

Enhancement of a Two-Phase Partitioning Bioreactor System by Modification of the Microbial Catalyst: Demonstration of Concept

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Abstract: Application of two-phase partitioning bioreactors (TPPB) to the degradation of phenol and xenobiotics has been limited by the fact that many organic compounds that would otherwise be desirable delivery solvents can be utilized by the microorganisms employed. The ability to metabolize the solvent itself could interfere with xenobiotic degradation, limiting remediation efficiency, and hence represents a microbial characteristic incompatible with process goals. To avoid the issue of bioavailability, previous TPPB applications have relied on complex and often expensive delivery solvents or suboptimal catalyst-solvent pairings. In an effort to enhance TPPB activity and applicability, a genetically engineered derivative of *Pseudomonas putida* ATCC 11172 mutated in its ability to utilize medium-chain-length alcohols was generated (AVP2) and applied as the catalyst within a TPPB system with decanol as the delivery solvent. Kinetic analysis verified that the genetic alteration had not negatively affected phenol degradation. The volumetric productivity of AVP2 (0.48 g/L h⁻¹) was equivalent to that seen for wild-type ATCC 11172 (0.51 g/L h⁻¹), but a comparison of initial cell concentrations and yields revealed an improved phenol-degrading efficiency for the mutant under process conditions. Yield coefficients, cell dry weight, and viable count determinations all confirmed the stability of the modified phenotype. This work illustrates the possibilities for TPPB process enhancement through a careful combination of genetic modification and solvent selection. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* 79: 587–594, 2002.

Keywords: two-phase partitioning bioreactor; metabolic engineering; process enhancement; biodegradation; solvent

INTRODUCTION

The release of various toxic compounds into the environment can often result in considerable costs to human health and the environment. The detrimental consequences of this release have included immediate toxicity to exposed biological populations, accumulation and

degeneration of local sites, and pollutant transport leading to contamination of air, soil, groundwater, and waterways at locations far removed from the original site of discharge. Phenol is a toxic, highly water-soluble compound utilized in nylon production, and commonly found in the effluents from coking and petroleum manufacturing plants. The extensive production and use of phenols in industry has resulted in wastewaters containing phenolic concentrations ranging as high as 3 g/L (Annadurai et al., 2000) and, consequently, phenol has been classified as a priority pollutant by both Canadian and U.S. environmental protection organizations (Canadian Environmental Protection Agency, 2001; U.S. Environmental Protection Agency, 1998).

Despite being toxic, various contaminants, including phenol, can be utilized by microbes as carbon and energy sources (Gibson, 1968). Harnessing this degradative potential has been an area of considerable study and numerous biodegradative remediation approaches have been developed with impressive success at eliminating xenobiotics from the environment (Garbisu and Alkorta, 1999). A key issue in biodegradative approaches to pollutant remediation is delivery of the toxic compound to the microbial catalysts at an appropriate concentration to avoid nutrient limitation while not exceeding the microbes' exposure threshold. This is a considerable challenge because contaminant levels in the environment are often several orders of magnitude greater than microbial tolerance and target environmental limits (Singleton, 1994). As a result, treatments of concentrated wastes through biodegradative approaches have often been multistep processes requiring contaminant dilution prior to degradation.

One strategy that has been developed to address issues of contaminant delivery is the two-phase partitioning bioreactor (TPPB) system (Collins and Daugulis, 1996). In this scheme, an immiscible and biocompatible organic solvent phase is used to deliver toxic substrates to cells

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in the aqueous phase at subinhibitory levels. The concentration of the target molecule in each phase is specifically determined by the partition coefficient of the organic solvent. The presence of an appropriate second phase enables high levels of pollutant (substrate) to be introduced into the reactor while only subinhibitory concentrations result in the aqueous phase. As the catalyst degrades the pollutant in the aqueous phase, more substrate partitions from the organic phase to reestablish equilibrium, hence creating a demand-based pollutant delivery system. The key to the effectiveness of TPPB processes lies in systematic selection of the solvent phase that must, in addition to being immiscible, exhibit phase stability and have a high partition coefficient and low solubility in the aqueous phase (Bruce and Daugulis, 1991).

