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Characterization and optimization of a two-phase partitioning bioreactor for the biodegradation of phenol

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Abstract A two-phase partitioning bioreactor containing *Pseudomonas putida* ATCC 11172 was used to degrade high concentrations of phenol in batch and fed-batch mode. The 2-l (nominal volume) partitioning bioreactor employs a 1-l cell-containing aqueous phase, and a 500-ml immiscible and biocompatible second organic phase (2-undecanone), which partitions the toxic substrate into the aqueous phase at a rate based on the metabolic activity of the microorganisms. Using this reactor configuration, operated in batch mode, 10-g phenol was degraded to completion within 84-h. The system was, however, oxygen-limited during the rapid growth phase of the fermentation. A second experiment, using enriched air to prevent oxygen limitation, resulted in the complete degradation of 10-g phenol within 72-h. The use of a sequential feeding strategy, in which a 10-g phenol load was added in sequential 5-g aliquots, resulted in a significant reduction in the lag phase, from 36-h to 12-h, and the consumption of 10-g phenol in 60 h. Finally, fed-batch fermentation was used to attempt to determine the ultimate capacity of the system to degrade phenol. The organic phase was loaded with 10-g phenol, the microorganisms were allowed to consume this aliquot almost to completion, and a second 10-g aliquot was then added. The organic phase was spiked in this manner a total of four times, resulting in the degradation of 46.55-g phenol within 12 days. The system was also monitored for nutrient depletion, and a nutrient-feeding schedule was formulated, in response to the mass of phenol consumed.

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Introduction

Phenol is a toxic compound produced by many industrial processes, and its presence in the environment poses significant risks to indigenous biological populations. This aromatic compound is water-soluble and highly mobile, and as such is likely eventually to reach downstream drinking water sources where, even at very low concentrations, it can cause severe odor and taste problems. Various regulatory bodies have imposed strict limits on the phenol concentrations in industrial discharge streams (Singleton 1994). Phenol has traditionally been removed from industrial effluents by costly physical-chemical methods, but recently biodegradation has been studied as a low-cost alternative, offering the possibility of complete mineralization of this toxic organic (Kobayashi and Rittmann 1982).

A variety of contacting configurations for phenol biodegradation have been studied. Conventional batch fermentations have been used to degrade phenol, but are limited by the low initial concentrations required to prevent complete inhibition of microbial activity (Andrews 1968). Continuous-culture fermentations are unstable and unable to achieve high removal efficiencies at high inlet phenol concentrations (Pawlowsky et al. 1973). Immobilized cell reactors can achieve high conversions with better process stability, but they require high levels of aeration and agitation and can, therefore, be costly to operate (Molin and Nilsson 1985). In all of these instances, phenol toxicity is still a concern.

A two-phase partitioning bioreactor has been used to degrade styrene in a silicone oil/aqueous system (El Aalam et al. 1993). This configuration permits high loading of an inhibitory compound into a second phase, which is then partitioned into the aqueous phase at sub-inhibitory concentrations. We have successfully employed this reactor scheme, operated in batch and fed-batch mode, to degrade pentachlorophenol using diethyl sebacate as the organic phase (Munro and Daugulis 1997), and to degrade phenol using 2-undecanone as the organic

solvent (Collins and Daugulis 1996, 1997). The latter system has been shown to degrade high concentrations of phenol rapidly to completion with minimal operational control required. However, we have encountered several operational challenges, including oxygen limitation, and prolonged lag phases at high (above 20 g/l) phenol loadings. The present work has focused on reducing the time required to consume a given amount of phenol, by supplying enriched air to the fermentation broth, and through the use of sequential feeding strategies. We have also attempted to determine the ultimate capacity of the system to degrade phenol, and to characterize the nutrient requirements of the microorganisms as a function of the amount of phenol consumed during repeated fed-batch culture.

Materials and methods

Microorganism and medium formulation

Pseudomonas putida ATCC 11172 was used to degrade phenol in all experiments. The liquid maintenance medium was as previously described (Fujita et al. 1993), with 5 g/l glucose and 100 mg/l phenol provided as carbon sources and to maintain selection pressure. Cultures were grown up in 125-ml conical flasks containing 50-ml maintenance medium, incubated in a shaker bath at 30 °C for 36-h prior to inoculation of the fermentor. A total of 160-ml inoculum from four flasks was centrifuged, the supernatant decanted and the cell fraction resuspended in the remaining 40-ml medium, which was then used to inoculate the fermentor for both batch and fed-batch experiments.

