Expanded Application of a Two-Phase Partitioning Bioreactor through Strain Development and New Feeding Strategies

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This research demonstrated the microbial treatment of concentrated phenol wastes using a two-phase partitioning bioreactor (TPPB). TPPBs are characterized by a cell-containing aqueous phase and an immiscible and biocompatible organic phase that partitions toxic substrates to the cells on the basis of their metabolic demand and the thermodynamic equilibrium of the system. Process limitations imposed by the capability of wild-type *Pseudomonas putida* ATCC 11172 to utilize long chain alcohols were addressed by strain modification (transposon mutagenesis) to eliminate this undesirable biochemical characteristic, enabling use of a range of previously bioavailable organics as delivery solvents. Degradation of phenol in a system with the modified strain as catalyst and industrial grade Adol 85 NF (primarily oleyl alcohol) as the solvent was demonstrated, with the system ultimately degrading 36 g of phenol within 38 h. Volumetric phenol consumption rates by wild type *P. putida* ATCC 11172 and the genetically modified derivative revealed equivalent phenol degrading capabilities (0.49 g/L·h vs 0.47 g/L·h respectively, in paired fermentations), with the latter presenting a more efficient remediation option due to decreased solvent losses arising from the modified strain's forced inability to consume the delivery solvent as a substrate. Two feeding strategies and system configurations were evaluated to expand practical applications of TPPB technology. The ability to operate with a lower solvent ratio over extended periods revealed potential for long-term application of TPPB to the treatment of large masses of phenol while minimizing solvent costs. Repeated recovery of 99% of phenol from concentrated phenol solutions and subsequent treatment within a TPPB scheme demonstrated applicability of the approach to the remediation of highly contaminated "effluents" as well as large masses of bulk phenol. Operation of the TPPB system in a dispersed manner, rather than as two distinct phases, resulted in volumetric consumption rates similar to those previously achieved only in systems operated with enriched air.

Introduction

Industrial production of phenol and its derivatives is in excess of 1.5×10^9 kg/year in the U.S. alone (1996), and wastewaters containing phenolic concentrations ranging as high as 3000 mg/L have been reported (*1*, *2*). On the basis of severe chronic toxicity, the EPA has classified phenol as a high concern priority pollutant, and its release is regulated by both the Canadian and U.S. environmental protection agencies (*3*, *4*). Phenol contamination is of particularly great concern due to its high aqueous solubility, which presents a threat to water sources both locally and far-removed from the original site of release.

Bioremediation has become a powerful tool for dealing with the high degree of environmental pollution that has arisen from increased industrial activity and accidental or improper release of xenobiotics (*5*). Despite considerable advances in bioremediation approaches, however, the xenobiotic nature and concentrations at which industrial contaminants occur can lead to cell toxicity, and as a result, efforts employing biological treatment have often had to rely on prior chemical treatment or dilution. To address issues of toxicity and inhibition of microbial activity, successful implementation of biodegradative approaches for treatment require that the biocatalysts' xenobiotic exposure be maintained at subinhibitory levels. To meet this criterion, xenobiotic feedings to bioreactors have consistently been at very low levels and have consequently produced low volumetric productivities (*6*). A process that has enabled introduction of highly concentrated pollutants to reactors, while maintaining subinhibitory xenobiotic delivery to cells, is the two-phase partitioning bioreactor (TPPB). This scheme involves three components: an immiscible, biocompatible, but nonbioavailable solvent with a high capacity for xenobiotic compounds; an aqueous phase comprising cells and all non-carbon nutrients necessary for microbial growth; and the target xenobiotic(s). The organic nature of most xenobiotics leads to their preferential partitioning to the solvent phase, and appropriate contaminant−solvent−catalyst selections ensure an equilibrium partitioning resulting in subinhibitory xenobiotic concentrations within the aqueous phase. As xenobiotic is consumed by cells in

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the aqueous phase, more is transferred from the organic phase to reestablish equilibrium, presenting a stable, self-regulating system applicable to the treatment of highly concentrated xenobiotics but requiring monitoring only to determine the point of xenobiotic depletion.

The current work focused on enhancing xenobiotic degradation within TPPBs by addressing several challenges that have previously been encountered in implementation of these systems. Prior efforts to systematically identify and implement solvents with the appropriate characteristics for a specific TPPB application have been highly limited by the bioavailability of many compounds that otherwise exhibit desirable delivery solvent characteristics. To address this issue we have created a mutant no longer able to utilize alcohols as sole carbon substrates (*7*) and demonstrated its use in a TPPB scheme with decanol as the delivery solvent (*8*). In the current work we have tested a number of solvent−mutant pairings to identify a range of new solvent possibilities for operation of TPPB enabled by utilization of the modified strain as catalyst. In addition, this work has attempted to address the issue of oxygen limitation (*9*) through alterations in operating conditions, resulting in dispersion of the two phases within the reactor. The practical applicability of TPPB is assessed by evaluating system operation over repeated fed-batch applications of xenobiotic and with decreased solvent volumes. Furthermore, the possibility of applying the TPPB concept to partitioning/removal of phenol from highly contaminated effluents, these phases themselves constituting a two-phase bioreactor system in which phenol degradation can proceed biologically, is demonstrated.

