Indole-based assay to assess the effect of ethanol on *Pseudomonas putida* F1 dioxygenase activity

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Abstract Toluene dioxygenase (TDO) is ubiquitous in nature and has a broad substrate range, including benzene, toluene, ethylbenzene and xylenes (BTEX). *Pseudomonas putida* F1 (PpF1) induced on toluene is known to produce indigo from indole through the activity of TDO. In this work, a spectrophotometric assay previously developed to measure indole to indigo production rates was modified to characterize the effects of various ethanol concentrations on toluene aerobic biodegradation activity and assess catabolite repression of TDO. Indigo production rate by cells induced on toluene alone was $0.0012 \pm 0.0006\ \mathrm{OD}_{610}\ \mathrm{min}^{-1}$. The presence of ethanol did not fully repress TDO activity when toluene was also available as a carbon source. However, indigo production rates by PpF1 grown on ethanol:toluene mixtures (3:1 w/w) decreased by approximately 50%. Overall, the proposed spectrophotometric assay is a simple approach to quantify TDO activity, and demonstrates how the presence of ethanol in groundwater contaminated with reformulated gasoline is likely to interfere with naturally occurring microorganisms from fully expressing their aerobic catabolic potential towards hydrocarbons bioremediation.

Introduction

Biodegradation of target pollutants can be inhibited or slowed by the presence of easily degradable substrates (Duetz et al. 1994a, b). For example, the presence of ethanol can delay the aerobic biodegradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) (Hunt et al. 1997; Corseuil et al. 1998; Lovanh et al. 2002; Ruiz-Aguilar et al. 2002a). Furthermore, the degree of inhibition of toluene degradation exerted by ethanol was shown to increase linearly with the fraction of ethanol in the mixture (Lovanh and Alvarez 2004). However, the physiological effect of ethanol on BTEX biodegradation is not completely understood, and very few methods, if any, are available to describe this phenomenon.

A quantitative response to mixed chemical contaminants in environmental samples and the influence of these mixtures on cell physiology has been accessed using biosensors (Heitzer et al. 1998). Bioluminescence genes (*lux*) fused with catabolic genes in

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bacterial genome become active and emit light when the catabolic gene is induced. *Pseudomonas fluorescens* HK44 harboring the plasmid pUTK21 is a known *lux* biosensor organism used to evaluate the response of a specific genetic induction. However, the accuracy of the light emitted by this cell is sometimes confounded by physiological changes (e.g., pH, temperature, and the availability of aldehydes, ATP, and NADH) or the presence of non-specific inducers (e.g., glucose, yeast, peptone) (Heitzer et al. 1998; Neilson et al. 1999; Dorn et al. 2003). Thus, the use of this methodology may have limitations when evaluating the effects of ethanol on BTEX biodegradation.

All of the BTEX compounds have at least one aerobic pathway, which includes degradation to a substituted catechol prior to ring fission. In each case, the aromatic ring of the substituted catechol is later cleaved by a dioxygenase. *Pseudomonas putida* strain F1 (PpF1), a very well studied microorganism, is capable of degrading BTEX aerobically through metabolic reactions initially catalyzed by dioxygenases (Gibson and Parales 2000). The toluene dioxygenase (TDO) is responsible not only for the aerobic metabolism of BTEX, but also certain halogenated aromatics and various chlorinated aliphatic compounds such as trichloroethylene (TCE) (Wackett et al. 1988; Zylstra et al. 1989). TDO in PpF1 is constituted by three component enzyme system that oxides toluene to *cis*-toluene dihydrodiol through the addition of both atoms of molecular oxygen to the aromatic nucleus (Woo et al. 2000). This compound is then dehydrogenated to form methylcatechol. The methylcathecol is cleaved at the *meta* position to form *cis–cis*-hydroxy-6-oxohepta-2,4-dienoate, which is further broken down into pyruvate and acetaldehyde (Lau et al. 1994). The enzymes responsible for the degradation of toluene by PpF1 are encoded by the *tod* operon (Finette et al. 1984; Lau et al. 1994). The mechanisms by which TDO metabolizes toluene and its genetic regulation have already been characterized elsewhere (Zylstra et al. 1988; Zylstra et al. 1989).

