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Benzene degradation in a two-phase partitioning bioreactor by *Alcaligenes xylosoxidans* Y234

Sung-Ho Yeom [b], Andrew J. Daugulis [a,*]

[a] *Institute of Molecular Biology and Genetics, Seoul National University Seoul, 151-742, South Korea*
[b] *Department of Chemical Engineering, Queen's University, Kingston, ONT, Canada, K7L 3N6*

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Abstract

An aqueous-organic, two-phase bioreactor system was used for the biodegradation of benzene. Through a systematic solvent selection procedure considering biological, physical, operational, environmental and economic factors, hexadecane was chosen as the most promising solvent. The solvent has infinite solubility for benzene and shows very high phase stability with aqueous media. A*lcaligenes xylosoxidans* Y234 capable of degrading benzene without any other co-substrate was inoculated into the aqueous phase of the two-phase partitioning bioreactor, which consisted of a 1 l aqueous phase and 500 ml hexadecane. A feed of 7000 mg of benzene was loaded into the organic phase, which gave an initial equilibrium aqueous phase concentration of 100 mg/l. Over the course of 24 h, 63.8% of the benzene was degraded by the microorganism, and 36.2% was stripped by aeration. By installing a condenser and using a lower gas flow of pure oxygen to reduce stripping, more than 99% of a subsequent 7000 mg benzene addition was degraded by the organisms within 24 h.

Keywords: *Alcaligenes xylosoxidans*; Benzine degradation; Hexadecane; Partitioning bioreactor

1. Introduction

Toxic aromatic compounds such as benzene, toluene and xylene (BTX) are the starting materials for the synthesis of plastics, paints, pesticides, resins and dyes, and are also used as solvents for rubber and plastics, as well as being components of aviation and automotive fuels. Since they are suspected as being carcinogens, however, their release to the environment is strictly controlled and they are classified as priority environmental pollutants by the Environmental Protection Agency in the USA. Recently, biological treatment of BTX using microorganisms has been extensively explored as an alternative to physical or chemical treatment because it does not produce secondary effluent problems, and because biological treatment processes are probably the most cost–effective techniques for treating aqueous waste streams containing organic compounds [1].

Many researchers have undertaken fundamental investigations of the degradation of BTX by microorganisms. They have isolated microorganisms capable of degrading BTX [2–4], investigated the degradation kinetics [5,6] and observed the effect of environmental factors such as pollutant concentration, temperature, pH, substrate interactions, microbial competition and adaptation on the degradation of BTX [7–10].

One feature of benzene common to many other 'xenobiotics', is its toxicity to microorganisms, and the concomitant difficulty in degrading it when it is present at high concentrations. Choi et al. [11] developed a method to allow controlled, continuous addition of inhibitory aromatic compounds to an aqueous cell culture using silicon tubing. The rate of diffusion of the solvents through the silicon tubing was determined by the impeller speed in the aqueous medium and as such could be controlled. However, this system has several disadvantages such as difficulty of scale-up, fixed diffusion rate and biofouling as previously noted [12,13]. Yeom and Yoo [13] suggested a hybrid bioreactor that was comprised of a bubble column bioreactor in series

* Corresponding author. Fax: +1-613-5336637.
E-mail address: daugulis@chee.queensu.ca (A.J. Daugulis).

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with a biofilter. When a high concentration of BTX was fed to the bubble column bioreactor section, some of it was degraded and some was stripped by aeration. In this way, the BTX burden could be shared between the two sections by manipulating operating variables such as air flow rate and liquid residence time. In spite of these advantages, this system had drawbacks, often associated with biofilters, including cell overgrowth and plugging, and difficulties maintaining proper temperature, pH and moisture [14].

Our attention has recently focussed on an alternative method of 'delivering' high concentrations of inhibitory substrates, such as benzene, through the use of a second, distinct organic phase. In this scheme, very large amounts of xenobiotic substrate can be dissolved in a hydrophobic organic phase, and can then partition at appropriately low concentrations by means of equilibrium to cells. Such a system is self-regulating in the sense that the metabolic activity of the cells determines the rate of transfer of the xenobiotics, and the processing concept has been successfully applied to the degradation of very large amounts of phenol [12,15] and pentachlorophenol [16]. In this study, a very systematic procedure to select an appropriate solvent in the two-phase partitioning bioreactor system is presented. The degradation of benzene was used as a model system with an organism capable of utilizing benzene as sole carbon source, and loadings which would otherwise be completely toxic to the cells were shown to be readily and quickly consumed.

