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The use of *Enterobacter cloacae* ATCC 43560 in the development of a two-phase partitioning bioreactor for the destruction of hexahydro-1,3,5-trinitro-1,3,5-s-triazine (RDX)

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Abstract

Research on the biodegradation of explosives has focussed exclusively on the treatment of contaminated soil and water. In the present work the anaerobic degradation of hexahydro-1,3,5-trinitro-1,3,5-s-triazine (RDX) by *Enterobacter cloacae* ATCC 43560 was investigated, and a two-phase partitioning bioreactor (TPPB) was developed for the destruction of pure, past-date munitions. TPPBs are characterized by a cell-containing aqueous phase, and an immiscible and biocompatible organic phase into which very large amounts of toxic and/or insoluble substrates can be dissolved. Based on equilibrium partitioning, the substrate is then transported to the cells, in response to their metabolic requirements, providing a means of demand-based substrate delivery, and high bioreactor productivity. Through consideration of the critical log *P* of *E. cloacae*, whether various classes of solvents could be used as sole carbon and energy sources, the capacity of various organics to dissolve RDX, and solvent cost, 2-undecanone was ultimately selected as the delivery solvent for the TPPB. Using this solvent, both batch and fed-batch operation of the TPPB were undertaken, and the volumetric degradation rate of RDX was found to be higher in this arrangement than any previous values reported in the literature. This work has demonstrated the potential of a method for the destruction of decommissioned munitions involving the dissolution of RDX in 2-undecanone, the use of the RDX-rich solvent as the second phase in a TPPB to degrade this explosive, and the subsequent recycling and re-use of the solvent.

Keywords: Partitioning bioreactor; RDX; Biodegradation

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-s-triazine (RDX) is a nitroaromatic explosive used extensively since WWII in detonators, primers, mines, rocket boosters and plastic explosives (Yinon, 1990). For the biological treatment of RDX-contaminated soil and water, the conditions predominantly studied

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for biodegradation have been anaerobic (McCormick et al., 1981; Kitts et al., 1994; Young et al., 1997; Ronen et al., 1998; Freedman and Sutherland, 1998). It is believed that anaerobic degradation is required to first reduce the highly-oxidized nitro-groups, followed by spontaneous ring cleavage. Some work has shown aerobic degradation to be feasible (Binks et al., 1995), but there is little other evidence supporting this contention. It is also postulated that a two-step anaerobic-aerobic system would improve degradation; the anaerobic period would allow for the reduction of the nitro-groups, followed by an aerobic degradation of the products of ring cleavage (Ronen et al., 1998; Freedman and Sutherland, 1998). It is important to note that, with the exception of basic studies on the biochemistry of RDX degradation, most of the work to date on the biodegradation of RDX has focused on the remediation of contaminated soil and water.

The decrease in the stockpiles of 'active weapons' at military installations worldwide has led to a corresponding increase in storage for 'past-date' energetic materials from munitions and detonators. These stockpiles of explosives are a major concern, due to the potential for human harm. It would be beneficial if these compounds could be biologically treated, mitigating their mass-storage, or their release into the environment. The biodegradation of explosives is generally limited by the toxicity of the energetic materials, and their limited solubility in water (Kaye, 1980), and to make bioremediation a viable treatment option, these constraints must be overcome. A two-phase partitioning bioreactor (TPPB) has been developed to overcome these limitations.

TPPBs were initially used in extractive fermentations (Barton and Daugulis, 1992; Jones et al., 1993). An immiscible and biocompatible organic phase was used to selectively withdraw inhibitory end products from the aqueous phase based on equilibrium partitioning. This partitioning concept increases reactor productivity by eliminating product inhibition, and reduces water use as the inhibitory product can be produced at higher concentrations without requiring large volumes of water for dilution (Daugulis et al., 1994). In an analogous fashion, a system has been developed for the delivery of a toxic substrate using a second (organic) phase. The inhibitory substrate can be loaded into an organic phase at high concentrations, but partitions into the aqueous phase at sub-inhibitory levels. This TPPB has been successfully used for the biodegradation of styrene (El Aalam et al., 1993), pentachlorophenol (Munro and Daugulis, 1997a,b), phenol (Collins and Daugulis, 1996, 1997a,b), benzene, toluene and *p*-xylene (Collins and Daugulis, 1999), and polyaromatic hydrocarbons (Janikowski et al., 2002). The two-phase system has also been incorporated as one unit in a multiple unit process in the treatment of gaseous benzene streams (Yeom et al., 2000).