Materials and Methods

Strains and Medium Formulation. The two strains utilized in this work were *Pseudomonas putida* ATCC 11172 and a transposon mutant derivative, AVP2, no longer able to utilize a variety of alcohols as sole carbon sources (*7*). Cells were grown for 24 h in mineral salts medium (6 g of K_2HPO_4, 4 g of KH_2PO_4, and 2 g of $(NH_4)_2SO_4$ per liter of distilled water), augmented with a divalent salt solution (0.66 g of $MgCl_2$ and 0.25 g of $CaCl_2$ per liter of distilled water), 0.5% (v/v) trace element solution (0.3 g of H_3BO_3, 0.089 g of $ZnSO_4 \cdot 7H_2O$, 1.1 g of $FeSO_4 \cdot 7H_2O$, 0.024 g of $NiSO_4$, 0.018 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.003 g of $CuSO_4 \cdot 5H_2O$, 0.050 g of $MnSO_4$, and 0.190 g of $CoCl_2$ per liter of distilled H_2O) and 0.0006% $FeCl_3$ (v/v). Plates were prepared by addition of 1.5% (w/v) Bacto agar (Difco Laboratories, Detroit, MI). The carbon source for maintenance cultures was pyruvate (11 g/L). Unless otherwise indicated, incubations were for 24 h at 30 °C. Stock cultures for inoculum preparation were prepared by subculturing 1/10 in mineral salts medium containing 5 g/L glucose and 250 mg/L phenol as carbon sources. Inocula for fermentations were prepared by an additional subculture (1/10) in an appropriate volume of mineral salts/glucose/phenol medium to produce a (1/5) inoculum in the reactor aqueous volume. This inoculum was concentrated to 50 mL prior to addition to the reactor.

Bioavailability and Solvent Selection. The extractant screening program (ESP) contains physical property data and utilizes the UNIFAC group contribution method to predict various solvent properties (aqueous solubility, log P) and phase behavior (partition coefficient, selectivity) as a first means of selecting solvents for use in TPPB systems (Bruce and Daugulis, 1991). ESP was used to identify a range of alcohols utilized by wild-type ATCC 11172 but not degraded by AVP2 and hence applicable as solvents with this organism within a TPPB by focusing on longer chain alcohols with predicted log P values above the experimentally determined critical log P for the organisms (log P = 3.3; *8*). The bioavailability of the solvents in this list, which included a range of medium-chain-length, branched, and unsaturated alcohols, was assessed as part of strain characterization and solvent selection. Erlenmeyer flasks were prepared with 50 mL of minimal salts medium lacking a carbon source and then seeded with 5 mL of each of the previously identified alcohols (Sigma-Aldrich Chemical Co., Oakville, ON; Sherex Industries, Burlington, ON). A positive control was prepared with 5 mL of corn oil, while a negative control contained no solvent. The flasks were incubated on a shaker at 30 °C for 4 days, with optical density assessed every 24 h. A change in biomass concentration greater than the negative control indicated solvent consumption by the cells.

Fermentation with Adol 85 NF as Delivery Solvent. A 5-L Bioflo III Fermentor (New Brunswick Scientific Co., Edison, NJ) was prepared with 2 L of mineral salts medium. The unit was fitted with oxygen and pH probes, a condenser to prevent substrate and solvent losses, and two Rushton impellors to provide mixing. The vessel was autoclaved and then inoculated with either the wild-type or AVP2 as laid out above. The reactor was operated at 400 rpm, with 1 vvm (vol air/vol aqueous phase/min) aeration, conditions under which the two phases were dispersed. The pH was automatically maintained at 7.0 using 1 M KOH. Initially, 2 L of Adol 85 NF (oleyl alcohol) loaded with 9 g of phenol was introduced into the reactor, which partitioned approximately 360 mg/L to the aqueous phase. When phenol in the aqueous phase decreased below approximately 100 mg/L as a consequence of cellular uptake, supplementary feeds of 9 g of phenol dissolved in 50 mL of fresh Adol 85 NF were introduced to the reactor. Three such feeds were provided, resulting in a total of 36 g of phenol being added to the system.