2. Materials and methods

2.1. Microorganism

A. xylosoxidans Y234 isolated from oil-contaminated soil was used in this study. It can individually degrade benzene, toluene and phenol as sole carbon sources [17,18].

2.2. Growth medium

A. xylosoxidans Y234 was precultured at 30°C in a 125 ml flask containing 50 ml of: 10 g/l glucose, 5 g/l yeast extract, 5 g/l $(NH_4)_2$ SO_4, 5 g/l KH_2PO_4 and 1 g/l $MgSO_4$ $7H_2O$.

2.3. Microbial adaptation

To eliminate a microbial adaptation period in the bioreactor, *A. xylosoxidans* Y234 was pre-adapted to benzene. After the cells were harvested from the culture grown in the medium mentioned above, they were resuspended in distilled water and then centrifuged twice. The cells were then transferred into a 160 ml serum bottle containing 30 ml of medium A, which consisted of: 5.0 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 2 g/l $(NH_4)_2SO_4$, 0.3 g/l $MgSO_4{\cdot}7H_2O$ and 200 μl/l of trace element solution. The trace element solution consisted of 16.2 g/l $FeCl_3{\cdot}6H_2O$, 9.44 g/l $CaHPO_4$, 0.15 g/l $CuSO_4{\cdot}5H_2O$ and 40.0 g/l citric acid. Benzene (3 μl) was added to the bottle as a sole carbon and energy source (giving a benzene concentration of 42.8 mg/l). The bottle was sealed with a butyl-rubber septum and aluminum crimp cap. After 20 h incubation, the cells were assumed to be fully adapted to benzene and were ready for inoculation into the two-phase bioreactor.

2.4. Bioreactor system and operation

The two-phase bioreactor was set up as previously described [12]. The working volumes of the aqueous and organic phases were 1.0 and 0.5 l, respectively. The composition of the aqueous phase (Medium B) was: 5.0 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 4.0 g/l $(NH_4)_2SO_4$, 0.6 g/l $MgSO_4{\cdot}7H_2O$ and 1.0 ml/l of trace element solution to prevent mineral limitation. Inlet and outlet gas flow rates, and exit gas benzene concentration were monitored to allow calculation of benzene stripping. The dissolved oxygen (DO) level was also monitored carefully by means of a DO electrode. The bioreactor was operated at 350 rpm and 30°C. Aeration was either by air at 0.25 vvm (based on aqueous volume) or by pure oxygen at 0.1 vvm.

2.5. Analytical procedures

Since the solvent that was ultimately selected for this system has excellent phase stability (i.e. did not form a dispersion or an emulsion, but remained as a distinct second phase) samples of each phase could be readily withdrawn without ceasing operation. Aqueous samples were taken from the two-phase bioreactor and centrifuged to remove cells. The liquid benzene concentration was analyzed by directly injecting 2 μl of the liquid sample into a Perkin–Elmer gas chromatograph equipped with a J and W DB-1 megabore 30 m capillary column. The concentration was determined from the peak area as compared with a previously prepared calibration curve. To measure the benzene concentration in the organic phase (hexadecane), 3 ml of organic sample was taken and mixed with the same volume of distilled water. The mixed solution was shaken vigorously and left for 4 h. Aqueous phase (2 μl; lower phase) was injected into the GC. The benzene concentration in the hexadecane was calculated from the known distribution coefficient of benzene between the aqueous and organic phases, which was previously measured to be 140.1. To measure the gas phase benzene concentration, 250 μl of bioreactor exit gas was sampled in a gas-tight syringe and injected into the GC.

The resulting area was also compared with a calibration curve of gaseous benzene to determine the actual gaseous benzene concentration. The operating conditions of the GC in all cases (liquid and gas benzene) were: 250°C for the injection port, 50°C for the oven and 200°C for the detection port.

Cell concentration was measured by optical density and a calibration curve using a Brinkman PC600 Colorimeter at 640 nm.

2.6. Critical log P

The critical log P (the log P above which solvents are biocompatible) of *A. xylosoxidans* Y234 was determined as previously described [18].

