Effect of Ethanol, Acetate, and Phenol on Toluene Degradation Activity and *tod–lux* Expression in *Pseudomonas putida* TOD102: Evaluation of the Metabolic Flux Dilution Model

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Abstract: The reporter strain *Pseudomonas putida* TOD102 (with a *tod-lux* fusion) was used in chemostat experiments with binary substrate mixtures to investigate the effect of potentially occurring cosubstrates on toluene degradation activity. Although toluene was simultaneously utilized with other cosubstrates, its metabolic flux (defined as the toluene utilization rate per cell) decreased with increasing influent concentrations of ethanol, acetate, or phenol. Three inhibitory mechanisms were considered to explain these trends: (1) repression of the *tod* gene (coding for toluene dioxygenase) by acetate and ethanol, which was quantified by a decrease in specific bioluminescence; (2) competitive inhibition of toluene dioxygenase by phenol; and (3) metabolic flux dilution (MFD) by all three cosubstrates. Based on experimental observations, MFD was modeled without any fitting parameters by assuming that the metabolic flux of a substrate in a mixture is proportional to its relative availability (expressed as a fraction of the influent total organic carbon). Thus, increasing concentrations of alternative carbon sources "dilute" the metabolic flux of toluene without necessarily repressing *tod*, as observed with phenol (a known *tod* inducer). For all cosubstrates, the MFD model slightly overpredicted the measured toluene metabolic flux. Incorporating catabolite repression (for experiments with acetate or ethanol) or competitive inhibition (for experiments with phenol) with independently obtained parameters resulted in more accurate fits of the observed decrease in toluene metabolic flux with increasing cosubstrate concentration. These results imply that alternative carbon sources (including inducers) are likely to hinder toluene utilization per unit cell, and that these effects can be accurately predicted with simple mathematical models. © 2004 Wiley Periodicals, Inc.

Keywords: bioluminescence; catabolite repression; chemostat; metabolic flux dilution; substrate interactions; toluene dioxygenase

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INTRODUCTION

Toxic organic pollutants are often present with alternative carbon sources, which can influence the rate and extent of bioremediation. Monoaromatic hydrocarbons, for example, are often present in gasoline-contaminated aquifers together with ethanol (which is increasingly being used as an additive to meet Clean Air Act and renewable fuel requirements), acetate (a common metabolite), and phenol (a frequently co-occurring aromatic compound) (Lerner et al., 2000; Powers et al., 2001a, 2001b).

Numerous substrate interactions have been identified that affect monoaromatic hydrocarbon degradation. These include positive effects exerted by other aromatic substrates on enzyme induction (Alvarez and Vogel, 1991), proliferation of specific degraders (Alvarez et al., 1998), and co-metabolism (Alvarez and Vogel, 1991, 1995; Chang et al., 1993), as well as negative effects such as catabolite repression by labile compounds (Duetz et al., 1994a, 1997) and additional consumption of electron acceptors and nutrients that would otherwise be available to support hydrocarbon degradation (Corseuil et al., 1998; Da Silva and Alvarez, 2002). Quantifying and predicting the overall effect of alternative substrates on a target biocatalytic reaction is important for biological treatment process design and operation, including bioremediation and natural attenuation. However, this is a complex task that requires a better understanding of the effect of alternative carbon sources on gene expression and the resulting metabolic and population shifts. Yet, studies quantifying catabolic gene expression under mixed substrate conditions have been infrequent.

Several researchers have addressed the effect of alternative carbon sources on the induction or repression of specific catabolic enzymes (Dijkhuizen and Harder, 1979; Egli et al., 1982; Harder and Dijkhuizen, 1982; Law and Button, 1977; Schmidt and Alexander, 1985; Turner et al., 1988). However, most of these studies have reported only

qualitative aspects of gene expression or the resulting enzyme activity, which can be confounded by microbial growth and competitive inhibition. Except for a few studies that addressed the dynamics and regulation of enzyme synthesis in binary substrate mixtures (e.g., nitrilotriacetate monooxygenase expression in *Chelatobacter heintzii* fed glucose and nitrilotriacetate [Bally and Egli, 1996] and transketolase and transaldolase induction in methylotrophic yeast *Kloeckera* sp. 2201 fed methanol and glucose [Egli et al., 1983]), few data are available characterizing the effect of alternative substrates on catabolic enzyme induction.

