

Detection of Benzene, Toluene, Ethyl Benzene, and Xylenes (BTEX) Using Toluene Dioxygenase-Peroxidase Coupling Reactions

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We have developed a simple, whole-cell bioassay for the detection of bioavailable benzene, toluene, ethyl benzene, and xylenes (BTEX) and similar compounds. A genetically engineered *E. coli* strain expressing toluene dioxygenase (TDO) and toluene dihydrodiol dehydrogenase (TodD) was constructed, enabling the conversion of BTEX into their respective catechols, which were quickly converted into colored products by a horseradish peroxidase (HRP)-coupled reaction. The intensity of the color formation was correlated to concentrations of the BTEX compounds. Under the optimized conditions, a detection limit (defined as three times the standard deviation of the response obtained from the blank) of 10, 10, 20, and 50 μM was observed for benzene, toluene, ethyl benzene, and xylene, respectively. The bioassay was selective toward BTEX-related compounds with no interference observed with commonly used pesticides, herbicides, and organic solvent. The bioassay was very stable with little change in response over a 10-week period. The excellent stability suggests that the reported bioassay may be suitable for field monitoring of BTEX to identify and track contaminated water and follow the bioremediation progress.

Introduction

Benzene, toluene, ethyl benzene, and xylenes (BTEX) are common environmental pollutants often found together at contaminated sites resulting from spillages of fuels, solvents, or chemicals. Because they are all highly toxic and soluble in water, they represent a significant environmental hazard for humans. According to the U.S. Environmental Protection Agency (U.S. EPA), there is sufficient evidence from both human and animal studies to believe that benzene is also a human carcinogen. To protect the public from exposure to these chemicals and to conduct necessary bioremediation efforts, a variety of methods have been developed to detect BTEX. Gas/liquid chromatography, often in combination with other techniques such as mass spectrometry (1–2), membrane extraction (3), solid-phase microextraction (1, 2, 4), and immunoaffinity extraction (5), are widely used to detect BTEX and their derivatives in contaminated water because of their accuracy and sensitivity. Although very sensitive, the cost associated with analysis makes the overall monitoring efforts very expensive. In addition, bioluminescent bacterial reporters (6–7) and UV spectrophotometric detection plus in situ corona reactions (8) have also been employed to meet different challenges. The use of bioluminescent bacterial reporters has significant benefits over conventional analytical methods because of the ability to determine the bioavailable BTEX, which is necessary to determine whether the field conditions are favorable for biodegradation to occur.

In this paper we present a simple, sensitive, whole-cell-based bioassay system for detection of bioavailable BTEX compounds based on a method developed for screening of oxygenase activity (9). This method takes advantage of the flexibility of toluene dioxygenase

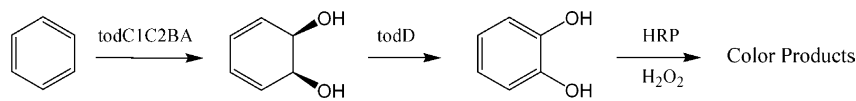
(TodC1C2BA) and toluene dihydrodiol dehydrogenase (Tod D) in converting BTEX compounds into their corresponding catechols in the presence of O_2 and NADH (10). Subsequent addition of hydrogen peroxide and horseradish peroxidase (HRP) enables the transformation of the respective catechols into colorimetric products that can be monitored easily at an emission maximum of 420 nm (Figure 1).

Materials and Methods

Reagents. Atrazine was purchased from Chem Service Inc. (West Chester, PA). IPTG (isopropyl- β -D-thiogalactopyranoside), HRP (horseradish peroxidase), and simazine were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from Fisher Scientific (Fair Lawn, NJ). A mixture composed of 60% *m*-xylene, 9% *o*-xylene, and 14% *p*-xylene was used as the xylene sample. All organic reagents were dissolved in ethanol and were stored in vials with Teflon septa at 4 °C, except IPTG, which was dissolved in double distilled water and stored at –20 °C.

Construction and Cultivation of Recombinant Strain. Plasmid pXTD13, which harbors *todC1C2BA* (encoding toluene dioxygenase) and *todD* genes (encoding *cis*-toluene dihydrodiol), was obtained from Dr. Frances H. Arnold (California Institute of Science and Technology, Pasadena, CA; unpublished result), and was transformed into *E. coli* DH5 α to construct recombinant strain DH5 α -pXTD13. Bacterial cells were cultivated in Luria-Bertani medium supplemented with 50 μg of ampicillin per mL. Cells were grown to an OD_{600} of 0.6 at 37 °C when IPTG was added to a final concentration of 1 mM. The cultivation was continued for an additional 3 h at 30 °C. Cells were harvested by centrifugation at 4 °C. After being washed once with 50 mM sodium phosphate buffer (pH 7.3), the cell pellet was resuspended in the same buffer containing 20% (v/v) glycerol to a final OD_{600}

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Benzene **Dihydrodiol** **Catechol**

Figure 1. Coupled dioxygenase-peroxidase reaction system.