Extended Fed-Batch Fermentation with Decanol as Delivery Solvent. The fermentor was prepared and inoculated as described above except that the aqueous-to-solvent ratio was altered to 3:1. The initial feed for both the wild-type and mutant fermentations was 8.5 g of phenol dissolved in 1 L of decanol. Dissolved oxygen concentration together with measured phenol concentration were used to determine the appropriate feeding schedule. The concentration of phenol in the initial feed was low to provide a nonstressful environment enabling cells to adapt and grow with limited inhibition. In later phases when adaptation and high biomass in the systems were evident, more aggressive feeds were introduced in an effort to "push" the system toward maximum capacity. Feeds consisted of the chosen phenol concentration dissolved in 35 mL of fresh decanol, and a 30-mL concentrated bolus of nutrients (for approximately every 10 g of phenol consumed). The additional volumes approximately balanced the volumes removed through sampling and hence the overall volume and phase ratio of the fermentor remained essentially constant. In the end, 84 g of phenol were added to the fermentor with AVP2 as catalyst (additional feeds of 8.5, 10, 12, 10, 20, and 15 g) over 82.5 h. The wild-type fermentation consumed a total of 60 g (additional feeds of 10, 9, 9, 8.5, and 15 g) of phenol within 77 h.

Cyclic-Batch Fermentation with Decanol as Delivery Solvent. In an effort to simulate a concentrated aqueous phenol waste stream, 9 g of phenol were dissolved in 3 L of mineral salts medium resulting in a

concentrated solution of 3000 mg/L and corresponding to the higher end of reported phenol concentrations within contaminated industrial effluents (*1*). This concentration is clearly substantially higher than the level (500 mg/L) reported as being completely toxic to *P. putida* (*11*). The 3 L was introduced into the reactor, and 1 L of decanol solvent was added to act as a "sponge" to extract the majority of the phenol from the aqueous phase and establish a subinhibitory concentration within the aqueous phase. The organic phase then behaved as a delivery pool, continually repartitioning phenol back to the aqueous phase in response to cell demand.

When the phenol concentration in the aqueous phase dropped below 100 mg/L, agitation and aeration were stopped, the two phases were allowed to separate, and 1.5 L of the aqueous medium and associated cells were removed using a Masterflex pump. This volume was replaced by addition of 1.5 L of the concentrated phenol solution (hence another 3 g/L in the total aqueous volume of 3 L), with mixing, to allow the organic phase to again act as a sponge to take up the majority of the phenol and reduce the aqueous phenol concentration to subinhibitory levels. This supplementation was repeated just prior to phenol depletion, resulting in treatment of a total of 27 g of phenol.

Analytics. OD_{600} of the aqueous phase and phenol concentration in both organic and aqueous phases were followed throughout the course of the fermentation. At chosen time points, samples were collected and centrifuged for 5 min to separate the two phases. Phenol concentration was determined by the colorimetric 4-aminoantipyrene method (*13*) and measurement of absorbance at 510 nm. The organic phase phenol concentration was determined by contacting an equal volume of solvent and mineral salts medium (Vortex Genie; 1 min); centrifuging to separate the phases ($\sim$10000 $\times$ *g* for 5 min); analyzing 1 mL of the aqueous phase; and using mass balance and solvent partition coefficients to determine phenol concentration in the organic phase. When samples were centrifuged, cell material occurred in the pellet and at the solvent−aqueous interface, both of which were redispersed in the aqueous phase and included in the estimation of cell dry weight (CDW) based on changes in OD_{600} and comparison to a standard curve relating optical density to CDW. To verify stability of the mutant phenotype throughout the course of fermentations, cell samples from fermentors using ATCC 11172 and AVP2 were washed and plated to mineral salts medium containing the respective solvent as the sole carbon substrate.

Results

Bioavailability and Solvent Selection. Bioavailability revealed a list of six solvents no longer utilized by AVP2 as carbon sources (Figure 1) but readily consumed by the wild type. The more limited growth of wild-type 11172 on unsaturated versus saturated alcohols over 96 h was likely due to the very low solubility of these compounds, which prevents sufficient contact between cells and solvent and is often the limiting factor on the extent of growth on organic compounds, even by strains that are able to utilize them as carbon sources. From the perspective of solvent selection, the low solubilities of these compounds is beneficial, being one of the critical properties of a desirable solvent. Growth on octanol was likely hindered by the log P of this solvent (3.0), which is just below the critical log P of the cells, and hence growth was likely balanced by considerable cell death.