2.7. Bioavailability

The organic solvent used in the two-phase partitioning bioreactor system should not be used as a substrate by the microorganism to ensure that the benzene acts as sole carbon source. To determine bioavailability, 5 ml of each solvent from a short list (as described later) was added to 125 ml flasks containing 50 ml of medium A with an initial cell concentration of 100 mg/l. Solvent was not added to one flask, which was used as a negative control. In addition, 5 ml of corn oil was added to one flask, to serve as a positive control. The flasks were placed on a shaker water bath at 30°C for 6 days, and cell concentrations were measured every 24 h.

3. Results and discussion

3.1. Solvent selection

Interest in exploiting the potential of two-phase bioreactors has been increasing with recent examples having been published in the areas of biotransformation [19] and biodegradation [15]. Perhaps the key element in the success of these systems is the selection of an appropriate second phase, which is a significant challenge given the huge number of potential solvents available. Solvents have been chosen somewhat arbitrarily or with consideration of only a few criteria. El Aalam et al. [20], for example, considered capacity, biocompatibility and bioavailability of the solvent, leading to the choice of silicone oil for the degradation of styrene. Collins and Daugulis [12,15] additionally considered partition coefficient, volatility, solubility, selectivity and cost. Nevertheless, the consideration of additional factors can make solvent selection more systematic and effective, and this was the first thrust of the present work.

In selecting an appropriate solvent for this application biological factors (biocompatibility and non-bioavailability), physical factors (volatility, density, hydrophobicity, and possibility of emulsification), chemical factors (the solvent should not need special handling and must not harm personnel or the environment) and cost were all considered. Once an organism is selected to degrade a specific contaminant, the critical log P (above which a solvent has no toxic effect) of the microorganism must be first determined. The log P value (the logarithm of its standard octanol–water partition coefficient) of each solvent can be determined from available data [21] or calculated using established methods [22] and commercial software [23]. In this work, the critical log P of *A. xylosoxidans* Y234 was determined to be 3.5 [18]. Solvents with high partition coefficients were selected next through the use of predictions from the UNIFAC-based Extractant Screening Program (ESP) [15]. In addition to estimating phase behaviour, the ESP program also has the capability of providing 'filters' to include or exclude solvents, based for example, on log P, boiling point, melting point, molecular weight and carbon number as criteria. Since volatility data are not readily available, boiling point was used as an equivalent criterion, and thus the database of 1350 solvents was screened to include only those solvents with a log P and boiling point higher than 3.5 and 150°C, respectively. Through this process about 400 solvents passed these criteria and were ranked on the basis of partition coefficient. The higher the partition coefficient, the higher the concentration of benzene that can be loaded into the solvent without causing an inhibition effect on the cells as a result of benzene partitioning into the aqueous phase. Phase stability (a solvent property characterized by the tendency to remain as a distinct liquid phase relative to the aqueous phase, and not to be volatilized) was considered next, and the choice of solvents limited to those with densities less than 0.85 g/ml. In addition, solvents which may cause emulsions with water should be excluded. Thus, the suspected emulsion-forming solvents, which are either long chain esters or very polar compounds, were excluded. Through these considerations of physical characteristics, almost 250 solvents were excluded.

In taking into account environmental considerations, solvents containing chloro, phenyl or cyano groups were excluded due to possible toxic effects. Acids were also excluded due to the possibility of causing corrosion to metals. Compounds requiring special handling such as the need to be kept in darkness or in the presence of inert gas were also ruled out. In considering costs, solvents were considered further only if they were sold at less than US$75/l. After consideration of all these factors only four solvents remained on a short list, and their properties are listed in Table 1. The bioavailability of these solvents was tested as a final step and only hexadecane and 1-decanol appeared not to be used as a

Table 1
Physical properties of selected solvents for the two-phase partitioning bioreactor for the degradation of benzene [a]

Solvent	Partition coefficient (ESP)	Partition coefficient (Exp)	Selectivity (ESP)	Log P	Benzene solubility (Exp)	Density (g/ml)	Boiling point (°C)	Molecular weight	Cost (US$/l)
1-Octadecene	341.6	162.5	1.26×10^7	9.34	∞	0.789	179	252	15.40
n-Dodecane	329.6	152.7	1.55×10^7	6.60	∞	0.748	216	170	75.00
n-Hexadecane	301.3	140.1	1.52×10^7	8.67	∞	0.773	280	226	19.80
1-Decanol	273.4	118.2	1.42×10^4	3.90	∞	0.839	228	158	11.20

[a] Prices are from Sigma-Aldrich or Alfa Aesar as at December, 1999.

substrate by *A. xylosoxidans* Y234. Since hexadecane has a higher log *P* and boiling point and lower density than 1-decanol, it was chosen as the most promising solvent for this bioreactor system.