The present work focuses on the development of a TPPB system for RDX degradation utilizing *E. cloacae* ATCC 43560. The organism was originally isolated and characterized for its ability to degrade pentaerythritol and trinitrotoluene aerobically (French et al., 1998), and subsequent investigations into the oxygen-insensitive nitroreductase enzyme of *E. cloacae* has shown the enzyme's ability to also degrade RDX (Kitts et al., 2000). Our study included a determination of the conditions required for the organism to degrade RDX, and careful solvent selection based on the interactions between the solvent and the organism, and the affinity of the solvent for RDX. The final stage was a 'demonstration of concept' in a batch and fed-batch system, allowing for a biologically-based destruction method potentially applicable to energetic materials collected from past-date munitions.

2. Materials and methods

2.1. Chemicals

RDX was provided by the Defense Research Establishment Valcartier (Val Belair, PQ). All other chemicals were obtained from Sigma–Aldrich Chemical Company Ltd. (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

2.2. Liquid chromatography

High-performance liquid chromatography (HPLC) measurements for RDX concentration were performed using a Waters 515 HPLC pump, a Waters 2487 Dual λ Absorbance Detector (at 254 nm) and a Waters Spherisorb® 5 μm ODS2 4.6 × 250 mm Analytical Cartridge. A mobile phase of methanol in water (50:50 v/v) was used at a flow rate of 1 ml min^{-1}. Analysis of the measurements were performed using WATERS MILLENNIUM Software.

2.3. Cell concentration

Total cell concentrations were measured using a Brinkmann PC800 Colorimeter (Mississauga, Ont.). The optical density of the suspension was measured at 650 nm and correlated to cell dry weight by means of a calibration curve.

2.4. Beardsmore medium composition

The 'Beardsmore Medium' (Beardsmore et al., 1982) was used in the culturing of three different species of Enterobacteriaceae by Kitts et al. (1994). This medium was tested for use with *Enterobacter cloacae* ATCC 43560 due to it also being of the family Enterobacteriaceae.

The medium consisted of 1.9 g l^{-1} K_2HPO_4, 1.56 g l^{-1} $NaH_2PO_4 \cdot H_2O$, 1.8 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 5×10^{-4} g l^{-1} $FeCl_2 \cdot 6H_2O$, 1×10^{-4} g l^{-1} $CuCl_2 \cdot H_2O$, 5×10^{-5} g l^{-1} $MnSO_4 \cdot H_2O$, 5×10^{-5} g l^{-1} $ZnSO_4$, 1.3×10^{-3} g l^{-1} $CaCl_2 \cdot 2H_2O$, 1×10^{-5} g l^{-1} $CoCl_2 \cdot 6H_2O$, 7×10^{-6} g l^{-1} H_3BO_3, 1×10^{-5} g l^{-1} $Na_2MoO_4 \cdot 2H_2O$ and 8.0 g l^{-1} yeast extract in distilled water.

2.5. Glycerol medium composition

A mineral salts medium with glycerol as the sole carbon source was used as an alternative to the Beardsmore Medium. This medium was comprised of 5.00 g l^{-1} K_2HPO_4, 0.04 g l^{-1} $CaCl_2 \cdot 2H_2O$, 2.88 g l^{-1} $NaH_2PO_4 \cdot H_2O$, 0.02 g l^{-1} Na_2SO_3, 0.10 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.69 g l^{-1} $NaNO_3$, 5.00 g l^{-1} glycerol, 4.8×10^{-4} g l^{-1} $Na_2B_4O_7 \cdot 10H_2O$, 9.2×10^{-4} g l^{-1} $Na_2MoO_4 \cdot 2H_2O$, 1.3×10^{-2} g l^{-1} $FeCl_2 \cdot 6H_2O$, 3.64×10^{-3} g l^{-1} $MnSO_4 \cdot H_2O$, 9.8×10^{-4} g l^{-1} $CuCl_2 \cdot H_2O$, 5.08×10^{-3} g l^{-1} $CoCl_2 \cdot 6H_2O$ and 1.56×10^{-3} g l^{-1} $ZnSO_4$ dissolved in distilled water.