Monoaromatic hydrocarbons, such as toluene, are degraded by inducible enzymes that are subject to positive gene control mechanisms (Wackett and Gibson, 1988). Such catabolic genes can be repressed when easily degradable (alternative) substrates are abundant (Epps and Gale, 1942). Ethanol, for example, has been reported to inhibit aromatic hydrocarbon degradation in aerobic batch studies (Corseuil et al., 1998; Ruiz Aguilar et al., 2002). This was hypothesized to be due to the repression of oxygenase enzymes that initiate catabolism, which would save cellular energy associated with the synthesis of inducible enzymes that would not be needed for growth on ethanol. Nevertheless, the effect of ethanol on the expression of specific catabolic genes has not been quantified, and it is unknown whether ethanol or its common metabolite, acetate (Da Silva and Alvarez, 2002), can exert catabolite repression under carbon-limiting conditions that are generally conducive to simultaneous utilization of multiple substrates (Daugherty and Karel, 1994; Egli, 1995; Harder and Dijkhuizen, 1982). Furthermore, the relationship between the degree of oxygenase enzyme induction and the resulting hydrocarbon metabolic flux has not been addressed in the literature.

In this work, carbon-limited chemostats were run with a bioluminescent reporter strain, *Pseudomonas putida* TOD102, to evaluate the effect of alternative substrates on the expression of a model catabolic gene (*tod*, coding for toluene dioxygenase) and the resulting effect on toluene metabolic flux. Experiments were conducted with two common labile substrates (ethanol and acetate) and a known inducer of the *tod* gene (phenol) (Applegate et al., 1998; Heald and Jenkins, 1996), and mathematical models were developed to characterize gene expression and toluene degradation kinetics as a function of the mixed substrate composition. Emphasis was placed on resolving the influence of three potential inhibitory mechanisms exerted by alternative carbon sources: (1) metabolic flux dilution; (2) catabolite repression; and (3) competitive inhibition.

EXPERIMENTAL METHODS

Bioreporter Strain

Pseudomonas putida F1 with a *tod–lux* fusion (also known as TOD102) was obtained from Jan Roelof van der Meer (EAWAG, Switzerland). The *tod* gene codes for toluene dioxygenase (an enzyme that initiates toluene degradation by dihydroxylation of the aromatic ring), whereas *lux* is a reporter gene coding for luciferase, an enzyme that produces bioluminescence from ATP hydrolysis in the presence of NADPH, oxygen, reduced riboflavin phosphate ($FMNH_2$), and aldehydes (Heitzer et al., 1998; Jaspers et al., 1999; Meighen, 1991). Thus, light production can be correlated with *tod* gene expression.

The central part of the *tod*X promoter contains a polymerase chain reaction (PCR) fragment between position 1813 and 2175 of the *tod*X sequence (Genbank U18304). SphI and XbaI sites were introduced in the PCR primers. This promoter fragment was then cloned into a *lux*ABCDE vector (pJAMA8) in the SphI and XbaI sites (Jaspers et al., 2000). The *tod*X–*lux*ABCDE fusion was then retrieved as a 2.8-kb NotI fragment and inserted into the NotI site of the mini-transposon vector pCK218. pCK218 contains another NotI insert, which was "replaced" by the *tod*X–*lux*ABCDE fusion. The orientation is such that the *tod*X promoter is closest to the kanamycin-resistance gene. This mini-Tn5 plasmid was then introduced by mating into *Pseudomonas putida* F1. The plasmid does not replicate in *Pseudomonas* and chromosomal transposon insertions can be obtained by selection for kanamycin resistance. The chromosomal insertion was verified by southern hybridization (Jaspers et al., 2000).

Oxygen and aldehyde availability can affect the bioluminescence emitted by bacteria with *lux* genes (Heitzer et al., 1998; Jaspers et al., 1999; Neilson et al., 1999). However, adequate oxygen supply (H_2O_2) and the short duration of our assays precluded oxygen limitation. Also, aldehyde was not limiting because a sufficient amount of *n*-decanal was added before sample measurement to enhance bioluminescence readings.