The nature of the solvent is critical not only in the substrate transfer between the two phases, but also in its interaction with the microbial catalysts being used in the system. The solvent must be biocompatible so as to not negatively impact on cell growth and metabolism. The solvent must not, however, be metabolized by the organism to prevent competition between degradative activities, and solvent losses in the system. This is a particular challenge in xenobiotic degradation wherein the organisms most effective at degrading a broad range of contaminating chemicals (e.g., *Pseudomonas*, *Arthrobacter*) exhibit a metabolic capability for a wide variety of organic compounds (Efroymsen and Alexander, 1991; Stanier et al., 1966), including a number of desirable candidate solvents. Metabolism of the delivery solvent itself may interfere with, or entirely outcompete, degradation of the toxic substrate, thereby limiting biodegradation efficiency. The bioavailability of solvents prevents their use as the delivery phase, and hence, despite the considerable success of TPPB systems, their application has been limited by the need to use complex, medium-chain-length compounds that are not biologically active, but may often be difficult to obtain, and possibly of considerable cost.

The objective of our work has been to genetically modify a catalyst to eliminate its ability to grow on a particular class of compounds exhibiting desirable solvent characteristics, hence enabling their use as the delivery phase within two-phase bioreactors. Medium-chain-length alcohols and *P. putida* ATCC 11172 were selected as the model system to demonstrate that metabolic engineering can be applied to alter a catalyst to be functional within a user-defined system configuration for the degradation of toxic substrates, with phenol being used as the target pollutant. The present work examines characteristics of a genetically modified derivative of ATCC 11172 from the perspective of various process considerations, and demonstrates its successful implementation as the biocatalyst within the TPPB system. Through this work we expand the options for solvent and catalyst selection, and demon-

strate that the organism itself can be a modifiable parameter in efforts to enhance TPPB system operation and applicability.

MATERIALS AND METHODS

Microorganisms and Medium Formulation

Pseudomonas putida ATCC 11172 and a mutant strain unable to use mid-chain alcohols (*P. putida* AVP2; Vrionis et al., 2002) were used to degrade phenol within a two-phase reactor system. The alcohol nonutilizing derivative was obtained by insertional inactivation using miniTn5*Km*::put, and selection for mutants no longer exhibiting growth on decanol as the sole carbon source. Transposon insertion in the identified mutant, AVP2, was within *agmR*, a response regulator influencing degradation of a variety of carbon compounds. The growth medium was a synthetic mineral salts medium comprised of (per liter): 6 g K₂HPO₄, 4 g KH₂PO₄, and 2 g (NH₄)₂SO₄, augmented with a divalent salt solution (0.66 g MgCl₂ and 0.25 g CaCl₂); 0.5% (vol/vol) trace element solution (0.3 g H₃BO₃, 0.089 g ZnSO₄ · 7H₂O, 0.024 g NiSO₄, 0.018 g (NH₄)₆Mo₇O₂₄ · 4H₂O, 0.003 g CuSO₄ · 5H₂O, 0.050 g MnSO₄, and 0.190 g CoCl₂ per 1 L distilled H₂O); 0.0006% FeCl₃ [vol/vol]; and supplemented with carbon sources as indicated in what follows. The various medium solutions were autoclaved separately to prevent precipitate formation. For maintenance, the carbon source was either 5 g/L glucose or 11 g/L sodium pyruvate. In inoculum build-up for fermentation runs, the mineral salts medium was supplemented with 5 g/L glucose and 250 mg/L phenol to ensure that cells were preacclimated to phenol, hence minimizing substrate-related lag phases. Solid agar was prepared by addition of Bacto-agar to 1.5% (wt/vol) (Difco Laboratories, Detroit, MI).

Cells were grown in Erlenmeyer flasks in a shaker bath overnight at 30°C on pyruvate, and subsequently subcultured in glucose/phenol medium for 24 h. This culture was considered the stock, and was used to inoculate starter cultures for fermentor runs. Starter cultures were prepared as 20% (vol/vol) inoculum (for fed-batch experiments) or 10% (vol/vol) (for kinetic experiments) and were also grown for 24 h. Prior to inoculation, the cells were centrifuged, a portion of the supernatant removed, and the pellet resuspended in 50 mL of medium, which was then used as the inoculum for fermentations.