Microorganisms expressing mono- or dioxygenases have demonstrated the ability to transform indole to dihydrodiol, which is dehydrated to form indoxyl (a soluble yellow dye compound) and, when dimerized, a dark blue compound (indigo) that can be detected visually (Ensley and Gibson 1983; Mermod et al. 1986; Murdock et al. 1993). Spectrophotometric analysis of indigo production, which has been mainly used to clone genes encoding oxygenases, has also been used as an indicator of TDO specific formation rates (O'Connor et al. 1997; Costura and Alvarez 2000). For example, a variety of styrene-degrading (e.g., *Pseudomonas putida* S12 and CA-3) or BTEX-degrading (*Pseudomonas putida* F1) strains are capable of transforming indole to indigo based on the structural similarity between the substrates styrene or toluene and indole (O'Connor et al. 1997). On the other hand, cells exposed to alternative sources of carbon such as ethanol, which is degraded via constitutively-expressed enzymes other than oxygenases, should not induce TDO and produce indigo from indole. Thus, the spectrophotometric indigo assay could be useful as a tool to determine the effect of ethanol on TDO activity and therefore complement our basic understanding on how ethanol affects the aerobic BTEX biodegradation.

In this work, a spectrophotometric assay previously developed to measure indole to indigo production rates was modified to assess TDO activity and characterize the effects of various ethanol concentrations on toluene biodegradation by *Pseudomonas putida* F1.

Materials and methods

Growth medium

PpF1 were grown in batch reactors using MSB-mineral salts basal medium. MSB was made from three stock solutions: buffer, nitrogen source, and metals solution. The buffer solution (solution A) contained one molar dibasic sodium phosphate and one molar monobasic potassium phosphate. The nitrogen solution (solution B) contained 200 g l^{-1} ammonium sulfate. The metals solution (solution C) contained (g l^{-1}): nitrilotriacetic acid (10), KOH (7.3), $MgSO_4$ (14.45), $CaCl_2 \cdot 2H_2O$ (3.33), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.00925), $FeSO_4 \cdot 7H_2O$ (0.099), and 50 ml l^{-1} of "Metals 44" solution. The "Metals 44" solution contained (g l^{-1}): EDTA (2.50), $ZnSO_4 \cdot 7H_2O$ (10.95), $MnSO_4 \cdot 7H_2O$ (1.54), $FeSO_4 \cdot 7H_2O$ (5), $CuSO_4 \cdot 5H_2O$ (0.392), $Co(NO_3)_2 \cdot 6H_2O$ (0.248), $Na_2B_4O_7 \cdot 10 \ H_2O$ (0.177), and 1 ml l^{-1} of sulfuric acid to retard precipitation. Each solution was autoclaved (20 min, 121°C). Subsequent aliquots of stock solutions were withdrawn aseptically and inside a laminar flow hood. The MSB

was made by combining 40 ml of the solution A, 20 ml of the solution B, 5 ml of the solution C, and sterile deionized water to make 1 l.

Batch reactors

Experiments were performed in sterile 250 ml glass bottle reactors containing MSB and toluene and/or ethanol as the only growth substrates. Toluene was chosen among the BTEX as a model compound since PpF1 was reported to transform indole to indigo when induced on toluene (O'Connor et al. 1997).