Chemostat Experiments

Small (300-mL) chemostats were constructed and run without a headspace to minimize volatile losses as described elsewhere (Lovanh et al., 2002), except that hydrogen peroxide (100 mg/L) was provided as an additional oxygen source for microbial growth. Unpublished batch studies conducted with TOD102 and previous column studies with mixed cultures (Anid et al., 1993) showed that this hydrogen peroxide concentration was not toxic to the microorganisms. Unless stated specifically, the dilution rate was set at 0.20 h^{-1}. This rate was sufficiently high to prevent cell attachment to chemostat walls and low enough to yield sufficient biomass for bioluminescence measurements. Note that this is an intermediate dilution rate in relation to the maximum specific growth rate for TOD102 on toluene ($\mu_{max} \approx 0.43\ h^{-1}$).

Toluene was initially fed alone, at 5 mg/L, as total organic carbon (TOC). Control runs without bacteria showed that toluene losses to volatilization and/or sorption were small (<5%). Multiple runs were conducted with binary substrate mixtures (i.e., with toluene and ethanol, acetate, or

phenol) by adding the cosubstrates incrementally at 1, 3, 5, and 10 mg/L as TOC. At least eight volume exchanges were allowed between runs to achieve steady state. Samples were collected during each run using 5-mL gas-tight syringes. Measurements were then taken to characterize the toluene metabolic flux and *tod* expression as a function of the influent mixture composition.

Toluene concentrations were measured by gas chromatography using a Hewlett-Packard (Model 5890) instrument equipped with an HP-19395A automatic headspace sampler, a flame ionization detector, and a 30-meter, 0.53-mm-diameter DB-wax column. Oxidation reduction potential (ORP) was measured to verify that aerobic conditions prevailed and oxygen was not limiting, using a Beckman Φ72 pH meter equipped with an ORP meter. The expression of *tod–lux* genes was quantified by bioluminescence using a luminometer (Model TD20e. Turner Designs), which reports it as relative light units (RLU). Decanal (0.1% by volume) was added to the sample earlier to obtain maximum light production from the tested microorganism. Specific bioluminescence (i.e., light per milligram of cell) was calculated by normalizing bioluminescence measurements to the microbial concentration, which was estimated from optical density measurements at 600 nm using the following correlation: milligrams per liter = 348 OD_{600} + 3.5; r^2 = 0.999 (Hunt et al., 1997). The relative induction of the *tod* gene was calculated by normalizing the specific bioluminescence of cells exposed to binary mixtures to the specific bioluminescence of cells fed toluene alone.

Model Development

Previous chemostat studies with binary substrate mixtures utilized to completion have shown that the specific utilization rate of a substrate is a function of the influent mixture composition (Egli et al., 1993; Lovanh et al., 2002). Kelly and colleagues (2000) modeled specific bioluminescence emitted by bioreporter strains *Pseudomonas putida* B2 and TBA8 (with *tod–lux* fusions) as a function of toluene utilization during trichloroethylene cometabolism, but this model did not consider the effect of alternative growth substrates on *tod* expression and toluene degradation. To evaluate potential inhibitory mechanisms exerted by alternative carbon sources, we developed mathematical models that describe metabolic flux dilution, competitive inhibition, and catabolic gene repression as a function of the inhibitory cosubstrate concentration.

Metabolic Flux Dilution

The metabolic flux of a compound can be defined as the rate at which it is metabolized per unit biomass. Therefore, the specific substrate utilization rate (U, expressed in grams substrate per gram cells per hour) is a measure of metabolic flux. In a chemostat, U can be calculated using experimental data as the product of the chemostat dilution rate (D = flow rate per unit volume) times the different between the influent (C_0) and effluent (C) concentrations, normalized to the biomass concentration (X):

$$U = \frac{D(C_0 - C)}{X} \tag{1}$$

The term *metabolic flux dilution* (MFD) was coined to describe a form of noncompetitive inhibition wherein the rate of utilization of one substrate decreases due to the metabolism of another that is not necessarily degraded by the same enzymes. Conceptually, the substrates could be initially transformed by different pathways that eventually converge into central metabolic intermediates (e.g., acetyl-CoA) and a common catabolic pathway (e.g., the Krebs cycle) (Fig. 1). This would create a bottleneck that decreases the degradation rate of a target compound (e.g., toluene) when the utilization of another (e.g., ethanol, acetate, or phenol) increases and common metabolic intermediates accumulate and exert feedback inhibition. Other causes for MFD include increased biomass concentration (X) due to the utilization of alternative substrates, which decreases U [Eq. (1)], as well as the increased availability of alternative substrates, which decreases the probability that a cell would come in contact with (and assimilates) the target pollutant.