Biocompatibility

Biocompatibility studies were conducted in 250-mL Erlenmeyer flasks containing 50 mL of growth medium with 5 g/L glucose and 5 mL of organic solvent. Prepared flasks were inoculated with either *P. putida* AVP2

or wild-type. Solvents were selected to span a $\log P_{\text{octanol}}$ range of 2.2 to 4.7. A control flask contained glucose with no added solvent. Utilization of glucose and cell growth were assessed to verify that the genetic alteration in AVP2 had in no way affected the cells' solvent tolerance. After 24, 48, and 72 h, 3-mL aqueous samples were removed from the flasks. Cell growth was assessed by measurement of optical density at OD_{600} (Ultraspec 3000 spectrophotometer, Biochrom, Cambridge, UK), and this value was compared with a standard curve to determine cell dry weight in grams per liter. The samples were then centrifuged to separate phases, and analysis of glucose concentration was conducted using the dinitro salicylic acid reagent (DNS) method (Miller, 1959). The color reaction was quantified by A_{650} measurement and glucose concentrations determined by comparison to a standard curve. Net changes in cell dry weight (CDW) and glucose concentration were used to calculate percent metabolic activity of the cultures relative to the control, and the critical $\log P$ value of the organisms was assessed.

Stability Analysis

To assess the long-term stability of the modified strain (AVP2), 250-mL Erlenmeyer flasks containing 50 mL of synthetic minimal medium and 5 mL of decanol were prepared and inoculated with 5 mL of either AVP2 or wild-type. Additional flasks were prepared containing 250 mg/L of phenol in addition to decanol, and inoculated; the phenol concentration in these flasks was allowed to go to zero. A second set of dual-substrate flasks was prepared, but in this case phenol supplementation was performed as necessary to maintain a nonzero phenol concentration within the flasks. Samples were taken from each flask daily, washed, serially diluted, and plated to Luria–Bertani broth (Difco) as well as synthetic medium plates, with decanol as the only carbon source (100 μ L added to Petri plate lids). Plates were incubated at 30°C overnight and colony forming units (CFU)/mL determined.

The stability of AVP2 under process conditions in two-phase fermentations was verified by continuing to provide essential growth nutrients for 1 to 2 days beyond phenol depletion, and measuring the OD_{600} . In addition, samples collected at various timepoints throughout fermentation (including point of phenol exhaustion and 36 h subsequently) were collected, washed, diluted, and plated on minimal/decanol plates. These plates were incubated at 30°C for 24 h and then evaluated for growth.

Kinetics

Kinetics analyses of both the wild-type and the genetically modified strain were performed in single-phase

fermentations with phenol concentrations ranging from approximately 180 to 735 mg/L. Agitation and aeration were initially set at 375 rpm, and 1 vvm, respectively, but were increased to a maximum of 500 rpm and 1.5 vvm as necessary to ensure the system did not become oxygen-limited (dissolved oxygen $\geq 10\%$). Plots of cell dry weight and phenol concentration vs. time were used to discriminate between the lag, exponential, and stationary phases of growth. $\ln(X/X_0)$ for the exponential period was calculated, graphed against time, and the specific growth rate was determined by the method of least squares from the slope of this graph. The specific growth rates were graphed against phenol concentration, and a comparison of the plots for the wild-type and modified strain was performed.

Fermentations

Fermentations with both wild-type and AVP2 were conducted in 5-L fermentor vessels (Bioflo III, New Brunswick Scientific Co., Edison, NJ) containing 2 L of synthetic medium. The head plate was fitted with dissolved oxygen and pH probes, as well as a condenser to limit solvent and substrate loss due to volatilization. The stir shaft of the fermentation vessel was fitted with two Rushton impellers to improve mixing. Sterilized fermentors were seeded with a concentrated cell inoculum as described earlier. For phenol delivery, 2 L of decanol loaded with 18 g of phenol was introduced into the reactor vessel. The fermentor was operated at 400 rpm and aerated at 1.5 vvm based on the aqueous volume. The pH of the fermentation broth was automatically maintained between 6.9 and 7.0 using 2N KOH, and the temperature was controlled at 30°C. Aqueous and organic phase phenol concentrations were sampled at least every 6 h to follow degradation and to ensure a nonzero phenol concentration within the reactor. An abiotic control was run to assess possible phenol loss due to volatilization.