3.2. Reactor operation

A batch fermentation was conducted with an initial cell concentration of 96.5 mg/l, and an initial loading of 7000 mg benzene in the hexadecane phase (14 000 mg/l), which partitioned into the aqueous phase to around 100 mg/l (Fig. 1). As the cells were previously pre-adapted to benzene, no extra adaptation or lag period was observed in this experiment and the cell concentration began to increase almost immediately. The medium turned light green for the first 5 h of operation probably due to the production of pigment. The DO level dropped sharply to 40% of the saturation level during this phase. During this exponential growth phase, the cells produced some foam, which formed at the top of the organic phase and a milk-coloured material, suspected to be an emulsifier, at the interface between the two phases. This material made the interface less distinct. After 20 h, when the benzene was exhausted in the aqueous phase, cell growth entered stationary phase and the DO level began to increase. No oxygen limitation was observed during the course of this fermentation (i.e. the DO did not drop below 20% of saturation). Throughout the entire experiment, samples were taken from both organic and aqueous phases to measure benzene concentration. Since the benzene concentration in the organic phase was affected by the presence of entrained cells and possibly an emulsifying material, some deviation existed between the measured benzene concentration in the organic phase and that calculated from the aqueous phase concentration and the distribution coefficient.

The benzene was not only degraded by the microorganisms but also stripped out by aeration. Therefore, the benzene leaving the bioreactor in the exit gas stream was monitored to calculate the amount of benzene lost due to stripping. At the start of the fermentation the benzene concentration decreased substantially despite a low cell concentration at the early stage of operation, which suggests that losses due to stripping can be important (Fig. 1). The stripped benzene concentration was proportional to the benzene concentration in the solvent [13]. The stripping rate of benzene was as high as 240 mg/h after 2 h of operation for the initially high benzene concentration in the organic phase (Fig. 2). As the experiment progressed, the exit benzene concentration decreased due to the decrease in the benzene concentration in the bioreactor. The amount of benzene lost to stripping was calculated by integrating the area below the curve shown in Fig. 2. The best fit equation for the curve was obtained by a second-order regression and integrated from 0 to 24 h. The resulting benzene amount was estimated to be 2531 mg, which meant that 4469 mg of benzene was degraded within 24 h (representing 63.8% biodegradation efficiency). Since the cell mass increase was 1579 mg, the cell yield was 0.353. Due to cell growth on the walls of bioreactor and some cells being entrained into the organic phase, the observed cell yield was thought to be lower than the true value. The overall degradation rate of benzene in this system is 186 mg/l/h.

To reduce the effect of stripping and to encourage more biological removal of benzene, a condenser was installed on the exit gas line of the two-phase bioreactor, and pure oxygen at a flow rate of 0.1 vvm was