MFD was modeled without any fitting parameters by assuming that the metabolic flux of toluene in a substrate mixture is proportional to its relative availability (Lovanh et al., 2002):

$$U = fU_0 \tag{2}$$

where U is the specific toluene utilization rate when present in a substrate mixture, U_0 is the specific toluene utilization rate when fed alone, and f is the relative availability of toluene, expressed as a fraction of the influent TOC. Thus, f is the independent variable. This empirical equation was tested experimentally with various substrates, including a known inducer (phenol) and potential repressors of the *tod* gene (acetate and ethanol) (Fig. 2). In all cases, a similar

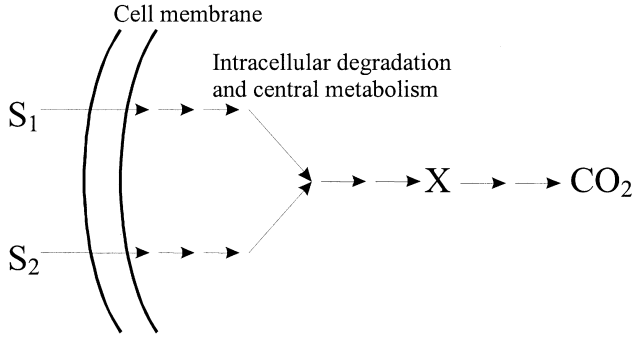

Figure 1. Conceptual diagram of metabolic flux dilution in a bacterium processing two substrates with converging catabolic pathways (modified after Hunt, 1999).

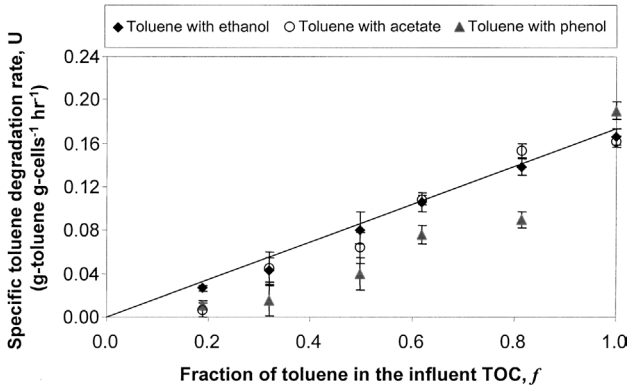

Figure 2. Toluene metabolic flux increased linearly with the fraction of toluene in the influent TOC for experiments with different binary substrate mixtures. Solid line depicts Eq. (2).

linear relationship between U and f was observed, which reflects that increasing the concentration of alternative carbon sources (i.e., decreasing f) "dilutes" the toluene metabolic flux, measured as U. However, the model (solid line in Fig. 2) overestimated U, especially at low values of f (i.e., high concentration of alternative carbon sources). This suggests that additional inhibitory mechanisms may be important, especially at higher cosubstrate concentrations. These are considered in what follows.

Competitive Inhibition

The competitive inhibition mechanism occurs when two substrates (e.g., toluene and phenol) are degraded by the same enzyme (e.g., toluene dioxygenase) and both substrates compete for binding to the active site. Thus, the rate of catalysis of the target substrate is diminished by reducing the proportion of enzymes that are bound to it. Competitive inhibition can be modeled by modifying Monod's equation (Alvarez-Cohen and Speitel, 2001; Chang et al., 1993):

$$U = \frac{k\,C}{K_S\left(1+\frac{I}{K_I}\right)+C} \tag{3}$$

where k is the maximum specific substrate utilization rate (grams substrate per gram cells per day), K_S is the half-saturation coefficient (milligrams per liter), C is the concentration of toluene to which cells are exposed to (milligrams per liter), I is the inhibitor concentration (taken here as the influent phenol concentration, in milligrams per liter), and K_I is the inhibition coefficient (milligrams per liter).