Phenol spikes were fed to each system by pumping in (Masterflex, Cole Palmer, Vernon Hills, IL) 50 mL of decanol containing 18 g of phenol over approximately 12 min. To avoid nutrient limitation, the fermentation broth was also supplemented with a nutrient solution, concentrated sufficiently to present a standard formulation in 50 mL, at the times of phenol feeding.

Analytcs

Samples (10 mL) from fermentors were centrifuged at $\sim 10,000 \times g$ (Jouan MR 1822, Canberra Packard, Montreal, PQ, Canada) for 5 min to separate aqueous and organic layers for sampling. Cells at the interface were redispersed in the aqueous phase. Biomass accumulation within the fermentor was determined by measuring changes in the OD_{600} of the aqueous phase

over time. Wall growth on the fermentor vessels and baffles was observed, and is recognized as a source of biomass underestimation.

Phenol concentrations in both the aqueous and organic phase were determined using the 4-aminoantipyrine method (der Yang and Humphrey, 1975) and measurement of absorbance at 510 nm. The phenol concentration in the organic phase was determined indirectly using a contacting protocol. Three milliliters of the solvent phase was contacted with an equal volume of synthetic mineral salts medium by vortexing for 1 min. The contacted samples were centrifuged at $\sim 10,000 \times g$ for 5 min to allow phase separation, and then 1 mL of the medium was analyzed. Mass balance and the previously determined partition coefficient were then used to determine the phenol concentration in the organic phase.

RESULTS

Biocompatibility and Solvent Selection

Figure 1 presents the results of metabolic and growth analysis of wild-type *P. putida* ATCC 11172 and the genetically modified derivative, AVP2, in the presence of solvents with a range of logPs from 2.2 to 4.7, which encompasses the predicted logP value of *P. putida* (3.1) obtained from the literature (Inoue and Horikoshi, 1991). Activity was assessed as a function of glucose consumed and increase in cell density relative to the control that contained no solvent. The experimental results identified a logP value of 3.3 for both wild-type 11172 and AVP2. This corresponded with the previously determined logP for *P. putida* ATCC 11172 of approximately 3.2 (Collins and Daugulis, 1997a), and revealed

that the genetic alteration in AVP2 had in no way affected the solvent tolerance of the organism.

Based on Extractant Screening Program (ESP) predictions, a program that combines UNIFAC parameters and system modeling equations to predict compositions of each phase in TPPB systems (Bruce and Daugulis, 1991), medium-chain-length alcohols have the desirable characteristics of a delivery solvent, including a high-partition coefficient, and logP values above the critical logP for the organism to be used in this work. Furthermore, these compounds are readily available in bulk and are less costly than the more complex organics often required in TPPB systems to avoid the issue of bio-availability.

Previous TPPB work with *P. putida* ATCC 11172 successfully employed 2-undecanone as the delivery solvent (Collins and Daugulis, 1997a, 1997b). A comparison of this solvent with decanol (a representative medium-chain-length alcohol) revealed similarly high boiling points (227.9° and 230.2°C), low aqueous solubility (20 and 37 mg/L), and equivalent logP values (3.91 and 3.90) for the two compounds (Yaws, 1999). Decanol exhibits a lower partition coefficient than 2-undecanone (24.7 vs. 46.5), but this value is sufficiently high to partition a large substrate load and supports application of decanol as the delivery phase in a TPPB system. The cost of 2-undecanone is currently \$158.50/L (Fisher-Acros, 2000/01 Catalog \$CAD), whereas the cost of an equivalent volume of decanol is only \$17.80. This comparison of costs of the two solvents reveals the real potential benefit of enabling utilization of medium-chain-length alcohols as the delivery phase in TPPB.

Bioavailability and Phenol Kinetics

As was known prior to the outset of this work (Collins and Daugulis, 1997a), *P. putida* ATCC 11172 is able to utilize medium-chain-length alcohols as growth substrates. This characteristic is undesirable if these chemicals are to be used as the second phase, because degradation of the solvent itself could negatively affect the kinetics of the desired degrading reactions, and could result in solvent losses within the system. Together, these effects would interfere with overall system functioning and equilibrium, leading to decreased efficiency in system performance and increased overall operational cost. Through genetic engineering we have generated AVP2, which no longer consumes medium-chain-length alcohols as a carbon source. In creating the alcohol nonutilizing derivative we did not intend to negatively affect the phenol-degrading capabilities of the *P. putida* strain. To ensure that AVP2 could degrade phenol with the same efficiency as wild-type ATCC 11172, kinetic analysis of the two strains over a range of phenol concentrations was performed and the specific growth rates for both microbes compared. As can be

