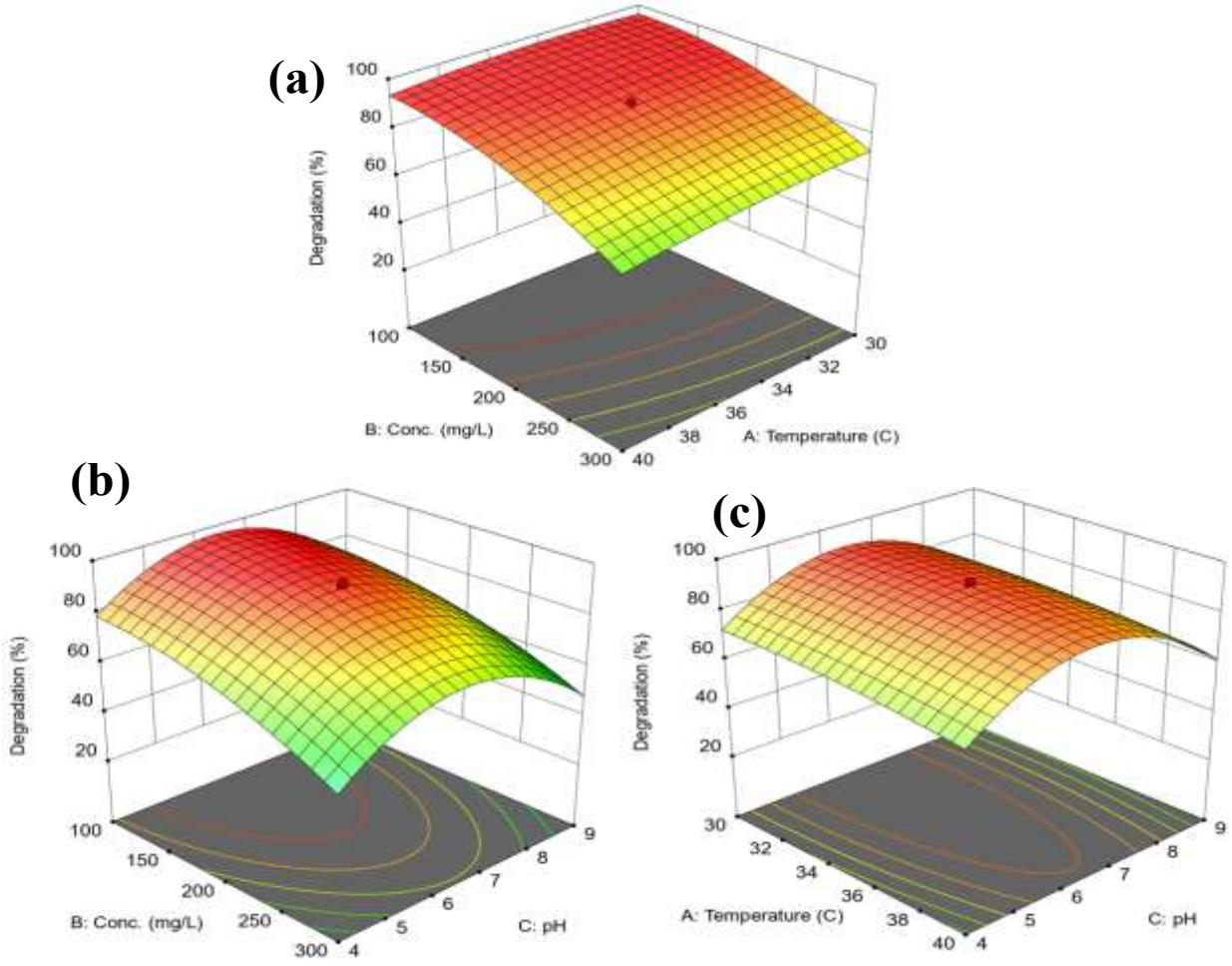


Fig.4. Three-dimensional response surface and contour plots illustrating the interactive effects of temperature, toluene concentration, and pH on toluene biodegradation efficiency.