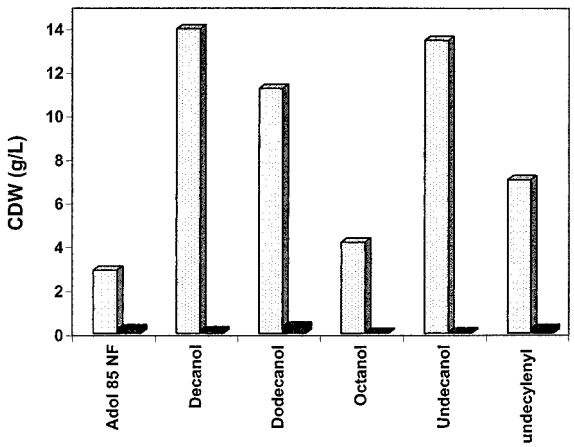

Figure 1. Bioavailability analysis of *P. putida* ATCC 11172 and AVP2 on various alcohol solvents as measured by cell dry weight (CDW): (□) 11172; (■) AVP2.

Fermentation with Adol 85 NF as Delivery Solvent. We have previously provided a demonstration of concept of the efficiency and stability of AVP2 as the catalyst in a two-phase partitioning system with decanol as the delivery solvent (*8*). This earlier work showed that AVP2 utilized phenol with the same efficiency as the wild type, while not degrading decanol, indicating that it is a superior catalyst option. Here, we attempted to demonstrate the expanded solvent potential of TPPB available through the use of AVP2 as the catalyst and a previously bioavailable solvent as the delivery phase. Although any of the newly nonbioavailable solvents in Figure 1 could have been chosen for use in an actual TPPB system with AVP2, Adol 85 NF was selected to demonstrate system application with a bulk, and hence cheaper, industrial grade solvent. To enable comparison with the decanol system, a similar total amount of phenol (36 g) was loaded into the TPPB over the course of the fermentation. Identical fermentation conditions (concentrations) could not be achieved because of the differences in the partition coefficients of the solvents, with decanol exhibiting a partition coefficient for phenol approximately double that of Adol 85 NF, hence requiring that the feeds comprise half the total amount of phenol to establish equivalent concentrations (approximately 400 mg/L) in the aqueous phase. Whereas with decanol, two feeds of 18 g were used, the Adol system utilized four feeds of 9 g each.

The volumetric productivities in the Adol system were 0.49 g/L·h for 11172, and 0.47 g/L·h for AVP2, reaching substrate depletion within 36.75 and 38.25 h, respectively (Figures 2 and 3). No lag phase for either fermentation was observed, with decrease in phenol levels in the organic phase occurring within the first 4 h in both systems. Degradation of phenol was further indicated by development of an intense yellow pigment in the fermentation broth, known to be associated with production of the metabolic intermediate, 2-hydroxymuconic semialdehyde. By 8 h into fermentation the dissolved oxygen (DO) concentration in the reactors was below 10% saturation, indicating a very active metabolic state for the cells. As previously mentioned, DO and actual phenol measurements were used to determine appropriate feeding times and proved to be an extremely accurate indicator of cell behavior within the fermentor. The DO remained low ($\sim$1%) until the majority of the original phenol feed had been consumed, at which point the DO concentration slowly began to increase. Addition of fresh feed immediately caused the DO to again drop to below 1% of saturation. DO increases at 20.25 and 29.25 h or

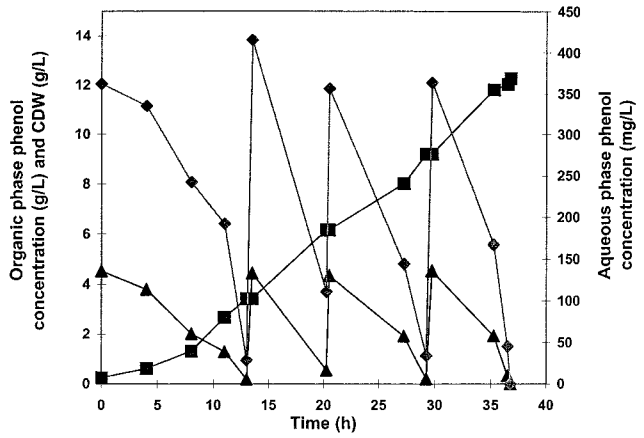

Figure 2. Time-course plot of organic-phase phenol (▲), aqueous-phase phenol concentration (◆), and cell dry weight (■) during fermentation with *P. putida* ATCC 11172 as the catalyst and Adol 85 NF as the solvent phase in a 1:1 phase ratio.

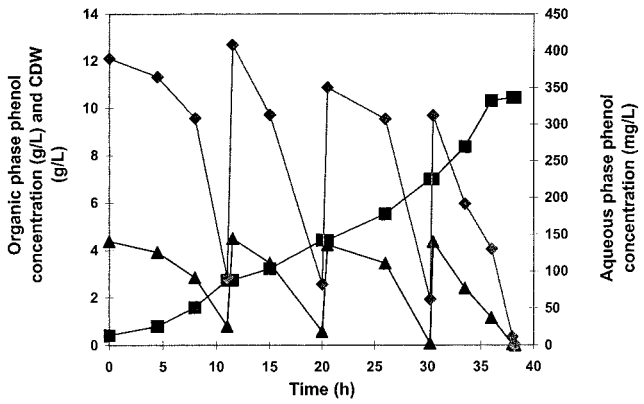

Figure 3. Time-course plot of organic-phase phenol (▲), aqueous-phase phenol concentration (◆), and cell dry weight (■) during fermentation with AVP2 as the catalyst and Adol 85 NF as the solvent phase in a 1:1 phase ratio.

