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# Multivariate optimization for the simultaneous bioremoval of BTEX and chlorinated aliphatic hydrocarbons by *Pseudomonas plecoglossicida*

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## HIGHLIGHTS

- Simultaneous bioremoval of BTEX, *cis*-DCE, and TCE mixture from soil.
- Response surface methodology, 2-D HCA, and PCA jointly applied.
- Factors influencing the mixture bioremoval optimized.
- Results extrapolatable to bioremediation of BTEX/chlorinated hydrocarbons mixture.

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## ABSTRACT

This study aimed to evaluate the effects of some major parameters on the cometabolic removal of *cis*-1,2-dichloroethylene (*cis*-DCE) and trichloroethylene (TCE), mixed with benzene, toluene, ethylbenzene, and xylenes, by an indigenous bacterial isolate *Pseudomonas plecoglossicida*. Such statistical methodologies as hierarchical cluster analysis heat map and principal component analysis were applied to better evaluate the effects of major parameters (soil pH, temperature, moisture, and *cis*-DCE/TCE concentrations) on the biological process. The bioremoval experiments were carried out in microcosms containing soil slurry, and the headspace concentrations of contaminants were analyzed by gas chromatography. The optimal bioremoval conditions for the mixture were soil water content >110%, pH 8–9, and temperature 15–20 °C, while the *cis*-DCE/TCE concentration did not significantly affect the mixture bioremoval within the tested range (∼10 mg per kg soil). Under the optimal conditions, benzene (97.7%), toluene (96.3%), and ethylbenzene (89.8%) were almost completely removed, while *cis*-DCE (24.5%), TCE (29.0%), *m*,*p*-xylene (36.3%), and *o*-xylene (29.6%) showed lower removal efficiencies. The obtained results would help to better design a remediation technology to be applied to the sites contaminated with mixed wastes, and the statistical methodologies used in this study appear to be very efficient and could serve as a template for optimization.

## 1. Introduction

Chlorinated aliphatic hydrocarbons (CAHs) and benzene, toluene, ethylbenzene, *ortho*-, *meta*-, and *para*-xylenes (BTEX) are among the most widespread contaminants in subsurface due to their increased use as well as accidental spillage [1]. The United States Environmental Protection Agency [2] reported many sites out of 976 listed in the National Priority List (NPL) were contaminated with both BTEX and chlorinated compounds. Trichloroethylene (TCE), ranked No. 1 contaminant in groundwater in U.S., is a representative CAH known for its toxicity, carcinogenic nature, and high resistance to degradation. Even though TCE can be degraded under the anaerobic condition by a sequential reductive dechlorination to ethane, the reductive dechlorination of TCE is often incomplete, resulting in the accumulation of *cis*-1,2-dichloroethylene (*cis*-DCE) and vinyl chloride (VC) [2]. The

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microbial oxygenases induced during the microbial growth on such substrates as methane, phenol, toluene, propane, isoprene, or ammonia have been reported capable of oxidizing and further mineralizing less chlorinated ethenes by the aerobic cometabolism [3]. Most *Pseudomonas* sp. strains have shown such characteristics as solvents and metal resistance, with some species capable of aerobically cometabolizing chlorinated solvents. TCE can be cometabolized by *P. fluorescens* and *P. putida* by supplying phenol and gasoline [4] and phenol as the growth substrates [5], respectively. *Cis*-DCE and TCE can be simultaneously cometabolized by *P. plecoglossicida* while growing on BTEX [6]. BTEX are naturally occurring compounds found in crude oil and are employed as industrial solvents in manufacturing processes [7]. They are among the most widespread contaminants in the environment and are frequently found in such contaminated sites as soil and groundwater, posing a severe risk to the human health [8]. Considering the co-contamination sites with CAHs and BTEX are often found, it would be meaningful to develop methodologies for their simultaneous degradation [6,9]. Shim et al. [10] evaluated the effects of different concentrations of both types of contaminants (BTEX, TCE, and total petroleum hydrocarbons) on the bioremoval efficiencies of indigenous microbial isolates from the soil samples potentially contaminated with gasoline compounds, and Chen et al. [9] found that the elevated TCE concentrations could inhibit the bioremediation of benzene and toluene. The natural attenuation methodology was also employed to treat the sites contaminated with BTEX and chlorinated solvents [1], and the diffusive fractionation of BTEX and CAHs mixture was investigated by Jin et al. [11]. Lu et al. [12] recently developed a hybrid process (adsorption and immobilization technologies) using waste scrap tyres to further improve the removal of a mixture of BTEX, TCE, and *cis*-DCE from the artificially contaminated water, while achieving high removal efficiencies for BTEX (95%), TCE (72%), and *cis*-DCE (62%).