Eq. (3) becomes Monod's equation when no inhibitor is present:

$$U_0 = \frac{k\,C_{\text{alone}}}{K_S + C_{\text{alone}}} \tag{4}$$

where C_{alone} is the toluene concentration in the chemostat when fed alone.

To model the combined effects of metabolic flux dilution and competitive inhibition, it is convenient to divide Eq. (3) by Eq. (4):

$$\frac{U}{U_0} = \frac{C/C_{\text{alone}}(K_S + C_{\text{alone}})}{K_S\left(1+\frac{I}{K_I}\right)+C} \tag{5}$$

This equation can be simplified and expressed in terms of the influent mixture composition by first incorporating Monod's equation into the metabolic flux dilution concept [Eq. (2)] as follows:

$$f = \frac{U}{U_0} = \frac{\frac{C}{K_S+C}}{\frac{C_{\text{alone}}}{K_S+C_{\text{alone}}}} = \left(\frac{C}{K_S+C}\right)\left(\frac{K_S+C_{\text{alone}}}{C_{\text{alone}}}\right) \tag{6}$$

Solving for C:

$$C = f\,C_{\text{alone}}\frac{K_S}{(1-f)C_{\text{alone}}+K_S} \tag{7}$$

Substituting Eq. (7) into Eq. (5) yields:

$$\frac{U}{U_0} = \frac{f(K_S + C_{\text{alone}})}{((1-f)C_{\text{alone}}+K_S)\left(1+\frac{I}{K_I}\right)+f\,C_{\text{alone}}} \tag{8}$$

Note that C_{alone} can be calculated using a common expression derived from mass balances for steady-state chemostats (Pirt, 1975):

$$C_{\text{alone}} = \frac{DK_S}{\mu_{\max} - D} \tag{9}$$

where $\mu_{\max}$ is the maximum specific growth rate and D is the chemostat dilution rate. Substituting Eq. (9) into Eq. (8) and simplifying we get an equation that describes a decrease in the specific toluene utilization rate as a result of the combined effects of metabolic flux dilution and competitive inhibition:

$$U = \frac{U_0 f}{\left(1-f\frac{D}{\mu_{\max}}\right)\left(1+\frac{I}{K_I}\right)+f\frac{D}{\mu_{\max}}} \tag{10}$$

Catabolite Repression

This mechanism refers to the repression of inducible enzymes (e.g., toluene dioxygenase) by the presence of a "preferred" carbon source (e.g., acetate or ethanol) degraded by constitutive enzymes through central metabolic pathways.

Catabolite repression was modeled as a modulated mechanism where the induction of the catabolic gene of interest (e.g., *tod*) decreases with increasing concentrations of the repressor (e.g., ethanol or acetate). Conversely, *tod* gene expression is assumed to increase with increasing relative availability of toluene in the mixture:

$$[\text{TDO}] = f[\text{TDO}]_{\max} \tag{11}$$

Table I. Biokinetic parameters used in Eq. (10) for modeling the inhibitory effects of phenol on the specific toluene biodegradation rate for TOD102 in steady-state chemostats.

Parameter	Value
Dilution rate (D) per hour	0.20
μ_{max} (per hour)	0.43
K_I (mg/L)[a]	3.5

[a]Reported values of K_I for phenol range from 0.2 to 10 mg/L (compiled in Alvarez-Cohen and Speitel, 2001).

where [TDO] is the level of induction of toluene dioxygenase in bacteria exposed to a toluene–repressor mixture and $[TDO]_{max}$ is the induction level when toluene is the only substrate.