30.25 h (11172 or AVP2, respectively) indicated the beginning of substrate limitation (less than 1 g of phenol remaining in the reactor) and additional 9 g phenol feeds were provided. After the initial decrease in dissolved oxygen concentration, DO measurements for the fermentors never increased above 15% of saturation, remaining between 0 and 2% during the majority of the fermentation. After phenol depletion the dissolved oxygen concentration in the fermentors with 11172 and AVP2 increased rapidly to 75% and 88%, respectively. A very modest amount of foam was produced in these experiments and the other fermentations reported in this work, and antifoam addition was not required. Cell yields on phenol were 0.67 g/g for 11172, and 0.56 g/g for AVP2, respectively. Only samples from fermentations with the wild type produced growth on mineral salts plates with Adol as sole substrate, indicating the stability of the AVP2 phenotype over the course of fermentation (i.e., AVP2 remained unable to use Adol as a substrate).

Extended Fed-Batch Fermentation with Decanol as Delivery Solvent. Several objectives were set for undertaking this experiment. A change in phase ratios within the system was utilized to see the impact of operating more cost-efficiently (i.e., with less solvent). The ability to operate over repeated batch feeds was tested to gauge the potential for long-term operation of the TPPB. Through these modifications, it was hoped that the system could be pushed to maximal capacity.

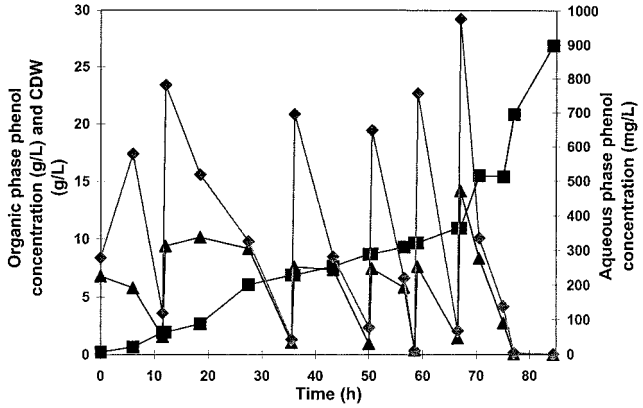

Figure 4. Time-course plot of organic-phase phenol (▲), aqueous-phase phenol concentration (◆), and cell dry weight (■) during extended fed-batch fermentation with 11172 as the catalyst and decanol as the solvent phase in a 3:1 (aqueous: solvent) phase ratio.

Six and seven phases (for 11172 and AVP2, respectively) were employed in these extended fed-batch fermentations, corresponding to the initial feed and five (or six) subsequent spikes (Figures 4 and 5). The final spike was added to each reactor at essentially the same time, 67 h for 11172 and 66 h for AVP2. The lower organic phase volume, 1 L (3:1 phase ratio) of this fermentation relative to the 2 L (1:1 phase ratio) in the Adol fermentation above and the previously described decanol solvent system (*8*), required that lower phenol feeds be used to obtain subinhibitory phenol concentrations in the aqueous phase.

The system with the wild type as the catalyst consumed 60 g of phenol within 77 h, corresponding to a volumetric rate of 0.26 g/L·h based on aqueous volume (Figure 4). In these runs the AVP2 system enabled loading of a greater total mass of phenol, 84 g, relative to the wild-type system. The volumetric productivity was 0.34 g/L·h, and the system required approximately 83 h to reach completion (Figure 5). The dissolved oxygen concentration was essentially 0 during the periods of rapid degradation. The cells exhibited adaptive behavior with more rapid degradation over time despite the fact that the phenol concentration of later feeds corresponded with anticipated aqueous phase concentrations as high as 730 mg/L, representing concentrations in excess of the inhibitory concentration for this organism (*11*, *12*). The potential that this provides in terms of enabling feeds that would previously have been considered toxic, the somewhat arbitrary choice for phenol concentration in the more concentrated spikes, and the observed oxygen limitation indicate that despite high metabolic activities, the maximal productivity of the system was not attained.