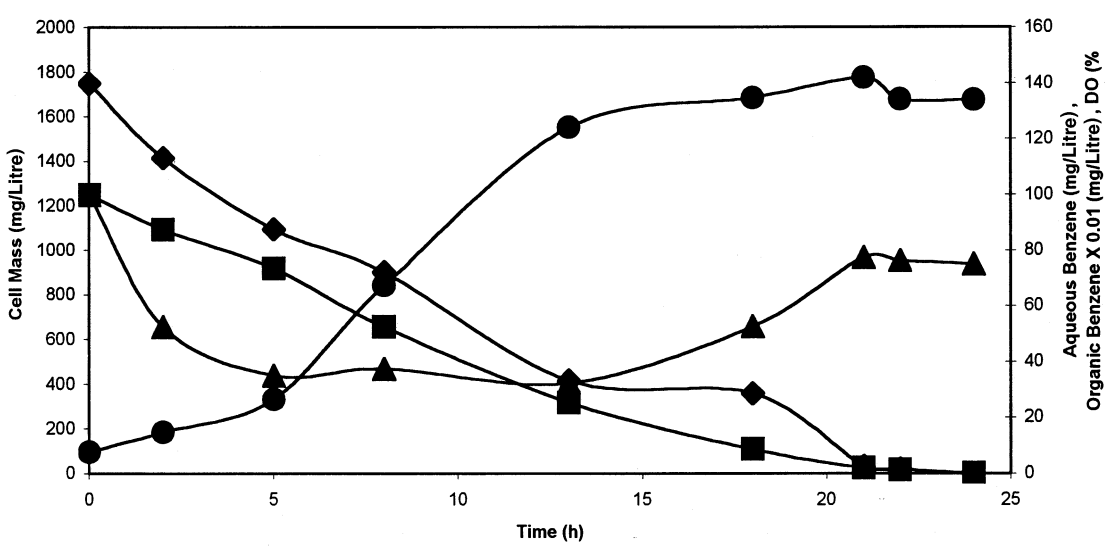

Fig. 1. Batch benzene degradation in the two-phase partitioning bioreactor. ●: cell mass, ■: benzene concentration in aqueous phase, ▲: DO level, ◆: benzene concentration in organic phase.

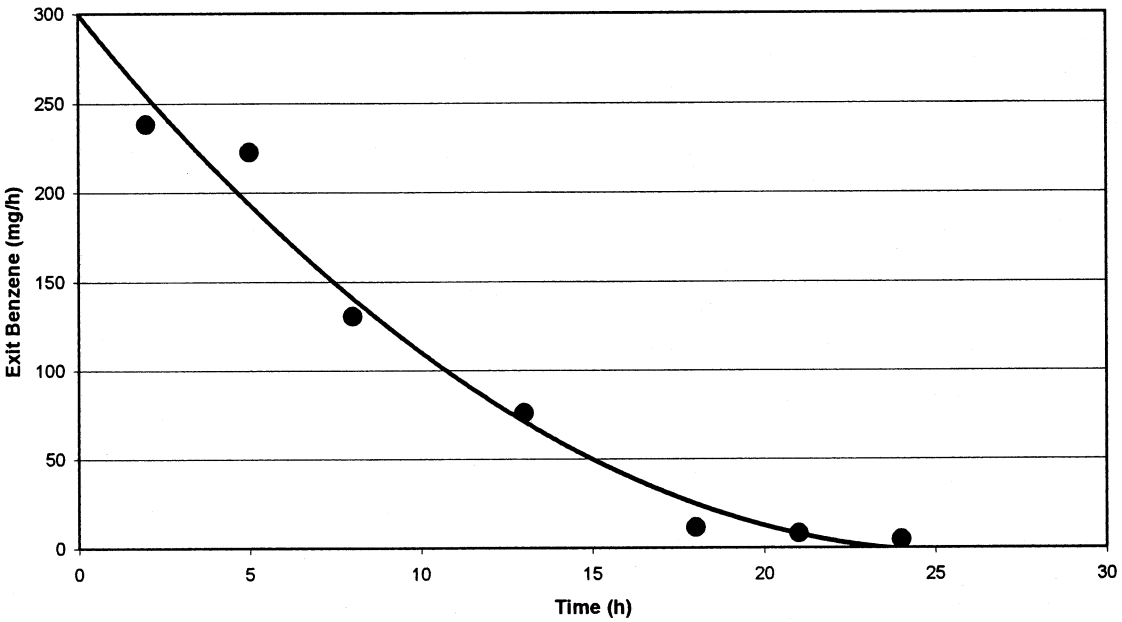

Fig. 2. Benzene stripping from the two-phase partitioning bioreactor by aeration.

used. The amount of benzene initially loaded was the same (7000 mg) and the initial cell concentration was somewhat higher (270 mg/l) due to more extensive inoculum growth. The water temperature entering the condenser was 9°C, which is 3.5°C higher than the melting point of benzene. During operation the benzene exiting the bioreactor appeared to condense and drop back into the organic phase. The cells entered exponential growth after 3 h of operation (Fig. 3). The medium turned light green before the exponential growth phase as observed in the previous batch experiment. The DO level began to drop sharply to 20% of the saturation level during the exponential phase, but increased again once the cells entered stationary phase. The foam, observed at the top of the organic phase in the former batch experiment, was completely depressed and cell growth on the glass walls of bioreactor was also much reduced during this experiment. Even though some material, suspected to be an emulsifier, was still produced and stayed at the interface of the two phases, the two phases were distinctly separate during the entire experiment. The final cell concentration reached 3150 mg/l.