Ethanol [80 or 120 mg l^{-1} (1.7 or 2.6 mmol l^{-1})], toluene [43 or 80 mg l^{-1} (0.5 or 0.9 mmol l^{-1})] or ethanol–toluene mixtures were used as growth substrate. The initial toluene concentration was chosen to be high relative to reported values of the half-saturation coefficient for toluene aerobic degradation [0.04–20 mg l^{-1} (0.43–217 µmol l^{-1})] (Button 1985; Robertson and Button 1987; Alvarez and Vogel 1991). Toluene and/or ethanol were added directly in the reactors containing 100 ml of MSB media previously autoclaved and sealed with Teflon (Mininert® valves) and aluminum crimps. Cells of PpF1 were transferred from a PIA (Pseudomonas isolation agar) plate to the 250 ml batch reactors using a sterile loop. The reactors were kept overnight inside an incubator-shaker (Brunswick Scientific C25KC) at 120 RPM and 28°C. Because TDO activity decreases exponentially with cell age (Costura and Alvarez 2000), all experiments were conducted using cells harvested in the exponential phase (after a period of approximately 8 h). More specifically, the MSB medium containing the cell suspension was transferred to a sterile 1.5 ml eppendorf and centrifuged at 10,000×g for 15 min (Eppendorf Centrifuge 5415D). After centrifugation, the supernatant was discharged and the cell pellet ressuspended in 1 ml phosphate buffer solution (pH = 7). The cells were then centrifuged and washed three times in the phosphate buffer to assure maximum cell purity and eliminate the potential for bacterial growth during analysis that could interfere with the results. Cell suspension was transferred from the eppendorf vial to a 1 ml polypropylene vial using a 1,000 µl sterile pipette. The initial biomass utilized in all experiments (OD_{610} = 1.95 ± 0.02) was measured in 1 ml polypropylene tube by specrophotometer analysis (Spectronic® Genesys™ 5). When necessary, the cell suspension was diluted with phosphate buffer solution to yield identical cell concentration in all sets of experiments.
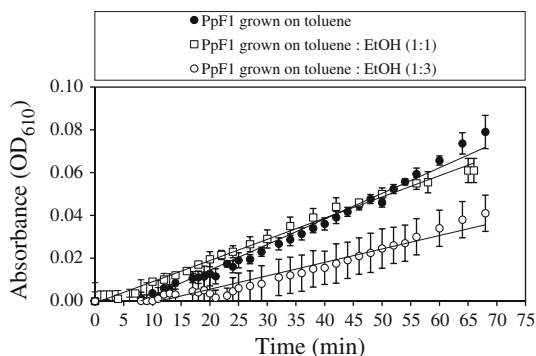
Spectrophotometer analysis

To determine the effect of ethanol on TDO activity, a previously developed spectrophotometric assay was used (O'Connor et al. 1997). Briefly, 300 µl of the concentrated cell suspension was transferred to 1 ml polypropylene vial containing 600 µl of phosphate buffer (pH = 7) and 0.25 mM indole. A 0.25 mM indole solution in 50 mM potassium phosphate buffer was made by dissolving indole in dimethylformamide (DMF) to a concentration of 100 mM and this was diluted in the same buffer to a final concentration of 0.25 mM indole. One polypropylene vial was analyzed per time in the spectrophotometer (Spectronic® Genesys™ 5) as the indigo produced come out of solution. The rate of indigo production was determined from the increase in optical density at wavelength of 610 nm (OD_{610}) over time normalized to initial absorbance at time zero. All experiments were performed in triplicate.

All chemicals used were of high purity ≥99% (Sigma®).

Results and discussion

The TDO system was selected because of its ubiquity in nature and its broad substrate range that includes several contaminants of concern, including BTEX. PpF1 cells induced on toluene are known to produce indigo from indole through the activation of TDO and its reaction with indole (Gibson and Parales 2000). The proposed pathway for indigo biosynthesis via TDO was previously demonstrated (O'Connor et al. 1997). The original indole to indigo method was modified in this work to accommodate ethanol as a co-substrate and to measure its effects on aerobic toluene biodegradation.

PpF1 harvested from toluene-fed reactors produced indigo from indole (Fig. 1). The TDO activity was estimated based on the OD_{610} readings measured during exponential (linear) phase of indigo production. Indigo production was not observed when ethanol was used as the sole source of carbon (Fig. 2). This was not surprising considering that

Fig. 1 Spectrophotometer readings of indigo production by *Pseudomonas putida* F1 after growth on toluene alone or toluene:ethanol (1:1 and 1:3 w/w) mixtures. *Error bars* represent ± one standard deviation from the mean of three replicates
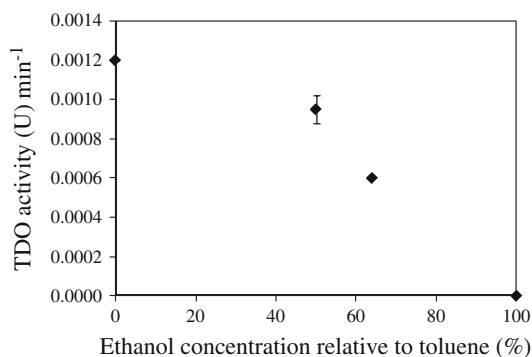
Fig. 2 Toluene dioxygenase activity (*U*) by *Pseudomonas putida* F1 after growth on toluene alone or toluene:ethanol (1:1 and 1:3 w/w) mixtures. No indigo formation was observed in cells grown on ethanol alone. *Error bars* represent ± one standard deviation from the mean of three replicates