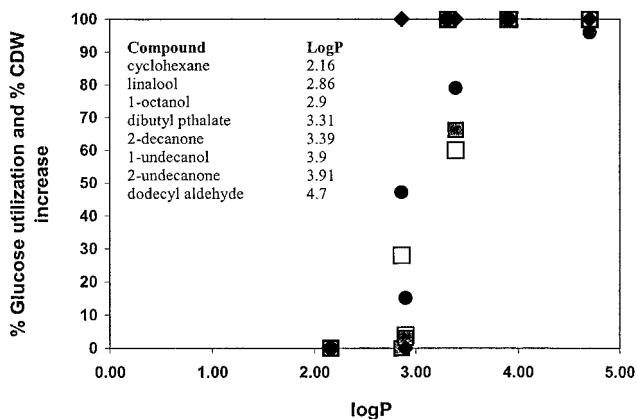


Figure 1. Biocompatibility of solvents of various log Ps with wild-type *P. putida* ATCC 11172 and the genetically modified derivative, AVP2, based on metabolic activity as evaluated by change in cell density and glucose consumption relative to a control. % CDW (■) 11172; (□) AVP2; % metabolic activity (◆) 11172; (●) AVP2.

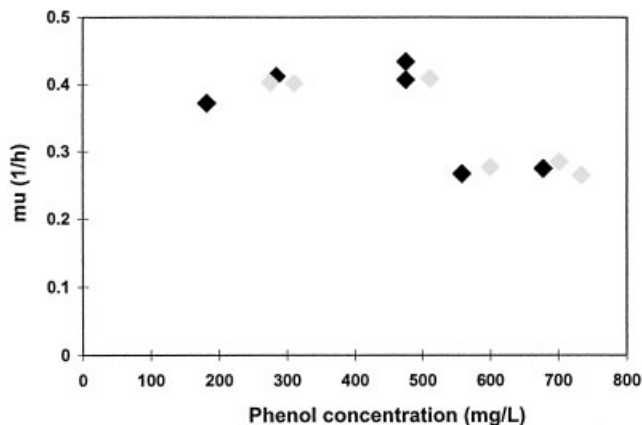


Figure 2. Comparison of phenol kinetics of *P. putida* ATCC 11172 (◆) and the genetically modified derivative, AVP2 (◆), based on specific growth rates over a range of substrate concentrations.

seen in Figure 2, the phenol kinetics of the genetically modified derivative mirrored the activity of the wild-type strain. The inhibitory nature of phenol can be seen in the inflection of the specific growth rate at substrate concentrations approaching approximately 500 mg/L, which is the inhibitory phenol concentration predicted for *P. putida* in the literature (Hill and Robinson, 1975; Kotturi et al., 1991).

Stability Analysis

In long-term (>4 days) incubation of flasks, genetically engineered AVP2 exhibited restored growth on medium-chain-length alcohols. Stability analysis was carried out to determine if this instability would present difficulty in our two-phase fermentations; that is, we sought to determine whether reversion to the alcohol utilizing phenotype would occur in the presence of an alternate carbon source (i.e., the toxic substrate). Figure 3 shows that, in cases in which decanol was the only carbon source available to the cells, the genetically modified strain (AVP2) exhibited a restored ability to grow on medium-chain-length alcohols after approximately 100 h; however, when phenol was also present, no reversion was observed over the 7-day experimental period. These results show that maintenance of a nonzero phenol concentration in the cells' environment prevents or slows reversion to solvent utilization, indicating that our genetically modified strain can effectively be applied as the catalyst in two liquid-phase fermentations with medium-chain-length alcohols as the delivery solvents.