Cyclic-Batch Fermentation with Decanol as Delivery Solvent. In this system the 3-L aqueous phase itself was the source of phenol (9 g), and the first step of the fermentation involved introduction of 1 L of decanol to extract the xenobiotic. Determination of the phenol concentration in the two phases revealed that solvent addition enabled removal of the majority of the xenobiotic from the aqueous phase and that the resulting concentrations in each phase reflected equilibrium partitioning. The initial decrease in aqueous phase phenol concentration from 3000 mg/L to approximately 380 mg/L enabled introduction of cells (AVP2), and the subsequent degradation of all of the phenol in the system by the repartitioning of phenol back into the aqueous phase at a rate controlled by cell demand and thermodynamic equilibrium.

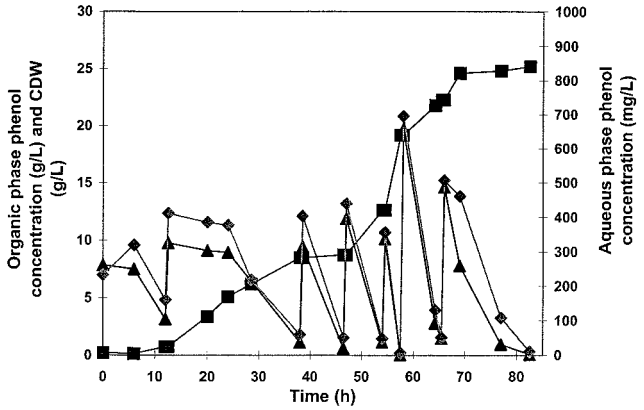

Figure 5. Time-course plot of organic-phase phenol (▲), aqueous-phase phenol concentration (◆), and cell dry weight (■) during extended fed-batch fermentation with AVP2 as the catalyst and decanol as the solvent phase in a 3:1 (aqueous: solvent) phase ratio.

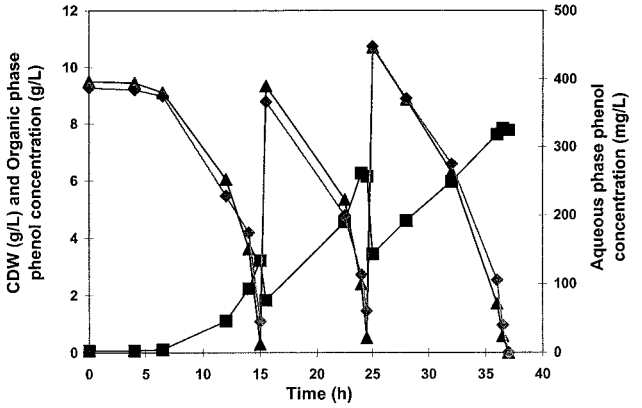

Figure 6. Time-course plot of organic-phase phenol (▲), aqueous-phase phenol concentration (◆), and cell dry weight (■) during cyclic-batch fermentation with AVP2 as the catalyst and decanol as the solvent phase in a 3:1 (aqueous:solvent) phase ratio.

In the first phase (0−15 h), an initial lag period of 6.5 h was observed followed by two distinct intervals: a period of modest degradation, followed by a more rapid phenol degradation stage (Figure 6). The shift between these two stages occurred when phenol in the system reached approximately 4.4 g (just under half of the initial phenol load) at 14 h. Within 15 h, approximately 8.5 g of the original 9 g feed introduced to the system had been degraded. At this point the system was allowed to settle, half of the aqueous volume (1.5 l) was removed, and 1.5 L of fresh high-concentration phenol solution (containing an additional 9 g of phenol) was introduced. The solvent in the system was again able to act as a sponge to extract the majority of the phenol from the aqueous phase, allowing degradation to proceed.

The majority (approximately 8.4 g) of the phenol was degraded within 9 h, and an additional cycle of removal of 1.5 L of "treated effluent" and addition of 1.5 L of contaminated aqueous solution was performed at 25 h. The resulting 3 g phenol/aqueous L was partitioned as previously observed, and complete degradation achieved by 37 h. In subsequent feeds, no lag phase was observed, but the pattern of degradation exhibited the two distinct intervals (modest and rapid degradation) described above. The overall volumetric productivity of the system was 0.24 g/L·h. Approximately 40% of the cells were removed at each cycle.

The phenol concentration in the spent aqueous volumes was less than 50 mg/L, a residual concentration that could be decreased even further by allowing a slightly longer treatment period or through a brief holding period to allow the cells present to degrade the modest amount of residual phenol (achieving compliance limits for release). This cyclic configuration made possible the treatment of a total of 6 L of a highly contaminated aqueous phase in a cyclic operation utilizing only 1 L of solvent. With minor adjustments it is possible to envision application of this concept to continuous treatment of large volumes of a concentrated waste stream or other highly contaminated aqueous solutions and removal of treated water that could be recycled or released to the environment with minimal solvent requirements.