ethanol metabolism occurs mostly by oxidation to acetaldehyde by an alcohol dehydrogenase enzyme (Kreb's cycle) (Madigan et al. 1997), which has no effect on the transformation of indole to indigo. Based on these results the indole to indigo spectrophotometric assay was used to infer on the potential effect of different concentrations of ethanol on toluene aerobic biodegradation.

Indoxyl spectrophotometric methods can be limited by interferences during OD measurements as a result of suspended biomass particles, or by limited amount of biomass available (Woo et al. 2000). In this work however, the interference of suspended biomass on indole to indigo transformation measurements was negligible. The increase in OD_{610} readings

were due to the transformation of indole to indigo and not by the suspended biomass as demonstrated by the negative controls (PpF1 induced on ethanol). Moreover, to avoid cell growth and biomass interferences during the analysis, the cells were repetitively washed in phosphate buffer to eliminate any carbon source that could serve as growth substrate during the assay.

The effect of ethanol on TDO was estimated based on the rates of indigo formation by PpF1 grown on toluene alone or toluene and ethanol mixtures (Figs. 1 and 2). Indigo production rate by cells induced on toluene alone was 0.0012 ± 0.0006 OD_{610} min^{-1}. Indigo production rates (i.e., TDO activity) decreased as with increasing ethanol concentration (Fig. 2). For the 3:1 (w/w) ethanol:toluene mixture, the rate of indigo production decreased by a factor of approximately 50%. This corroborates that the negative effect of ethanol on TDO activity increases with the content of ethanol in the substrate mixture (Lovanh and Alvarez 2004). Apparently, cells save energy by shutting the synthesis of enzymes such as TDO that are not needed for growth when ethanol is abundant (i.e., catabolite repression), because ethanol is metabolized via constitutive enzymes. Nonetheless, the presence of ethanol did not fully repress TDO activity when toluene was also used as carbon source, as previously reported by Lovanh and Alvarez (2004) for the bioluminescent reporter strain *Pseudomonas putida* TOD102. Whereas partial concentration-dependent repression of catabolic enzymes appears to be common for other substrate mixtures under carbon-limiting conditions (e.g., substrate ≪ biomass) (Egli 1995; Egli et al. 1982, 1983, 1993), the regulation of catabolite repression was beyond the scope of this work.

Figure 2 shows that the decrease in indigo production rate (U) is proportional to the increase in ethanol to toluene concentration ratio, and no indigo formation was observed for cells grown on ethanol alone. Since enzyme activities assays do not necessarily measure gene expression, these results mainly suggests that ethanol dilutes the metabolic flux of toluene. Metabolic flux dilution was previously defined as a noncompetitive inhibition of the specific utilization rate of a target substrate (e.g., toluene degradation rate per cell) due to the metabolism of an alternative substrate (e.g., ethanol) that is degraded by other enzymes (Lovanh et al. 2002; Lovanh and Alvarez 2004).

The high biochemical oxygen demand (BOD) exerted during ethanol biodegradation could interfere with the availability of oxygen, which is used as co-substrate for the oxygenases. The effect of the dissolved oxygen on indigo production (TDO activity) was not investigated in this work. However, previous studies showed that dissolved oxygen concentration during growth did not significantly affect TDO activity, which was observed even under hypoxic conditions (DO < 0.1 mg l^{-1}) (Costura and Alvarez 2000). Thus, the measured variations in specific rates of indigo production by PpF1 were not attributable to oxygen limiting conditions.

Although this spectrophotometric assay did not directly address gene repression, it served to demonstrate the interference of ethanol (a commonly found co-substrate in groundwater contaminated with reformulated gasoline) on aerobic toluene biodegradation. The TDO activity measured by the rates of indigo production decreased with increasing concentrations of ethanol. From a bioremediation perspective, the presence of ethanol in groundwater contaminated with aromatic hydrocarbons is likely to interfere with naturally occurring microorganisms from fully expressing their aerobic catabolic potential.

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