The stability of the modified strain was further observed in fermentation runs wherein optical density of the wild-type continued to increase 36 h beyond the time of phenol consumption, whereas growth of AVP2 appeared to cease (Figs. 4 and 5). Maintenance of stability throughout fermentations was also supported by viable cell analysis of washed cell samples from various sam-

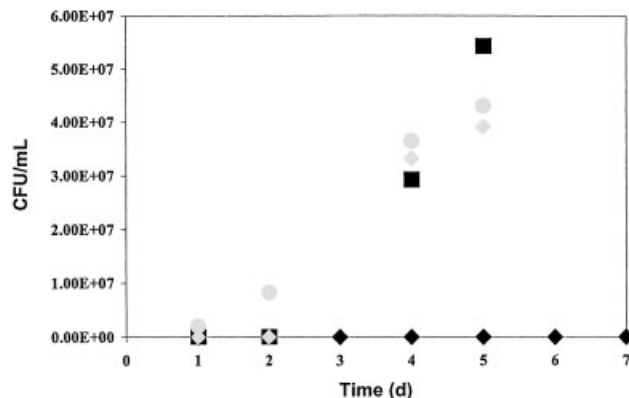


Figure 3. Stability analysis of AVP2 grown in the presence of decanol only (◆), decanol + phenol (phenol maintained at a nonzero concentration) (◆), and decanol + phenol (phenol utilized and allowed to go to zero) (■), as assessed by viable cell counts. 11172 (●) grown in decanol only is shown as a positive control.

pling times throughout phenol degradation. When plated to minimal decanol plates, only the wild-type samples exhibited growth (data not shown).

Fermentations

Eighteen-gram quantities of phenol dissolved in 2 L of decanol were introduced into each of two reactor vessels. At equilibrium, aqueous phase phenol concentrations were determined to be 400 mg/L and 460 mg/L for 11172 and AVP2, respectively. In the reactor with wild-type cells (Fig. 4), phenol transformation was rapidly initiated, as indicated by the development of a distinct yellow tint to the aqueous phase, which is known to correspond to the appearance of the phenol *meta*-pathway intermediate, 2-hydroxyomuonic semialdehyde (Léonard et al., 1999), within the first hour of the fermentation. This yellow color was maintained until about 12 h, at which point the first phenol feed was nearly depleted and the fermentation broth took on a deeper more orange pigmentation. A slight yellowing of the medium was again observed at approximately 15 h as

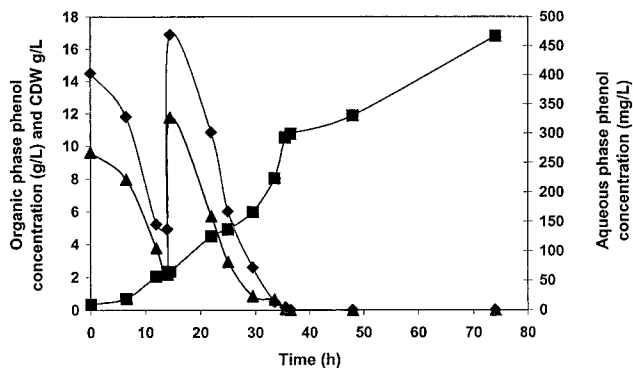


Figure 4. Time-course plot of organic phase phenol (▲), aqueous phase phenol concentration (◆), and cell dry weight (■) during fermentation with 11172 as the catalyst and decanol as solvent phase.

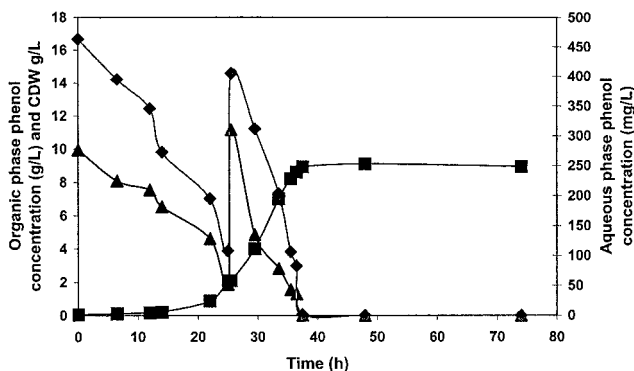


Figure 5. Time-course plot of organic phase phenol (▲), aqueous phase phenol concentration (◆), and cell dry weight (■) during fermentation with AVP2 as the catalyst and decanol as solvent phase.

the cells proceeded to degrade the additional spike of phenol introduced at approximately 14.5 h, although the orange pigmentation prevailed.