Soil parameters such as soil pH, temperature, moisture, nutrients, and oxygen supply were identified as possible determinants of the bioremediation success [13]. Among them, soil pH, moisture, and temperature were considered as the most crucial factors affecting microbial growth [14]. The concentration ratio of growth substrates (BTEX) to non-growth substrates (CAHs) has been considered a significant factor in determining the degradation extent of both substrates [6]. Therefore, it is essential to optimize these parameters regulating the bioremediation for a better performance on the contaminant removal. Response surface methodology (RSM) has been widely used for the process parameters optimization, mainly due to its easy operation [15–17]. It is a graphical statistical approach to identify the factor settings that produce the best response and satisfy the operation or process specifications. Principal component analysis (PCA) is used to reduce the data dimensions, thereby visualizing the overall patterns and trends within the dataset, and allows the evaluation of the relationships among numerous observed variables [18]. Hierarchical cluster analysis (HCA) is a multivariate approach to classify the variables with similar characteristics into groups [18,19]. The two-dimensional (2-D) HCA heat map simplifies the data and improves the ability to interpret a complex dataset and quickly identifies the groups of variables with similar patterns and/or trends, as well as clusters of observations which drive variable grouping. The combined utilization of PCA and 2-D HCA heat map facilitates a rapid data visualization and provides an efficient approach to explore the observation trends and understand the variable relationships. These methodologies have been applied to various research areas, including environmental and ecological studies [19], but there has been almost no report on their uses to evaluate/optimize the bioremoval of organic contaminants present in mixture, especially the investigation of relationships between variables and the integrated use of these techniques.

**Table 1**
Experimental ranges and levels of independent variables for the response surface methodological experiment.

| Factor | Range and level | | | | |
|---|---|---|---|---|---|
| | −2 | −1 | 0 | +1 | +2 |
| Initial pH ($X_1$) | 6 | 7 | 8 | 9 | 10 |
| Culture temperature (°C) ($X_2$) | 15 | 20 | 25 | 30 | 35 |
| Moisture (% of water holding capacity) ($X_3$) | 70 | 90 | 110 | 130 | 150 |
| Concentration of CAHs (mg kg$^{-1}$)[a] ($X_4$) | 0 | 2.5 | 5 | 7.5 | 10 |

[a] The BTEX concentration was 400 mg kg$^{-1}$.

This study focused on the simultaneous bioremoval of a BTEX/*cis*-DCE/TCE mixture from the artificially contaminated soil microcosm under the aerobic condition using an indigenous isolate *Pseudomonas plecoglossicida*. The objectives of this study were to evaluate the biodegradability of a mixture of BTEX, *cis*-DCE, and TCE, and to optimize the parameters (soil pH, temperature, moisture, and concentrations of *cis*-DCE and TCE) affecting the bioremoval for a better performance by employing RSM combined with 2-D HCA heat map and PCA. Many studies have been conducted on the optimization of the bioremediation process, while the current study would be the first that documents the aerobic bioremoval of mixed TCE/*cis*-DCE and BTEX in soil using *Pseudomonas plecoglossicida* under multivariate analysis statistical procedure. The results obtained in this study would help to better design a remediation technology for the hazardous waste sites where TCE and *cis*-DCE usually coexist with BTEX.

## 2. Materials and methods

### 2.1. Chemicals

Benzene (purity, 99.7%), toluene (purity, 99%), ethylbenzene (purity, 99%), *ortho*-xylene (purity, 99%), *meta*-xylene (purity, 99%), and *para*-xylene (purity, 99%) were purchased from the International Laboratory (U.S.A.). Both dimethylformamide (purity, 99%) and TCE (purity, 99%) were purchased from Damao Chemical Manufacture in Tianjin, China. *Cis*-DCE (purity, 99%) was purchased from the Tokyo Chemical Industry CO., LTD (Japan). All other chemicals used were also purchased from the International Laboratory. All the volatile organic compounds (VOCs) were dissolved in dimethylformamide to prepare the respective stock solutions.

### 2.2. Bacterial culture

The pure indigenous bacterial strain used, identified as *Pseudomonas plecoglossicida*, was isolated from a heavily petroleum-contaminated soil [20], capable of cometabolizing *cis*-DCE and TCE by the oxygenase induced during growth on toluene as a primary substrate [6,12]. Serum bottles (160-mL) containing 50 mL of mineral salts medium (MSM) [20] containing (in g/L) $KH_2PO_4$ 1.0, $K_2HPO_4$ 1.0, $NH_4NO_3$ 1.0, $MgSO_4{\cdot}7H_2O$ 0.2, $Fe_2(SO_4)_3$ 0.05, and $CaCO_3$ 0.02 were inoculated with *P. plecoglossicida* culture (inoculum size, 10%, v v$^{-1}$). The BTEX mixture (400 mg L$^{-1}$) was added as a carbon source for the routine subculturing once a week. In order to achieve high biomass, 48 h prior to performing the experimental runs (Table 1), the routine subculture (inoculum size, 10%, v v$^{-1}$) was transferred to MSM with 150 mg L$^{-1}$ toluene as sole substrate. The stock solution was prepared according to the mass fraction of each compound in crude oil [benzene (22.7%), toluene (48.3%), ethylbenzene (4.6%), *o*-xylene (6.3%), *m*-xylene (6.9%), *p*-xylene (11.1%)]. It should be noted that the physical properties and the exact chemical composition of crude oils vary greatly and depend on where and how the crude oil was formed [21]. Either NaOH (1 mol L$^{-1}$) or $HNO_3$ (1 mol L$^{-1}$) solution was used to adjust the pH