The exit benzene concentration and outlet gas flow were also monitored and the total amount of benzene lost to stripping was calculated to be 24 mg, which means that 6976 mg of the benzene was degraded with 99.7% biodegradation efficiency within 24 h. Considering substrate consumption and the increase in cell concentration, the cell yield was estimated to be 0.413. Since there was still some cell growth on the fermentor walls, and some entrainment of cells into the organic phase, the real cell yield was also thought to be higher than this value. Because it was not possible to measure the cell concentration accurately during operation, it is not possible to calculate characteristic biodegradation parameters such as specific degradation rate, half-saturation constant in the Monod equation and the exact cell yield. The overall degradation rate of benzene in this batch experiment was 291 mg/l/h or 56.1% higher than the former case, and five times higher than in a previous study [24].

4. Conclusions

This paper has attempted to utilize an extremely promising organism for benzene degradation along with a rigorous and systematic procedure for the identification of a superior organic solvent for use in a two-phase partitioning bioreactor configuration consisting of a 1 l aqueous phase and 500 ml of solvent. A very high benzene loading (7000 mg) was applied to the system, but because of the equilibrium partitioning between the two phases, the cells were readily able to completely consume this material within 24 h with a removal efficiency of 99.7%, and high volumetric productivity. The presence of a suspected emulsifying agent warrants further investigation, however. We are currently considering the application of this two-phase system to the removal of organic contaminants in gas streams, and their biodegradation in the partitioning bioreactor. That is, we will be using our high boiling solvent as a contacting fluid in a gas stripping column, before sending it to the bioreactor, and recycle back to the stripper. This may provide an attractive alternative

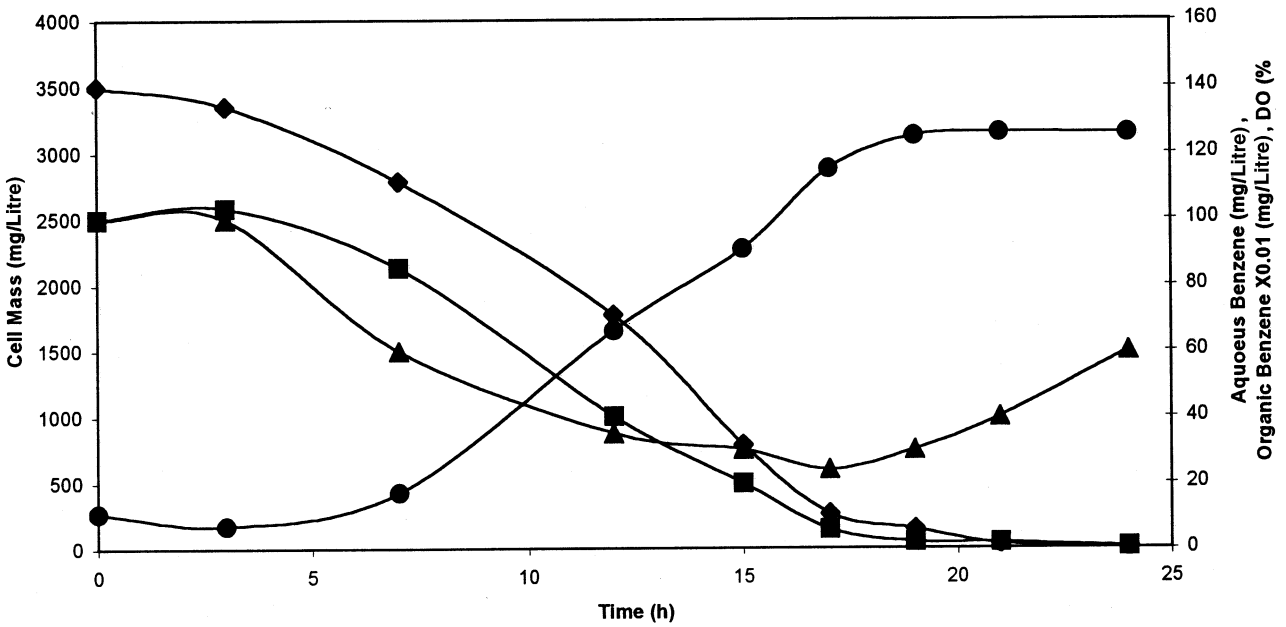

Fig. 3. Batch benzene degradation in the two-phase partitioning bioreactor with a condensor installed and with pure oxygen aeration. ●: cell mass, ■: benzene concentration in aqueous phase, ▲: DO level, ◆: benzene concentration in organic phase.

biological means to biofilters for the removal of volatile organics in gaseous effluents.