Fermentation of 10-g phenol

Fermentation was carried out in a 2-l New Brunswick Scientific BioFlo bioreactor modified for aeration with two sintered glass spargers. The fermentation broth was maintained at 30 °C, agitated at 250 rpm and aerated at 0.5 vvm. The pH was automatically controlled at 6.8 using 2M KOH. The fermentor, containing 1-l doubly concentrated carbon-free medium, was autoclaved, and the sterilized medium was then inoculated. A 500-ml sample of organic phase (2-undecanone), containing 10-g phenol, was then added to the fermentor, and the system was allowed to equilibrate for 30 min before initial measurements were taken.

In an effort to overcome the oxygen limitation seen in previous experiments, a second experiment was undertaken with enriched air. In this instance, 0.25 vvm air was blended with 0.25 vvm pure O_2, so that the fermentor was aerated with enriched air at a total gas flow of 0.5 vvm. The dissolved oxygen concentration in the fermentation broth was maintained at or above 80% throughout the fermentation, with a dissolved-oxygen electrode being used to monitor the level of oxygen in the aqueous phase. The fermentor was prepared and inoculated as above, and 10-g phenol was again loaded into the organic phase.

Fermentation of 10-g phenol using a sequential feeding strategy

The fermentor was prepared and inoculated as described above, but the 10-g phenol load was divided into two 5-g aliquots. The first 5-g phenol was added at the initiation of the fermentation. When the microorganisms had consumed almost all of the initial aliquot, after 35-h, 250-ml organic phase was removed, and spiked with the second 5-g phenol. The goal of this feeding strategy was to reduce the length of the lag phase by exposing the organisms to lower concentrations of the inhibitory substrate. The fermentor was aerated at 0.5 vvm with air.

Ultimate capacity of the system

The BioFlo fermentor was prepared as above, with 10-g phenol dissolved in the organic phase. The phenol concentration in the reactor was allowed to drop to near zero, then the organic phase was spiked with phenol as described above, such that the total amount of phenol in the organic phase was once again 10-g. The system was spiked four times in this manner, so that the system ultimately consumed 46.55-g phenol (including the initial phenol load). The system was aerated with air at 0.5 vvm.

During this experiment, the fermentation broth was expected to become nutrient-depleted as the cells consumed multiple aliquots of phenol. By observing when this occurred (e.g. by a flattening of the phenol-consumption curve), a nutrient supplementation schedule could be determined, corresponding to a known mass of phenol consumed, which could then be used for subsequent feeding cycles. This strategy was followed throughout the course of the fermentation.

Analytical methods

Samples were taken from the organic and aqueous phases at least every 12-h for the duration of the experiments, and were centrifuged to isolate the desired components. The cell fraction was resuspended and cell density estimated by measuring the absorbance of the samples at 650 nm. Phenol concentrations in the aqueous phase were measured by the 4-aminoantipyrine method (Yang and Humphrey 1975) and a Bausch and Lomb Spectronic 2000 spectrophotometer. The organic-phase samples were contacted with medium buffer using the Mixxor contacting system. The resulting suspension was then separated by centrifugation, and the phenol concentration in the buffer phase measured using the 4-aminoantipyrine method mentioned above. The phenol concentration in the organic phase was then calculated by mass balance using an experimentally determined partition coefficient.

Results

The two-phase partitioning bioreactor is based on the concept of selective partitioning of a third component between an immiscible organic phase and a cell-containing aqueous phase. By proper selection of the organic solvent (on the basis of considerations of, *inter alia*, biocompatibility, immiscibility, and partition coefficient), it is possible to deliver substrate concentrations to the aqueous phase at levels that are sub-inhibitory. Moreover, such a system is self-regulatory in response to the cells' metabolic activity, with substrate delivery being provided from the organic phase in response to consumption in the aqueous phase. Such a system is readily operated in batch and fed-batch mode as described below.