Assuming that the specific toluene utilization rate, U, is proportional to the level of *tod* expression when a repressor is present, Eq. (10) implies that catabolite repression would cause U to be proportional to f. Recalling that MFD separately implies that U is also proportional to f [Eq. (2)], a simple empirical equation was postulated to combine the effects of metabolic flux dilution and catabolite repression:

$$U = f^2 U_0 \tag{12}$$

Model Parameters

Table I summarizes the biokinetic parameters used in this modeling exercise. The maximum specific growth rate (μ_{max}) was determined from batch incubations with acclimated cells (amended with 100 mg/L toluene) as the slope from the increase in $\ln(OD_{600})$ versus time during the exponential growth phase. K_S was measured as the effluent steady-state concentration of toluene (in the absence of cosubstrates) when the chemostat dilution rate (D) is set at 50% of μ_{max}. The phenol inhibition coefficient (K_I) was obtained by fitting U versus f data with Eq. (10) using nonlinear regression techniques (Berthouex and Brown, 1994) with a Microsoft Excel spreadsheet. Specifically, the selected K_I value was that which minimized the sum of squared differences between simulated [Eq. (10)] and experimental data [Eq. (1)]). Note that K_I was the only fitting parameter used in this exercise as all other parameters were independently obtained. Furthermore, the selection of K_I was constrained to a range of values reported in the literature (Alvarez-Cohen and Speitel, 2001).

RESULTS AND DISCUSSION

Effect of Cosubstrates on *tod–lux* Induction

Toluene (5 mg/L as TOC) was initially fed alone to chemostats seeded with TOD102. This resulted in a steady-state, specific bioluminescence of about 50 RLU mg cell L^{-1} (100%). Adding acetate at low concentrations (1 mg L^{-1} as TOC) decreased the specific bioluminescence by about 80% (Fig. 3). Note that hydrogen peroxide was added to preclude oxygen limitation, which was confirmed by positive ORP (data not shown). Therefore, acetate repressed the expression of the *tod* gene under carbon-limiting conditions.

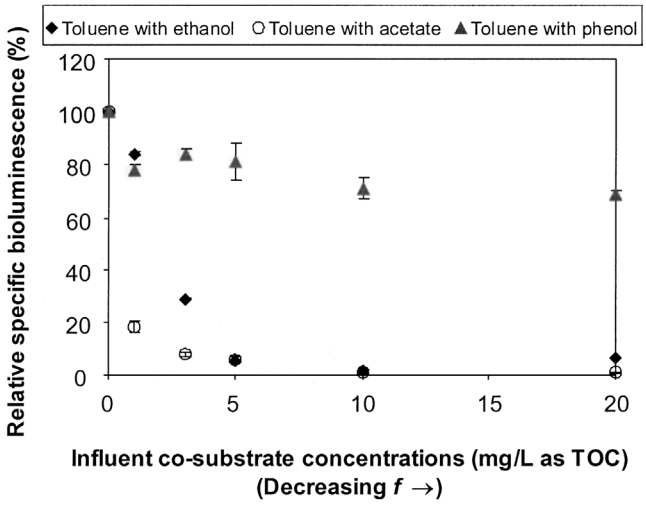

Figure 3. Comparison of the effect of ethanol, acetate, and phenol on *tod* expression by *Pseudomonas putida* TOD102 in carbon-limited, steady-state chemostats.

Many studies have reported catabolite repression in pseudomonads by Krebs cycle intermediates and by acetate, which can enter the Krebs cycle directly through the glyoxylate shunt (Madigan et al., 2002). These studies include inhibition of oxalate degradation by acetate (Harder and Dijkhuizen, 1982), of glucose and mannitol utilization by succinate (MacGregor et al., 1991), of aniline metabolism by organic acids (Helm and Reber, 1979), and of protocatechuate degradation by succinate (Zylstra et al., 1989). All these reports correspond to batch studies with carbon sources supplied in abundance. However, Duetz et al. (1994a, 1997) also reported repression of the upper TOL pathway (which initiates toluene degradation by oxidizing the methyl group) by glucose and succinate in carbon-limited chemostats with *P. putida* pWW0. Catabolite repression was attributed to a high-energy status of the cells rather than directly to the growth rate or the presence of alternative growth substrates (Duetz et al., 1996). These previous studies provided a valuable framework for understanding the biochemical and genetic basis of catabolite repression, but did not quantify how the substrate mixture composition affects catabolic gene expression and specific degradation activities.

Ethanol also exerted catabolite repression, although the decrease in relative specific bioluminescence was not as pronounced as with acetate (Fig. 3). In both cases, the degree of *tod* repression increased with increasing repressor concentration (i.e., decreasing relative availability of toluene). However, complete repression did not occur under carbon-limiting conditions, and there was a basal induction

level (5 ± 2 RLU mg cells^{-1} L) even at the highest ethanol and acetate concentrations tested. This is consistent with previous observations of concurrent toluene degradation with ethanol in chemostat settings (Lovanh et al., 2002).