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References

[1] Thayer AM. Biodegradation: innovative technology for cleaning up hazardous waste. Chem Eng News 1991;Aug:23–44.
[2] Baggi G, Barbieri P, Galli E, Tollari S. Isolation of a *Pseudomonas stutzeri* strain that degrades *o*-xylene. Appl Environ Microbiol 1987;53:2129–32.
[3] Edwards EA, Wills LE, Reinhard M, Grbic-Galic D. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl Environ Microbiol 1992;58:794–800.
[4] Yadav JS, Reddy CA. Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl Environ Microbiol 1993;59:756–62.
[5] Chang MK, Thomas CV, Craig SC. Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and *p*-xylene by two *Pseudomonas isolates*. Biotechol Bioeng 1993;41:1057–65.
[6] Oh YS, Shareefdeen Z, Basil CB, Bartha R. Interactions between benzene, toluene, and *p*-xylene (BTX) during their biodegradation. Biotechnol Bioeng 1994;44:533–8.
[7] Alvarez PJJ, Vogel TM. Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. Appl Environ Microbiol 1991;57:2981–5.
[8] Arvin E, Jensen BK, Gundersen AT. Substrate interaction during aerobic biodegradation of benzene. Appl Environ Microbiol 1989;55:3221–5.
[9] Duetz WA, DE Jong C, Williams PA, VAN Andel JG. Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl Environ Microbiol 1994;60:2858–63.
[10] Rozich AF, Colvin RJ. Effects of glucose on phenol biodegradation by heterogeneous populations. Biotechnol Bioeng 1986;28:965–72.
[11] Choi YB, Lee JY, Lim HK, Kim HS. A novel method to supply the inhibitory aromatic compounds in aerobic biodegradation process. Biotechnol Tech 1992;6:489–94.
[12] Collins LD, Daugulis AJ. Use of a two phase partitioning bioreactor for the biodegradation of phenol. Biotech Techniq 1996;10:18–22.
[13] Yeom SH, Yoo YJ. Removal of benzene in a hybrid bioreactor. Proce Biochem 1999;34:281–8.
[14] Hodge DS, Devinny JS. Modelling removal of air contaminants by biofiltration. J Environ Eng 1995;121:21–32.
[15] Collins LD, Daugulis AJ. Biodegradation of phenol at high initial concentration in two-phase partitioning batch and fed-batch bioreactors. Biotechnol Bioeng 1997;55:155–62.
[16] Munro DR, Daugulis AJ. The use of an organic solvent and integrated fermentation for improved xenobiotic degradation. Resour Environ Biotechnol 1996;1:207–25.
[17] Yeom SH, Lee JH, Yoo YJ. Cometabolic removal of xylene isomers by *A. xylosoxidans* Y234. J Microbiol Biotechnol 1998;8:222–8.
[18] Yeom SH, Daugulis AJ. A new method for the determination of microbial activity and critical log P in the presence of organic solvents. Biotechnol Techniq 1999;13:549–53.
[19] Chae HJ, Yoo YJ. Mathematical analysis of an enzymatic reaction in aqueous/organic two-phase system: tyrosinase-catalyzed hydroxylation of phenol. J Chem Technol Biotechnol 1997;70:163–70.
[20] El Aalam SE, Pauss A, Lebeault JM. High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. Appl Microbiol Biotechnol 1993;39:696–9.

[21] Inoue A, Horikoshi K. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J Ferment Bioeng 1991;71:194–6.
[22] Rekker RF, de Kort HM. The hydrophobic fragmental constant: an extension to a 1000 data point set. Eur J Med Chem-Chemical Therapeutica 1979;14:479–88.
[23] ACD. ACD/Log P Calculator, version 4.0 for Microsoft Windows, Toronto. Canada. 1998.
[24] Collins LD, Daugulis AJ. Benzene/toluene/*p*-xylene degradation. Part II. Effect of substrate interaction and feeding strategies in toluene/benzene and toluene/*p*-xylene fermentation in a partitioning bioreactor. Appl Microbiol Biotechnol 1999;52:360–5.