Air and enriched air fermentation of 10-g phenol

Figure 1 shows the consumption of 10-g phenol in the BioFlo reactor, operated in batch mode, aerated with air. With a 10-g loading in 500-ml organic phase, the initial phenol concentration delivered to the aqueous phase under these conditions was 420 mg/l, well below the inhibitory value of 500 mg/l for this organism (Kotturi et al. 1991). The system experienced a lag phase of 36-h, after which it passed into a rapid growth phase,

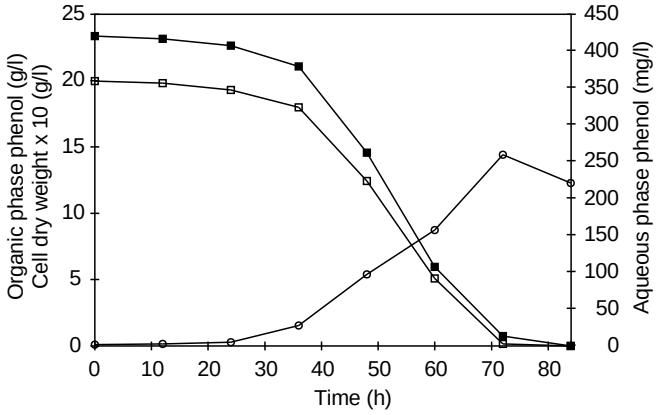

Fig. 1 Time-course plot of organic-phase phenol concentration (□), aqueous-phase phenol concentration (■), and cell dry weight × 10 (○) during the batch fermentation of 10-g phenol

during which the majority of the phenol was consumed. However, activity during this phase did not follow a typical exponential curve. Instead, the increase in cell density and the consumption of phenol were approximately linear. During this period, the dissolved oxygen concentration in the fermentation broth was 0%, indicating that growth in the system was oxygen-limited. Phenol was consumed to completion within 84-h, after which the dissolved oxygen concentration rapidly climbed to 100%.

In the next experiment, we attempted to overcome the oxygen limitation by blending pure oxygen with air, to supply enriched air to the microbes. The system was provided with enriched air once the dissolved oxygen concentration fell below 50% of saturation, which occurred at the end of the lag phase. The organic phase was again loaded with 10-g phenol, which partitioned into the aqueous phase at an initial concentration of 420 mg/l. As shown in Fig. 2, the lag phase again lasted 36-h, after which the dissolved oxygen concentration dropped below 50%, and oxygen was added to the air supply to bring the dissolved oxygen up to 80% saturation. The dissolved oxygen was maintained at this level throughout the exponential growth phase. The phenol in the system was consumed to completion within 72-h.

Fermentation of 10-g phenol using a sequential feeding strategy

In an attempt to reduce the length of the lag phase, 10-g phenol was divided into two 5-g aliquots and delivered to the system serially. The organic phase was loaded with 5-g phenol, which delivered phenol at a concentration of 210 mg/l to the aqueous phase, resulting in a 12-h lag phase, as shown in Fig. 3. At the end of the lag phase, the dissolved oxygen concentration dropped to 0%, and phenol was consumed at a rapid rate. After 35-h, the initial 5-g phenol had been consumed almost to exhaustion, and a 5-g spike of phenol was added to the organic phase. At this point, the microbes had become acclimated to this level of phenol, so the fermentation was able to continue at a rapid rate without a lag period. The 10-g phenol was consumed to exhaustion within 60-h. It should be noted that the system was oxygen-limited throughout the two rapid growth phases.

Ultimate capacity of the system

To try to determine the ultimate capacity of the system, multiple 10-g spikes of phenol were added to the BioFlo reactor, as shown in Fig. 4. After the initial 10-g phenol had been added to the organic phase, there was a 36-h lag phase, after which the dissolved oxygen concentration dropped to 0% saturation, where it remained until the conclusion of the experiment. The initial phenol load was consumed almost to exhaustion within 84-h, at which point the organic phase was spiked with enough phenol to return the overall amount of phenol in the system to 10-g. The system rapidly consumed this second spike until it became nutrient-depleted, at about 120 h. At this point, the phenol concentrations in the aqueous

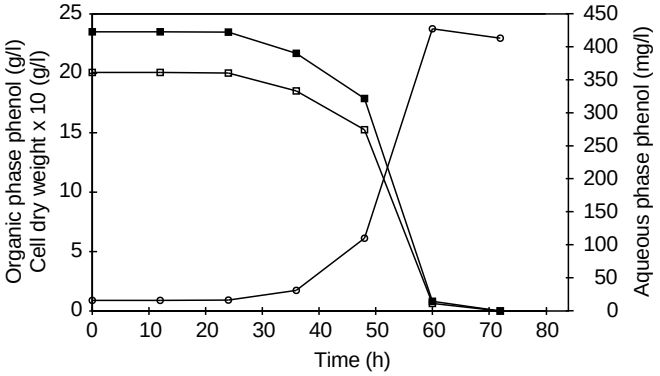

Fig. 2 Organic-phase phenol concentration (□), aqueous-phase phenol concentration (■), and cell dry weight × 10 (○) as a function of time in the enriched-air batch fermentation of 10-g phenol