2.6. Replication of Kitts et al. (1994) work

Work was undertaken with ATCC 43560 utilizing the method of Kitts et al. (1994) (who had worked with three other species of Enterobacteriaceae) to determine if *E. cloacae* could degrade RDX under identical conditions. Inoculum was grown in 50 ml of Beardsmore Medium in a 125 ml Erlenmeyer flask aerobically for 24 h. 5 ml of inoculum was then transferred to 100 ml of Beardsmore Media (containing 9 mg l^{-1} RDX) in a 200 ml anaerobic jar, grown aerobically for 24 h to obtain a high cell density, then plugged to initiate anaerobic conditions. The culture was exposed to 5 ml of air every 24 h due to the 5 ml sampling volume taken from each flask. Samples were analyzed for RDX.

2.7. Requirement for yeast extract

Two BioFlo III bioreactors (New Brunswick Scientific, Edison, NJ), one containing 3 l of Beardsmore Medium and 65 mg l^{-1} RDX and the other containing 3 l of Glycerol Medium and 60 mg l^{-1} RDX, were inoculated with 50 ml of *E. cloacae* grown for 24 h in Beardsmore Medium containing no RDX. The reactors were operated aerobically for 24 h by sparging air at 1 vvm (volume of air per volume of aqueous phase per minute) to obtain a high cell density, and then switched to anaerobic operation by sparging with nitrogen at 0.5 vvm based on aqueous volume. The reactors were sampled periodically, and the samples analyzed for RDX concentration.

2.8. Effect of aerobic/anaerobic cycling

A BioFlo III bioreactor containing 3 l of Beardsmore Medium and 60 mg l^{-1} RDX was inoculated with 50 ml of *E. cloacae* grown for 24 h in Beardsmore Medium containing no RDX. The reactor was operated aerobically for 24 h by

sparging air at 1 vvm to obtain a high cell density and then switched to anaerobic operation for 100 h by sparging with nitrogen at 0.5 vvm. The attainment of maximum cell density corresponded to the point of glycerol depletion. The reactor was then switched back to aerobic operation for 36 h, and a bolus of 50 ml of a 10 × concentrated media was added to the reactor. This cyclic aerobic/anaerobic/aerobic cycling was repeated. Samples were taken approximately every 12 h and analyzed for RDX concentration.

2.9. Solvent selection—critical log P

The critical log P of an organism is determined by exposing a cell line to solvents encompassing a range of log P (logarithm of the octanol-water partition coefficient) values, and determining the point (log P) at which the cell is seemingly unaffected by the presence of a solvent. In this work solvents with a wide range of log P values (0.27–9.43) were used, and the growth of the cells in the presence of the solvents was compared with the growth of the cells in the absence of any solvent. Thus, in each of several 125 ml Erlenmeyer flasks, 50 ml of sterilized Beardsmore Medium (containing yeast extract) was inoculated with 5 ml of liquid culture (same medium). Each flask also contained 5 ml of solvent, with the control having no solvent. After 24 h of incubation at 30 °C, the solvent was removed by aspiration, and the optical density of the broth was measured. The solvents employed in this work, and their log P values were: 2-propanol (0.27), 1-butanol (0.79), hexanal (1.76), chlorobenzene (2.61), dibenzyl ether (3.10), cyclohexane (3.11), 1-nonanol (3.38), pentanoic acid (3.56), 1-undecanol (3.90), *cis*-jasmone (4.15), myrcene (4.50), 1-decene (5.19), *n*-dodecane (6.60), oleyl alcohol (7.50) and polypropylene glycol (9.43).

2.10. Solvent selection—use as sole carbon and energy source

In each of several 125 ml Erlenmeyer flasks, 50 ml of Beardsmore Medium (modified by containing only 0.5 g l^{-1} yeast extract) was inoculated with 5 ml of inoculum prepared in the same medium. To each flask, 5 ml of a solvent was added as a sole carbon source, with the two controls being no solvent (negative control) and corn oil (positive control). After 24 h of incubation at 30 °C, the change in optical density was measured. The optical density of the negative control was subtracted from all values, and these values were then represented as percentages of the growth relative to the positive control. Solvents showing comparable growth to the positive control are bioavailable to the organism, and as a result are not suitable candidates for the second phase of a TPPB.