Phenol did not significantly hinder *tod* expression when added concurrently with toluene. This corroborates previous reports that phenol can induce the *tod* gene (Applegate et al., 1998; Heald and Jenkins, 1996). However, phenol was not as effective as toluene for inducing *tod* (e.g., 30 versus 80 RLU mg cell^{-1} L in batch reactors fed at 50 mg TOC L^{-1}). Applegate and colleagues (1998) also found that the specific bioluminescence from another bioreporter strain with a *tod–lux* fusion was lower with phenol than with toluene (70 versus 234 nA/OD_{546}). This would explain the lower specific bioluminescence for phenol–toluene mixtures relative to experiments with toluene alone (Fig. 3).

Effect of Alternative Carbon Sources on Toluene Metabolic Flux

Analytical models that consider different potential inhibitory mechanisms were developed to simulate the measured specific toluene utilization rate (U, a surrogate for toluene metabolic flux) as a function of the alternative carbon source concentration.

Phenol inhibited toluene metabolic flux (Fig. 4), but it did not repress the *tod* gene (Fig. 3). Because oxygen was not limiting, and all substrates were added at below toxic levels, this inhibitory effect was attributed to MFD and/or competitive inhibition. The MFD model, which does not include any fitting parameters [Eq. (2)], slightly overpredicted the specific toluene degradation rate (Fig. 4) (R^2 = 0.88), and incorporating competitive inhibition [Eq. (10)] improved the goodness of fit (R^2 = 0.97). Note that the biokinetics parameters used for Eq. (10) (Table I) were independently determined (except of K_I), as previously described. All model parameters were within the range of values reported in the literature (Alvarez-Cohen and Speitel, 2001; Button, 1985; Duetz et al., 1994b; Robertson and Button, 1987).

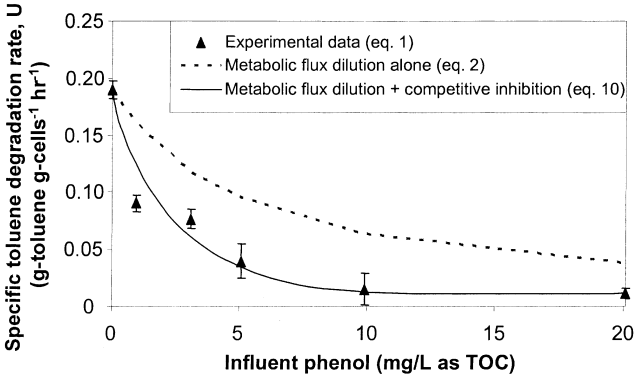

Figure 4. Decrease in toluene metabolic flux with increasing influent phenol concentration.

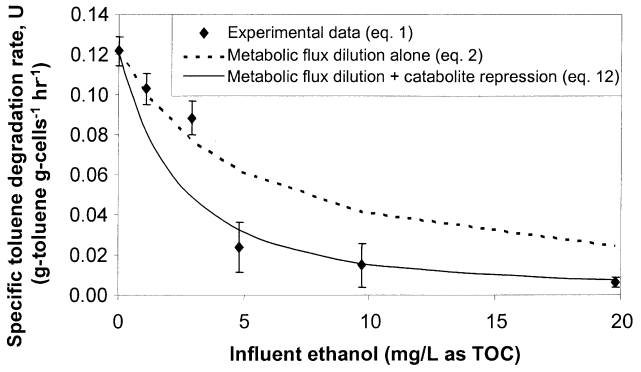

Figure 5. Decrease in toluene metabolic flux with increasing influent ethanol concentration.

A similar decrease in toluene metabolic flux was observed with increasing concentrations of ethanol (Fig. 5) or acetate (data not shown). Similar to experiments with phenol, MFD simulations with Eq. (2) slightly overpredicted the observed specific toluene degradation rates, suggesting that other inhibitory mechanisms may have been important. Incorporating an empirical relationship for catabolite repression, which also does not contain any fitting parameters (Eq. (12)], improved the goodness of fit (e.g., R^2 increased from 0.79 to 0.91 for toluene–ethanol mixtures) (Fig. 5).