Discussion

The applicability of the TPPB concept for the degradation of a variety of xenobiotics including benzene, phenol, and pentachlorophenol has been previously demonstrated (*9*, *10*, *14*, *15*); however, certain challenges have been encountered during these applications. The foci of this work have revolved around efforts to improve and enhance TPPB operation by addressing the limitation of bioavailability on solvent selection and testing different system configurations to identify strategies with beneficial influences on phenol degradation and system function. Furthermore, modifications that would increase efficiency and hence expand potential applicability of TPPB schemes were evaluated.

In the past, process issues have primarily been addressed by altering operating conditions to circumvent the specific constraint. In the case of solvent bioavailability within TPPBs, alternative solvents were merely selected, and for some applications, this may remain the simplest option. By specifically defining a system involving *P. putida* as the catalyst and long chain alcohols as the desired class of delivery solvents and then eliminating the biochemical characteristic of solvent bioavailability that would otherwise interfere with system function, we demonstrated the possibility of overcoming process constraints from the perspective of the catalyst. Ability to manipulate the catalyst as a "parameter" of the system presents great possibilities for process enhancement in terms of defining optimal solvent−catalyst pairings and selecting low cost solvents. Furthermore, as the composition of pollutant streams becomes increasingly complex, so will the matter of defining appropriate solvent−catalyst pairings. Solvent selection for TPPB applications will need to address issues of chemical partitioning for numerous xenobiotics and accommodate threshold and toxicity limits for each of the xenobiotic substrates, while also not being bioavailable. The ability to eliminate even a single one of these variables simplifies the solvent selection process, increasing the potential for successfully defining and applying a specific TPPB system. Other possible process issues that could be envisioned to be solved through catalyst modification are oxygen transfer and solvent and cell losses due to emulsification, potentially through alteration of oxygen-regulating or biofilm/LPS determining genes, respectively.

Through construction and application of AVP2, a TPPB scheme that enabled application of cheap and easily obtainable delivery solvents was delineated. Similarity in the volumetric productivities of AVP2 and the wild type, 0.47 vs 0.49 g/L·h, respectively, for the Adol system, indicated that the alteration was performed without negatively affecting the organisms' phenol-degrading capabilities. This was also previously confirmed in phenol kinetic analysis experiments (*8*). The consistently higher

yield coefficients in fermentations utilizing the wild type as catalyst, 0.67 g cells/g phenol and 0.57 g/g in equivalent oleyl (Figure 2) and decanol (*8*) fed-batch systems, compared to 0.56 g/g (Figure 3) and 0.49 g/g with the mutant, suggest the utilization of both solvent and phenol by the wild type. This was further confirmed by evaluation of cell viability, which showed growth for 11172 but not AVP2 when cells during the course of experiments were inoculated on plates with solvent as the sole carbon source. It should be noted that wall growth during the rapid phase of growth presented a difficulty in obtaining accurate quantifications of total biomass in the fermentors, and hence the absolute values of the cell yields determined in this work represent estimates. The method of quantification was consistent throughout all fermentations, however, and therefore the relative differences indicate a real distinction between the cell yields for 11172 and AVP2. The advantage of AVP2 over 11172 thus stems from the fact that solvent losses are minimized during the course of TPPB operation, particularly during periods of phenol depletion.

Although the system with Adol 85 NF involved a lower total phenol load to the system (9 g/feed) relative to the use of decanol (18 g/feed) as the delivery solvent, the efficiency of degradation of 36 g of phenol in the two systems was almost indistinguishable: 0.49 and 0.50 mg/L·h for the wild type on Adol (Figure 2) and decanol (*8*), respectively, and 0.47 mg/L·h (Figure 3) and 0.48 mg/L·h for AVP2. This similarity in system function confirms that the cells were influenced only by the concentration of xenobiotic (phenol) in the aqueous phase, which was essentially equivalent. The results with Adol reveal that even systems employing modest solvent partitioning coefficients can result in high volumetric consumption rates, though their effectiveness will need to be balanced against increased requirements for monitoring and feeding. This is not likely to be a major deterrent to application, however, since the technologies for automating sampling and substrate provision through a feedback signal are available (*13*, *16*).

Despite previous successes with TPPB, volumetric productivity has been constrained by the inability to meet the cells' oxygen demand during periods of rapid phenol utilization (*9*). As a result, the maximal volumetric consumption rates achievable within the TPPB system have been determined by the rate of oxygen rather than substrate transfer, or cell specific substrate consumption rate, and consequentially reflect suboptimal cell growth. This is confirmed by experimental work indicating that substrate transfer in the reactor is several orders of magnitude greater than oxygen transfer (*17*). The oxygen limitation during the rapid phase of growth during the present work was reflected in the essentially zero dissolved oxygen concentration measured in the reactor. Previous efforts to address oxygen limitation using enriched air improved volumetric productivity by greater than 50% from 0.25 to 0.37 g/L·h (calculated without incorporating the lag phase; *9*). The use of enriched oxygen in this earlier work arose as a consequence of the modest aeration and agitation conditions employed, which were intended to maintain two distinct phases within the bioreactor. In the present work, the TPPB system was operated as a dispersion through more aggressive mixing and aeration and produced similar or greater improvements in volumetric rates to those seen in systems operated with enriched air.