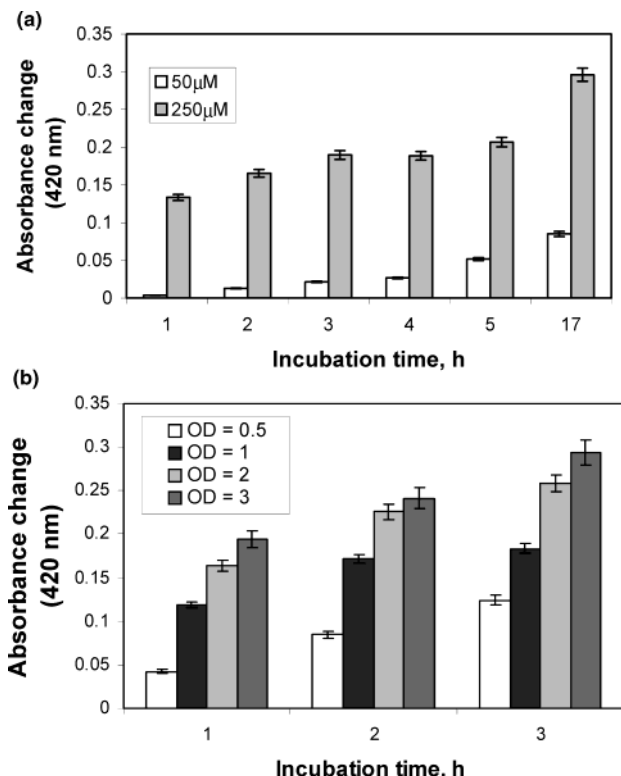


Figure 2. (a) Cells of 1.0 OD_{600nm} were incubated with 50 or 250 μM benzene for various times. (b) Cells of 0.5, 1.0, 2.0, and 3.0 OD_{600} were incubated with 250 μM benzene for 1–3 h. The supernatants were collected, allowed to react with peroxidase, and measured spectrophotometrically. The efficiency of biotransformation was presented as absorbance at 420 nm.

of 50. The cell slurry was then aliquoted and stored at -20°C for further investigation.

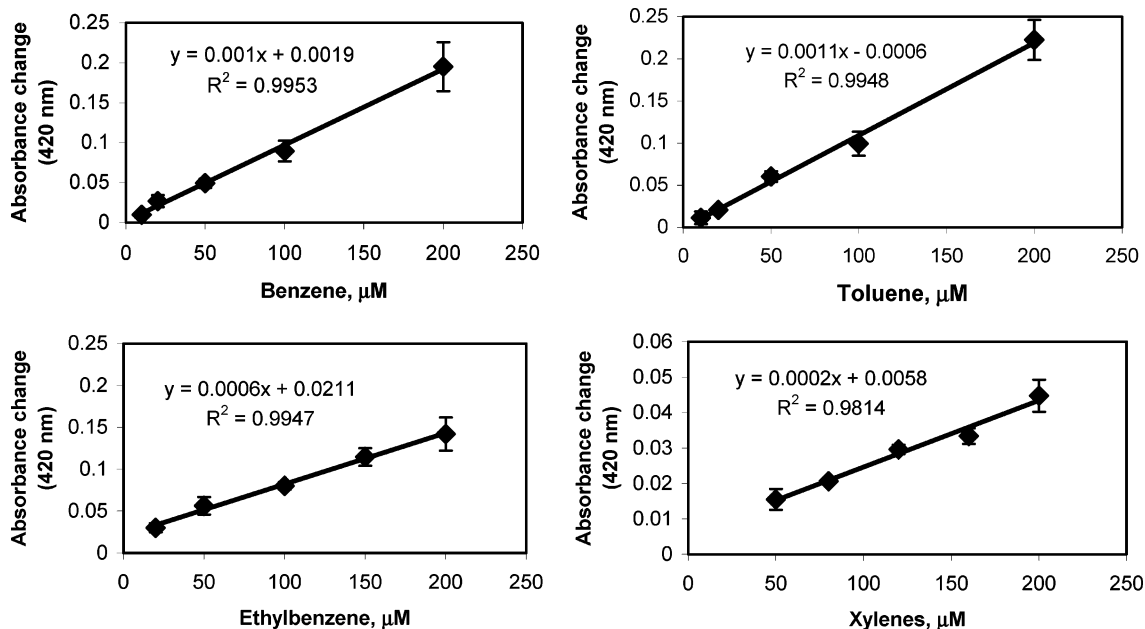


Figure 3. Calibration plots for (A) benzene, (B) toluene, (C) ethyl benzene, and (D) xylenes.