Rapid degradation of both the initial 18 g of phenol and the additional 18 g of feed was observed with complete phenol removal being observed by 36.5 h. The rapid degradation was indicated by both the slope of the phenol depletion and a drop in dissolved oxygen concentration, which was below 1% by 8 h into the fermentation and did not increase again until approximately 33 h when the phenol concentration in the system had been decreased to < 2 g. At the point of phenol exhaustion, the cell concentration in the reactor was determined to be about 10.7 g/L (formation of approximately 10.2 g/L of cells over the course of the fermentation), corresponding to a yield of 0.29 g of cells per gram of phenol. Approximately 36 h after phenol depletion, the cell density in the reactor was determined to be approximately 16.8 g/L, indicating continued cell proliferation (approximately 6.1 g/L of additional cells) on decanol, the only remaining carbon source in the reactor. Streaking of washed cells to minimal/decanol plates during and at the end of the run produced a lawn of growth, indicating the cells' use of decanol as a carbon source.

A slightly slower degradation of the first 18 g of phenol was observed in the fermentor with AVP2 as the catalyst (Fig. 5). This lagged degradation may have been due to the higher initial phenol concentration in the aqueous phase, approximately 460 mg/L, which is close to the inhibitory concentration for *P. putida*, and the lower initial cell concentration. Development of the yellow pigment indicative of degradation intermediates was not observed until approximately 11 h into the fermentation. The more gradual degradation was mirrored by a less drastic decrease in dissolved oxygen concentration, which remained at > 25% during degradation of the first phenol feed.

Degradation of the additional 18 g of phenol introduced at 25.5 h into the fermentation proceeded very rapidly, with phenol exhaustion occurring at 37.5 h. This corresponded to degradation of a total of 36 g

of phenol in a time period only 1 h longer than that observed for the wild-type. The cell yield on phenol was determined to be 0.25 g cells per gram substrate. Subsequent to phenol depletion, no increase in cell density was observed despite nutrient (other than carbon) supplementation. Cell death upon exhaustion of phenol in the system was indicated by an increase in pH, a known consequence of cell lysis. No growth was detected on minimal/decanol plates streaked with washed AVP2 cells from points throughout the fermentation run, or from 36 h after phenol depletion. Assessment of phenol in an abiotic (control) fermentation revealed no loss due to volatilization, and hence the observed phenol depletion was a direct result of biological transformation.

DISCUSSION

The present work was initiated to demonstrate the applicability of metabolic engineering in eliminating the bioavailability of certain organics, hence enabling their application as the delivery solvent in two-phase partitioning bioreactors. We chose medium-chain-length alcohols as the solvent class of interest with decanol as the representative organic. The results indicate that a selectively modified microbe can continue to degrade toxic substrates at similar rates as the wild-type strain, while functioning more efficiently (improved specific substrate utilization rate) in a user-defined system optimized from a catalyst and solvent selection perspective.

Direct observation of decanol utilization is not possible (short of using radiolabeled decanol), and hence indirect indicators of decanol utilization—yield coefficient, volumetric productivity, off-line growth on decanol during fermentation, and CDW evaluation subsequent to phenol depletion—were used to analyze cell activity and provide distinctions between fermentations with the wild-type and genetically modified strains. The lower cell yield for AVP2 relative to wild-type 11172 on equivalent phenol concentrations indicates additional growth of the wild-type at the expense of phenol as well as solvent. This was further exhibited by the 36% increase in cell concentration within the 36 h subsequent to depletion of phenol within the system (Fig. 4), which was not observed for the modified strain (Fig. 5). The growth of ATCC 11172 on minimal/decanol plates at all sampling times provides additional support for the hypothesis that dual-substrate utilization was occurring.

Despite the higher initial cell concentration in the fermentation with 11172, there was no significant difference (values within 5%) in the overall volumetric rate relative to AVP2 (0.51 g/L h⁻¹ vs. 0.48 g/L h⁻¹), and the two fermentations reached completion within 1 h of each other. This indicates that the phenol-degrading

activity of AVP2 is equivalent to that of the wild-type. If the lower initial cell concentration and accumulation in the system is accounted for, however, it becomes obvious that, under the reactor conditions applied in this work, AVP2 is in fact the more efficient catalyst.