In addition to the inherent influences of greater aeration and agitation on the performance of the system, the oxygen capacity of solvents (which is higher than that of water; *18*) is also believed to have improved overall oxygen transfer in this system. The cost of the increased energy demand involved in operating the TPPB system at the higher agitation and aeration rates (400 rpm and 1 vvm vs 250 rpm and 0.5 vvm, respectively) relative to the cost(s) associated with use of enriched oxygen should be substantially lower, and therefore these operational modifications present an alternate and potentially cheaper option to enriched air for improving oxygen transfer within the TPPB system.

As outlined above, 1:1 and 3:1 (total volume 4 L) aqueous to organic phase ratios were tested in fermentations in this work. The higher volumetric productivities in fermentations with a 1:1 ratio, 0.49 and 0.47 g/L·h for 11172 and AVP2, respectively, indicate a greater efficiency of this configuration. This is likely a reflection of the increased oxygen transfer rate associated with the higher solvent volume. The above values represent a 35% improvement over the previous "best": volumetric rates for phenol in the TPPB, which were achieved through utilization of enriched air (*9*). If the lag phases observed in those cases are considered in calculation of productivity, fermentations in this work present a way of doubling the volumetric productivity and decreasing the overall length of treatment, without the extra expense of enriched air. Though a 1:1 configuration represents a greater initial solvent cost, the recyclable nature of the system and the balance of a more efficient system operation could in the end likely offset this initial cost. Although improvements in volumetric rates have been shown, which are likely due to enhanced oxygen transfer, the fact that oxygen limitation can still occur means that the values reported may not be the intrinsic kinetic rates associated with these systems.

The difference in the volumetric productivities of the extended fed-batch fermentations (0.26 and 0.34 g/L·h for wild type and AVP2, respectively) suggests that, over longer time periods, the dual substrate behavior of the wild type negatively interferes with phenol degradation. More work is needed to quantify this potential impediment, but even with similar volumetric productivities, the cost benefit of decreased solvent losses and associated improvements in long-term phase stability clearly identify AVP2 as the superior catalyst. The genetic stability of AVP2 in terms of its inability to utilize alcohols has been preciously shown (*8*).

The continued partitioning and phenol degradation through repeated feeds in this work demonstrate the possibility for adapting the TPPB scheme to stable long-term remediation applications. The lower volumetric rates (0.24−0.34 g/L·h) observed for systems with a 3:1 configuration reflect the lower oxygen transfer within the fermentation broth of these systems, but as a result of dispersed operation these fermentations still exhibited competitive volumetric rates (i.e., equivalent to, though not higher than, those with enriched air). In cases where the prescribed solvent is of especially high cost, the 3:1 system may represent the more cost-effective alternative with the savings in solvent balancing the potentially longer operating period.

The cyclic batch operation most clearly demonstrated a valuable practical application by providing a means of directly extracting dissolved contaminants and releasing remediated water, while also providing a means of complete biological degradation of the concentrated contaminants without having to resort to either complex, multistep treatments involving dilution or incineration and landfill and the potentially hazardous repercussions associated with these technologies. The concept of solvent

extraction is certainly not new, but with careful solvent selection it becomes possible to define an overall remediation process starting from extraction and environmental restoration and culminating in complete xenobiotic degradation within a TPPB. A well-mixed continuously fed system would be an alternative means of dealing with excessively high concentrations of substrates, and the choice of method would be determined by the relative economics and ease of operation.

Conclusion

Through alterations in system configuration and feeding approaches we have laid the groundwork for defining an optimal phase ratio and system operation to obtain efficient treatment of xenobiotics over extended periods of time. Furthermore, we have examined a specific application of TPPB, namely, treatment of highly contaminated water and evaluated possibilities for operating cost-effectively. Through strain modification we have demonstrated the ability to overcome limitations in solvent selection by eliminating undesirable bioavailability characteristics and have suggested additional process issues that could be addressed through catalyst manipulation. Future work will test some of these possibilities. Other considerations will involve defining optimal catalyst systems for treatment of more complex phenols and mixed contaminant sources. Finally, the issue of oxygen transfer will be revisited and efforts made to quantify the specific influences of phase ratio and dispersed operation on dissolved oxygen concentrations and, by extension, volumetric efficiency within TPPB systems.

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