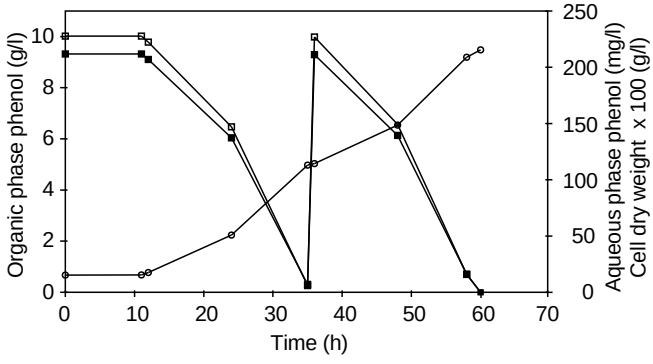

Fig. 3 Time-course plot of the fermentation of a total of 10-g phenol, using a sequential feeding strategy, showing organic-phase phenol concentration (□), aqueous-phase phenol concentration (■), and cell dry weight × 100 (○)

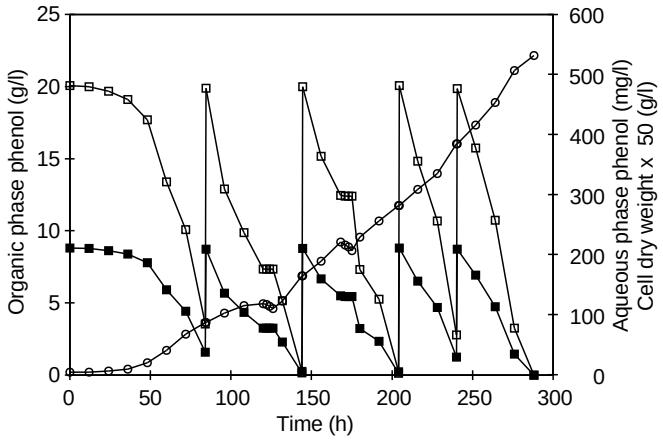

Fig. 4 Organic-phase phenol concentration (□), aqueous-phase phenol concentration (■), and cell dry weight × 50 (○) during the fed-batch fermentation of a total of 46.55-g phenol

and organic phases leveled-off, and the cell density began to drop. With the original double-salts medium used, this occurred after approximately 14.6-g phenol had been consumed. A single-salts concentration spike was then added, and phenol degradation rapidly resumed. A second phenol spike was added at 144-h, as described above. As expected, phenol consumption stopped and the cell density began to drop after a further 7.3-g phenol had been consumed, at 171-h, again in response to perceived nutrient depletion. For the remainder of the experiment, a nutrient bolus was added for every 7.25-g phenol consumed to prevent nutrient depletion, which adversely affects the performance of the microorganisms. Additional phenol spikes were delivered, as needed, at 204-h and 240-h. The system ultimately consumed 46.55-g phenol in 288-h.

Discussion

Oxygen-enriched fermentation of 10-g phenol

When phenol degradation by the microorganisms is oxygen-limited, the growth rate and substrate consumption rate during the rapid growth phase are approximately linear, rather than exponential, as expected. Since oxygen is transferred to the microorganisms at an essentially constant rate (given constant aeration and agitation conditions), the cell growth and substrate consumption rate are expected to be linear as well. Enriched air can be supplied to the reactor so that the specific growth rate of the organism, rather than oxygen-transfer rate becomes the limiting factor. This is seen in Fig. 2 as near-exponential changes in cell and substrate concentrations.

Since the system is only oxygen-limited during the exponential growth phase, the lag phases are the same length in the oxygen-limited and oxygen-enriched fermentations. However, the length of the growth phase is favorably affected by the addition of enriched air. The enriched air system is able to consume 10-g phenol to completion in 72-h, as opposed to 84-h in the oxygen-limited system. Accordingly, the volumetric productivity of the system (based on phenol consumption) is significantly improved by the use of enriched air. The oxygen-limited system is able to consume 0.248-g phenol l^{-1} h^{-1} from the end of the lag phase to depletion, while the enriched air system is able to consume 0.373-g phenol l^{-1} h^{-1} for the same period. This represents a 50% increase in the volumetric productivity for the enriched-air system over the oxygen-limited system. The addition of pure oxygen to the air supply can therefore be used to reduce the length of the growth phase and to increase the overall rate of phenol consumption.