Previous work with two-phase partitioning systems using *P. putida* ATCC 11172 as the catalyst and 2-undecanone as the delivery solvent in batch and fed-batch mode exhibited significant phenol degradation levels with overall volumetric productivities of 0.12 g/L h⁻¹ and 0.17 g/L h⁻¹, respectively (Collins and Daugulis, 1996, 1997a, 1997b). These results represented a significant improvement in volumetric consumption rates relative to the degradation observed in traditional single-phase reactors. In the current work, this productivity was approximately tripled as a result of changes in system parameters.

Oxygen limitation has been a recurring problem in TPPB systems. In previous work, agitation and aeration were maintained at low levels to retain two distinct liquid phases within the bioreactor. In the current work, agitation and aeration in the reactors were increased, resulting in a dispersion of the two phases and a subsequent improvement in oxygen transfer within the reactor. The benefit of increased oxygen transfer was reflected in the increased slope of degradation and the enhanced volumetric rates. Oxygen transfer remains an issue, however, as indicated by the substantially low (and in many cases 0%) dissolved oxygen concentrations during exponential phenol degradation, and the linear nature of the increase in cell density. The integral role of oxygen transfer has been predicted in modeling of phenol degradation by *P. putida* and emphasizes the reality that dissolved oxygen concentration is the limiting factor controlling cell growth and substrate consumption rates (Cruickshank et al., 2000). The decision to operate the system as a dispersion to enhance oxygen transfer allowed the cells to more efficiently degrade phenol, but was still insufficient for the cells to reach their maximum rates of phenol degradation and cell growth during the fermentation. In addition to benefits for oxygen transfer, we believe the dispersed system enabled improved estimation of cell accumulation in the reactor by allowing sampling and enumeration of "entrained" as well as aqueous phase cells.

Metabolic engineering is increasingly being developed as a powerful means by which to optimize industrial fermentation processes. The earliest of these efforts involved introduction of recombinant DNA into cells, but numerous other applications can now be envisioned. These various approaches can be grouped into seven categories: heterologous protein production; extension of substrate range; pathways leading to new products; pathways for xenobiotic degradation; engineering of cellular physiology for process improvement; elimination or reduction of byproduct formation; and improvement of yield or productivity (Cameron and Tong, 1993; Nielsen, 2001). In addition to the benefits that each of these approaches

presents in their own right, a single alteration may produce multiple improvements in overall bioprocessing. In the current work, for instance, the mutation in *agmR* altered cell physiology, enabling application of the recombinant strain as the catalyst within a prescribed two-phase system without solvent loss, and has consequently created a metabolic flux (phenol degradation only), leading to improved system efficiency.

Intentional or consequential targeting of regulatory proteins in metabolic engineering efforts can be beneficial by allowing several objectives to be met simultaneously, but can also present difficulties if all functions of the control element are not known (Nielsen, 2001; Olsson and Nielsen, 2000). Furthermore, the adaptive nature of cells poses the potential for secondary alterations leading to auxiliary or regulatory independent activity and instability of the desired phenotype. This adaptive characteristic is believed to be responsible for the observed reversion of AVP2 to decanol utilization in the absence of an alternate carbon source. We have shown, however, that provision of a phenol selection pressure stabilizes the mutant phenotype and prevents reversion under process conditions over the period of investigation.

Until now, efforts at TPPB process enhancement, scale-up, and industrial application have been greatly restricted by the limitations imposed by the biochemical nature of the microbial catalysts. The bioavailability of a wide range of organics is the greatest deterrent to their application as delivery solvent within TPPB systems. In this work, we have shown that metabolic engineering presents a viable option for overcoming this obstacle and enhancing biodegradation efficiency and cost from a solvent/catalyst selection perspective. It has been demonstrated that, by directed metabolic alteration, it is possible to eliminate undesirable biochemical features while not negatively affecting pathways of xenobiotic degradation. The resulting increased pool of potential solvents broadens the possibilities for system optimization from both process and cost perspectives, and further enhances the applicability of two-phase bioreactors for xenobiotic degradation.

The advantage of a fed-batch feeding strategy is evident in this work with acclimated cultures exhibiting unprecedented rates of phenol degradation. Future work will attempt to further enhance degradation by optimization of the feeding strategy. In addition, efforts will be made to quantify the mass transfer benefits of dispersed two-phase systems from the perspective of improved substrate and oxygen transfer.

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