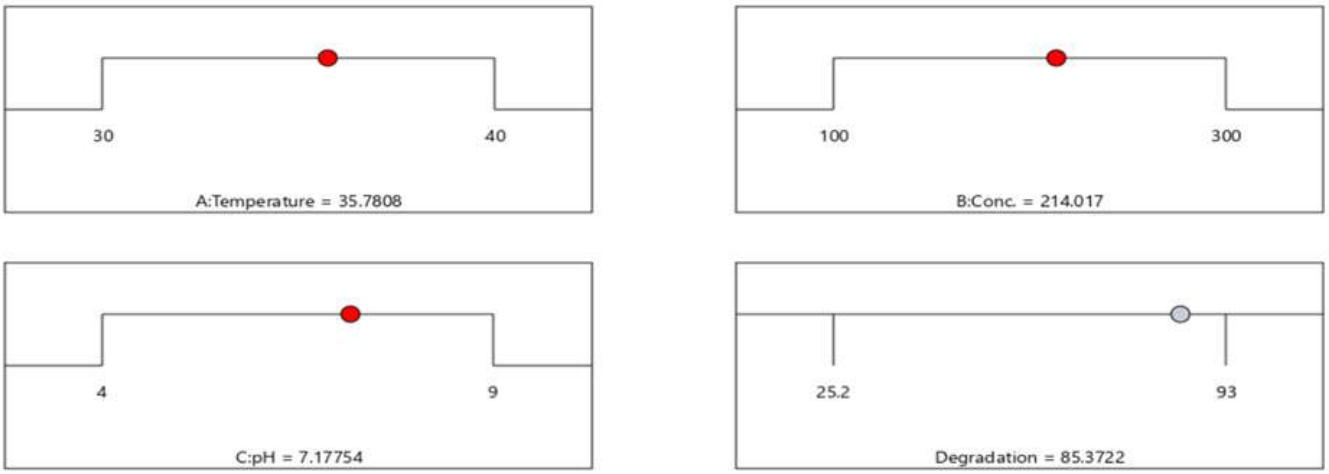


Fig.5. Optimization Results / Desirability Plot (1.00) from RSM

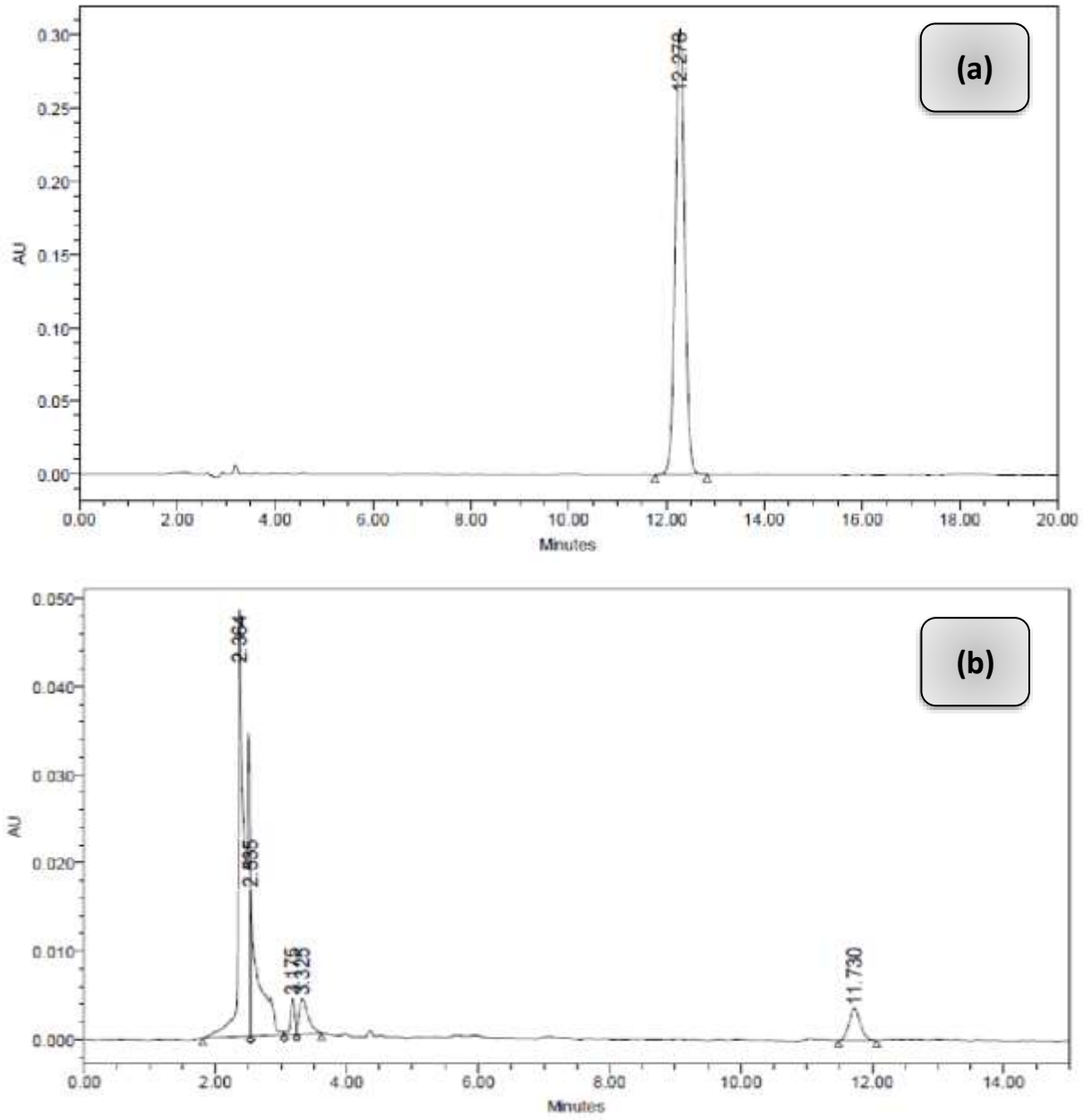


Fig.6. HPLC chromatogram of: a. Pure Toluene, b. *Bacillus haikouensis* treated toluene,