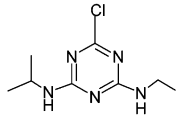
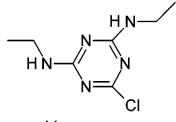
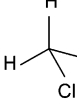
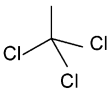
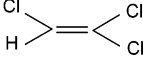
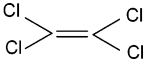
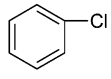
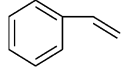
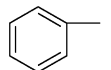
Biotransformation and Bioassay. For each bioassay, an appropriate amount of cell aliquot was resuspended in phosphate buffer and 10 μL of substrate. The final volume of the mixture was adjusted to 1 mL by the addition of phosphate buffer. Cell suspensions added with 10 μL of ethanol were set as the negative control. The mixtures were transferred to 25 mm \times 150 mm culture tubes and incubated at 30 $^\circ\text{C}$ with shaking for several hours. All tubes were screw-capped and were wrapped with Parafilm during incubation to prevent possible volatilization. Samples were transferred into microcentrifuge tubes after incubation and were centrifuged at maximum speed for 2 min to remove the cell pellets. Supernatants were then transferred into clean microcentrifuge tubes. A reddish brown color was visualized shortly after the addition of 5 μL of 0.1 M H_2O_2 and 10 unit of HRP. The color production was allowed to proceed at room temperature for 30 min, and the intensity was measured at 420 nm with a DU640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). For the selectivity experiments, a similar procedure was used except that BTEX compounds were replaced with 20 mM of tested chemicals (2 mM in the case of Simazine).

For the stability experiments, aliquots of frozen cells were used over a 10-week period as described above. Two different concentrations of toluene were used for testing in all the stability experiments.

Results and Discussion

Principle of Detection. The initial degradation of toluene by *Pseudomonas putida* F1 is carried out by a multicomponent enzyme, toluene dioxygenase (TDO), which is encoded by the *todC1C2BA* genes of the *tod* operon (Figure 1). This enzyme has a broad substrate specificity, recognizing benzene, toluene, ethyl benzene, and xylenes as targets. The diols generated from the

Table 1. Selectivity of the Bioassay System^a

Contaminants	Structures	Concentration (μM)	Toluene-equivalent concentration (μM)* (Means \pm SD, n=3)
Atrazine		200	-0.8 \pm 2.0
Simazine		20**	2.6 \pm 6.1
Dichloromethane		200	3.6 \pm 8.4
1,1,1-Trichloroethane		200	0.9 \pm 2.3
Trichloroethylene		200	8.2 \pm 4.8
Tetrachloroethylene		200	3.6 \pm 2.3
Monochlorobenzene		200	135.3 \pm 8.5
Styrene		200	130.4 \pm 10.3
Toluene***		200	194.2 \pm 7.2

^a (*) Toluene-equivalent concentration was calculated from the calibration equation obtained from Figure 3. (**) Higher concentration was not available because of solubility problem. (***) Toluene was involved as a positive control to test the reliability of the assay.

initial dioxygenation of BTEX are subsequently converted to their respective catechols by the toluene dihydrodiol dehydrogenase (Tod D). Using the HRP-coupled reactions, these catechols can be converted to colored compounds, forming the basis of the BTEX detection assay reported in this paper.

Optimization of the Assay Conditions. Plasmid pXTD13, coding for toluene dioxygenase and toluene dihydrodiol dehydrogenase under control of an IPTG-inducible *tac* promoter, was introduced into *E. coli* DH5 α . IPTG-induced cells were harvested and resuspended in phosphate buffer before the addition of BTEX compounds. Colored products generated by the HRP-coupling reaction were scanned from 300 to 500 nm, and a distinct peak common for all substrates was observed at 420 nm, whereas controls cells carrying no plasmid showed no visible color production (data not shown).