Significant biofilm formation occurred in this system. Cells attached themselves to the walls of the reactor, the baffles and the spargers. Biofilm growth has been shown to produce erroneously low estimates for cell yield coefficients in both batch and continuous cultures (Molin 1985). It is very difficult to prevent biofilm formation in this system and, as such, it is not possible to obtain true yield coefficients for this system. Although some foaming occurred during all of the experiments conducted, particularly during periods of rapid phenol degradation, it did not pose any significant operational difficulties, and no antifoam was used.

Fermentation of 10-g phenol using a sequential feeding strategy

By supplying enriched air to the fermentation broth, we were able to reduce the length of the exponential growth phase, thereby increasing the volumetric phenol consumption rate of the system. In examining sequential feeding strategies, we were seeking to reduce the length of the lag phase, further reducing the length of time required to consume 10-g phenol. By dividing the 10-g phenol load into two equal doses, the length of the lag phase was dramatically reduced from 36-h, for a standard 10-g load, to 12-h. This feeding strategy permitted the complete degradation of 10-g phenol in only 60-h. The overall volumetric productivity (including the lag phase) of this system was therefore 0.167 g l^{-1} h^{-1}, as compared to the overall volumetric productivity of 0.119 g l^{-1} h^{-1} for the standard system, which represents a 40% increase in productivity with the use of a sequential feeding strategy.

Ultimate capacity of the system

Operated in fed-batch mode, the two-phase partitioning bioreactor was able to consume 46.55-g phenol in 12 days. The performance of the system was consistent over the course of the fermentation, which indicates that the system can very effectively be operated in fed-batch mode without any significant accumulation of toxic or

inhibitory cellular metabolites. The use of a fed-batch mode of operation maximizes the percentage of time in which the fermentation is productive. The productivity of the system could be further improved through the use of an optimized feeding strategy, in which the microbes were exposed to lower aqueous-phase concentrations of phenol, through more frequent feedings of smaller aliquots of phenol, and through the use of enriched air. Moreover, although the original goal of the experiment was to determine the ultimate capacity of the system to degrade phenol, the limit has clearly not yet been reached and, with scheduled nutrient supplementation, it is expected that more extensive phenol consumption could be accommodated. This is further suggested by the fact that the cell-growth and phenol-consumption rates did not decrease as the fermentation progressed.

The results with the two-phase partitioning bioreactor demonstrate an unprecedented level of phenol removal, with the consumption of 10-g aliquots in batch fermentation, and 46.55-g phenol consumed in a 2-l bioreactor (1-l aqueous/cell volume) within 12 days in fed-batch mode. In contrast, conventional batch and fed-batch schemes are limited to the addition of low amounts of phenol (below 500 mg/l) to avoid substrate inhibition. In batch systems, such maximum initial loadings would likely produce lag periods of approximately the same length as was experienced in our two-phase batch configuration (which had an initial partitioned aqueous phenol concentration of 420 mg/l), but would be limited to the consumption of only 500 mg/l, rather then the 20-fold higher level (10-g) that we employed. In conventional fed-batch fermentations, multiple feedings of a maximum of 500 mg/l could be used, while we were readily able to add 10-g on a repeated basis. Such large feeding amounts ultimately resulted in high, and increasing, cell concentrations that facilitated phenol degradation even further. Operationally, the two-phase partitioning bioreactor required very little intervention, with far fewer feedings required to deliver the same overall amount of phenol than would be needed for conventional systems. Rational selection of the substrate-containing solvent phase provides opportunities for the provision of large substrate loadings, as well as for the application of the process concept to other biodegradation systems.

In conclusion, the biphasic bioreactor continues to provide an excellent alternative to conventional phenol biodegradation schemes. The system requires only pH and temperature control, and has very low energy requirements for agitation and aeration. Phenol is delivered on a demand basis to the aqueous phase, at a rate determined by the metabolic rate of the microorganisms. Our recent research has successfully reduced the duration of both the lag phases and the rapid-growth phases through the use of sequential feeding strategies and enriched air, and has demonstrated the large capacity of the system for phenol degradation. In addition, a nutrient-feeding schedule was established, which further permits optimal operation of the bioreactor. Future research will utilize modeling tools to improve system performance further, and will examine the application of the two-phase partitioning bioreactor concept to other xenobiotics such as benzene, toluene and xylene.

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