HPLC chromatogram of: a. Pure Toluene, b. *Bacillus haikouensis* treated toluene,

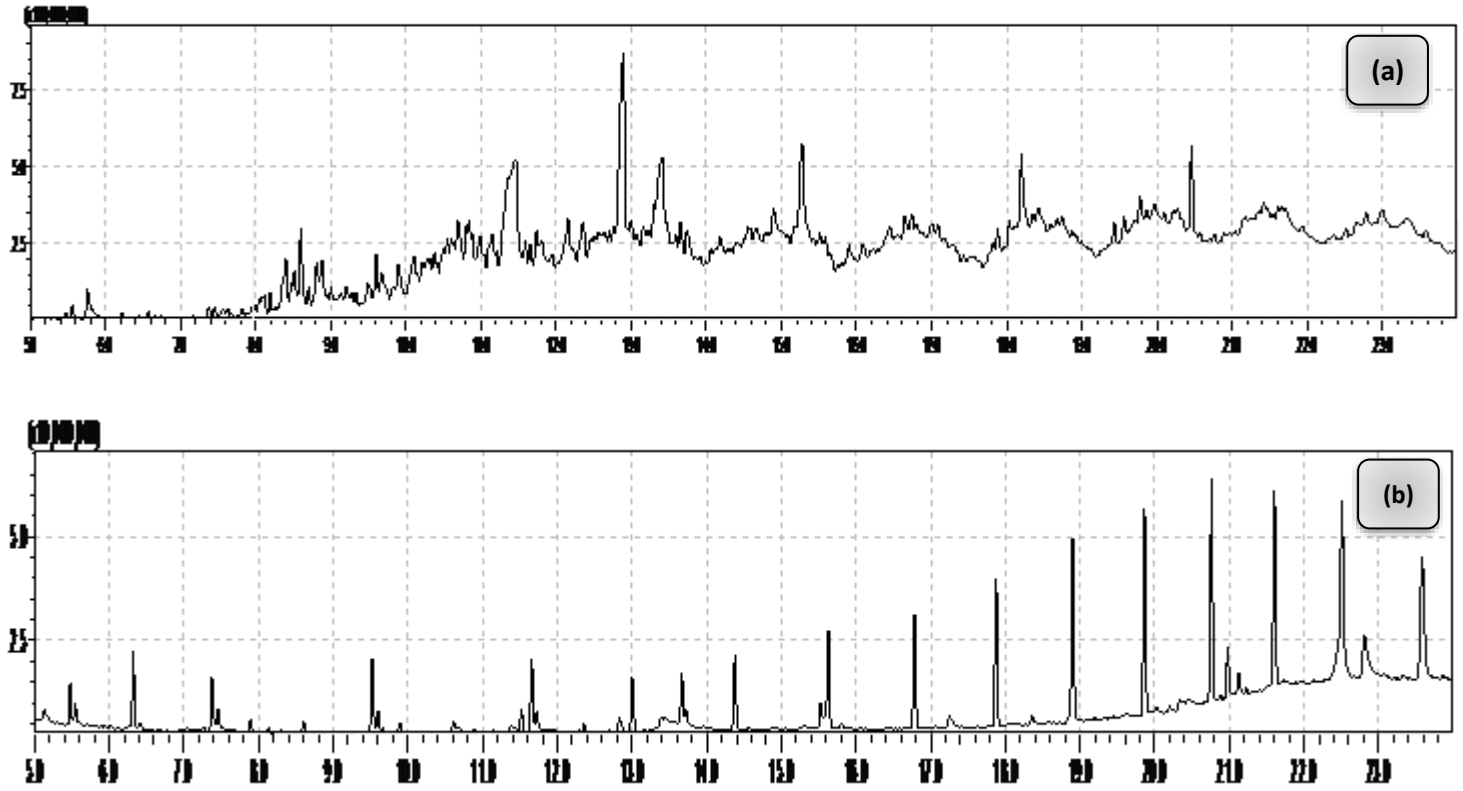


Fig.7. GC–MS chromatogram of: a. standard toluene, b. degraded sample,

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table.docx