Figure 6 depicts the relationship between toluene metabolic flux and *tod* expression in the presence of a repressor (ethanol) in a replicate experiment with toluene–ethanol mixtures. The data suggest that there should be no toluene utilization without *tod* induction (i.e., the Cartesian origin), and that the level of induction (i.e., the intracellular concentration of toluene dioxygenase) may be a rate-limiting factor in the absence of mass transfer limitations. Specifically, as f increases, higher levels of *tod* expression result in a higher toluene metabolic flux. Some proportionality between *tod* expression and toluene metabolism was also observed in experiments with acetate, especially at low

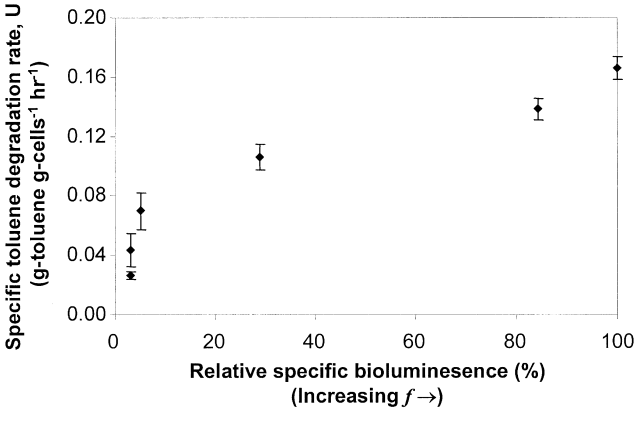

Figure 6. Specific toluene degradation rate as a function of relative specific bioluminescence in *Pseudomonas putida* TOD102 under carbon-limited, steady-state chemostats with toluene–ethanol mixtures.

f values (data not shown), but not in experiments with phenol, which does not exert catabolite repression but competitively inhibits toluene dioxygenase activity.

These results show that alternative carbon sources (including catabolic enzyme inducers) are prone to hinder the rate of toluene degradation per cell. The similar decrease in toluene metabolic flux due to increasing concentrations of alternative substrates that exert different effects on *tod* expression (Figs. 4 and 5) suggests that metabolic flux dilution might be a predominant inhibitory mechanism under carbon-limited conditions.

This work has focused on quantifying the expression of a model catabolic gene and related biodegradation processes at the cellular level in a reductionist effort to resolve how alternative carbon sources affect bioremediation. However, the effects of alternative substrates on other processes such as microbial growth and electron acceptor availability were not considered. Although such processes could exacerbate inhibition by alterative carbon sources (e.g., accelerated oxygen depletion), it is also plausible that some cosubstrates could enhance aromatic hydrocarbon bioremediation by fostering the proliferation of specific degraders (Alvarez et al., 1998). Therefore, future experimental and modeling efforts involving substrate interactions should consider effects on microbial community structure.

CONCLUSIONS

Chemostat experiments were conducted with a bioluminescent reporter strain (with a *tod–lux* fusion) to quantify the effect of alternative carbon sources on toluene degradation activity and toluene dioxygenase induction as a function of the binary substrate mixture composition.

The toluene metabolic flux decreased with increasing influent concentrations of ethanol, acetate, and phenol, and this decrease followed similar patterns despite the opposite effects that phenol (an inducer) and acetate or ethanol (repressors) exerted on *tod* expression. This phenomenon was postulated to be partly due to metabolic flux dilution, a form of noncompetitive inhibition that implies that the utilization of a carbon source in a mixture is proportional to its relative availability. The inhibitory effect exerted by acetate and ethanol was exacerbated by catabolite repression. Similarly, competitive inhibition exacerbated the inhibitory effect of phenol on specific toluene degradation rates. In all cases, the measured toluene metabolic flux was accurately simulated as a function of the influent cosubstrate concentration, using mathematical models that incorporated these three potential inhibitory mechanisms with independently determined parameters.

This work implies that alternative carbon sources (including catabolic enzyme inducers) are likely to hinder toluene metabolic flux, which does not necessarily preclude a potential enhancement in overall bioremediation rates due to additional growth of toluene degraders.

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