Because the sensitivity of the assay depends on the extent of catechol formation, a time course study was performed to determine the optimal incubation time required for complete biotransformation of benzene. Two parallel samples, containing 50 and 250 μM benzene, respectively, were tested with a fixed amount of cells (1 mL of OD₆₀₀ = 1.0). As shown in Figure 2a, the efficiency of biotransformation increased gradually with time during the initial 3 h, reaching the highest level only after overnight incubation. To reduce the time requirement for

optimal conversion, the effect of cell density on the conversion rate was investigated using a fixed benzene concentration (250 μM). As shown in Figure 2b, tripling the cell density resulted in a significant increase in the rate of biotransformation, and maximum product formation was achieved within 3 h. This condition was adapted for all subsequent analyses of the performance of the assay.

Characteristics of the Bioassay: Calibration, Sensitivity, and Stability. The relationship between concentration and absorbance changes for each BTEX compound was measured, and the individual calibration curves are shown in Figure 3. The responses are linear up to 200 μM for all substrates tested. Although the HRP-coupling reaction system could detect the individual BTEX compounds at a similar wavelength, the sensitivity of the assay varied among the substrates. As shown in Figure 3, the bioassay was the most sensitive toward toluene and benzene, followed by ethyl benzene and xylene. The lower detection limit (defined as three times the standard deviation of the response obtained from the blank) of benzene, toluene, ethyl benzene, and xylenes was 10, 10, 20, and 50 μM , respectively. The sensitivity of the bioassay is in line with the selectivity of toluene dioxygenase toward these substrates. This detection limit reported here is comparable to that reported for bioluminescent bacterial reporters utilizing BTEX-inducible

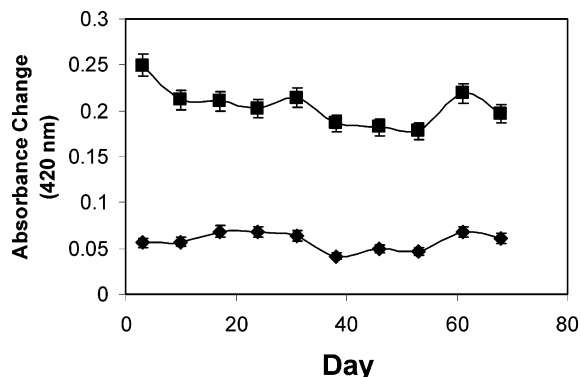


Figure 4. Activity of the frozen cells over a 10-week period of storage. Cells were tested with (■) 250 or (◆) 50 μM toluene.

Pu promoter from the TOL plasmid (β). However, the benefit with the reported bioassay is that resting cells rather than growing cells are needed, suggesting the possibility of long-term storage and usage of the bioassay for field applications.

The long-term storage stability of the bioassay was investigated by evaluating the response of engineered cells to benzene after extended storage at $-20\text{ }^{\circ}\text{C}$. As shown in Figure 4, the bioassay was very stable with little change in response over a 10-week period. The excellent stability makes the assay easy to perform, as a stock of cells can be produced once and used over a long period. Such a capability suggests that the reported bioassay may be suitable for field monitoring of BTEX to identify and track contaminated water and follow the bioremediation progress.

Selectivity of the Bioassay. The selectivity of the bioassay was tested with several compounds commonly identified as industrial or agricultural contaminants in groundwater. For the eight different contaminants tested, only monochlorobenzene and styrene responded positively (Table 1). Other pesticides, herbicides, and organic solvents including trichloroethylene, known to be degraded by TDO, produced no noticeable response. This high degree of specificity is again consistent with the substrate specificity of TDO (10) and HRP toward aromatic compounds. Since the bioassay responds to all BTEX compounds and other related aromatics, it may be used as a useful bioassay for assessment of fuel contamination, which contains most of these detectable compounds.

Conclusion

In this study, we developed a quick, simple, and reliable bioassay system for detection of bioavailable BTEX and related contaminants based on genetically engineered *E. coli* harboring the *todC1C2BAD* genes and HRP-coupled reactions. This assay requires less than 3

h to complete without the need for cell lysis, sophisticated equipments, and exquisite techniques. One major benefit over other promoter-based whole cell bioassays for BTEX is that only resting cells and not actively growing cells are necessary for the assay. Even frozen cells stored at $-20\text{ }^{\circ}\text{C}$ are shown to be stable over a 10-week period. The convenience of multiple sample-handling makes this whole cell assay an attractive candidate to be developed as a field diagnostic kit for on-site BTEX contamination.

Acknowledgment

This work was supported by a grant from the U.S. EPA (R829606).

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Accepted for publication July 18, 2003.

BP0341794