2.11. TPPB in batch operation

A BioFlo III bioreactor containing 3 l of Beardsmore Medium at triple the concentration was inoculated with 50 ml of *E. cloacae* grown for 24 h in Beardsmore Medium containing no RDX. 250 ml of 2-undecanone (the solvent ultimately selected) containing 600 mg of RDX was then added to the reactor. The reactor was operated aerobically by sparging air at 1 vvm based on the aqueous volume, and grown to the maximum cell density based on the medium concentration. The reactor was then switched to anaerobic operation by sparging with nitrogen at 0.5 vvm based on the aqueous volume. Samples, both aqueous and organic, were analyzed daily for RDX concentration as was the cell concentration in the aqueous phase.

2.12. TPPB in fed-batch operation

A BioFlo III bioreactor containing 3 l of Beardsmore Medium at triple the concentration was inoculated with 50 ml of *E. cloacae* grown for 24 h in Beardsmore Medium containing no RDX. The reactor was operated aerobically by sparging air at 1 vvm based on the aqueous volume, and grown to the maximum cell density based on the medium concentration. The reactor was then switched to anaerobic operation by sparging with nitrogen at 0.5 vvm based on the aqueous volume. A 2-undecanone solution of RDX was prepared with a final RDX concentration of 2.84 g l^{-1} to serve as the 'spiking' solvent. A 50 ml spike was

added upon switching the reactor system to anaerobic operation. Two subsequent spikes (one 50 ml and one 100 ml spike) were added when the aqueous phase RDX concentration had dropped below the equilibrium level (42 mg l^{-1}) that could be maintained by RDX loaded in the solvent phase. Aqueous samples were analyzed daily for RDX concentration and cell concentration, and an organic sample was analyzed at the end of the cultivation.

3. Results

3.1. Replication of Kitts et al. (1994) work

RDX degradation was observed in duplicate flasks at a rate of approximately 1 mg l^{-1} per day (data not shown). This rate was slightly less than those of the three isolates reported by Kitts et al. (average of 2.4 mg l^{-1} per day), but it was encouraging that the ATCC strain seemed capable of RDX degradation under conditions similar to those used for other species of Enterobacteriaceae. No other peaks (degradation products) were observed by HPLC analysis of samples for this experiment, or for any of the others reported in this work. Standards of RDX degradation products (e.g. 1-nitroso-3,5-dinitro-1,3,5-triazacyclohexane, and 1,3,5-trinitroso-1,3,5-triazacyclo-hexane) were tested, and were detectable by our HPLC but, as noted, did not appear on any of the chromatograms in this study. In addition, to ensure that no RDX was lost due to volatilization, uninoculated shake flask and bioreactor experiments were operated for several days without any change in RDX concentration.

3.2. Requirement for yeast extract

The initial 21 h of cultivation exhibited no RDX degradation as the reactors were operated aerobically to promote cell growth. After both reactors were switched to anaerobic operation by nitrogen-gassing, RDX degradation was observed only in the reactor containing the yeast extract (data not shown). The specific component(s) in yeast extract which facilitated the RDX-degradation by *E. cloacae* has not been identified.

3.3. Effect of aerobic/anaerobic cycling

During the first period of anaerobic operation, RDX disappeared as observed in the investigation into the requirement for yeast extract (Fig. 1). No degradation was observed during the second aerobic phase. It is interesting to note that no subsequent degradation occurred after the second aerobic phase, which was operated under anaerobic conditions. The bolus of nutrients added prior to the second aerobic period (124 h) was intended to ensure that non-carbon nutrient limitation was not being experienced by the cells. Cell concentrations did not increase during the last two periods of cultivation.

3.4. Solvent selection—critical log P

Once the ability of *E. cloacae* ATCC 43560 to degrade RDX had been confirmed, the critical log P of this organism was determined. As shown in Fig. 2, the critical log P of *E. cloacae* is approximately 3.2. The only deviation was cis-jasmone ($\log P = 4.05$), where cell aggregation caused the optical density not to be representative of cell growth.

3.5. Solvent selection—use as sole carbon and energy source

A final test for solvent uptake by *E. cloacae* was conducted before final solvent selection was undertaken. The solvents tested as to their biodegradability were: 3-decanone, isophorone, fenchone, 8-cyclohexadecen-1-one, 2-undecanone, carvone, β-ionone, limonene, bis (3,5,5-trimethylhexyl) phthalate, dibenzyl ether, lauronitrile, trioctylamine, cyclooctanol, patchenol, and nerolidol. The results, based on percent growth relative to a positive and negative control, showed that alcohols are generally bioavailable, while ketones, ethers, amines and nitriles are not.

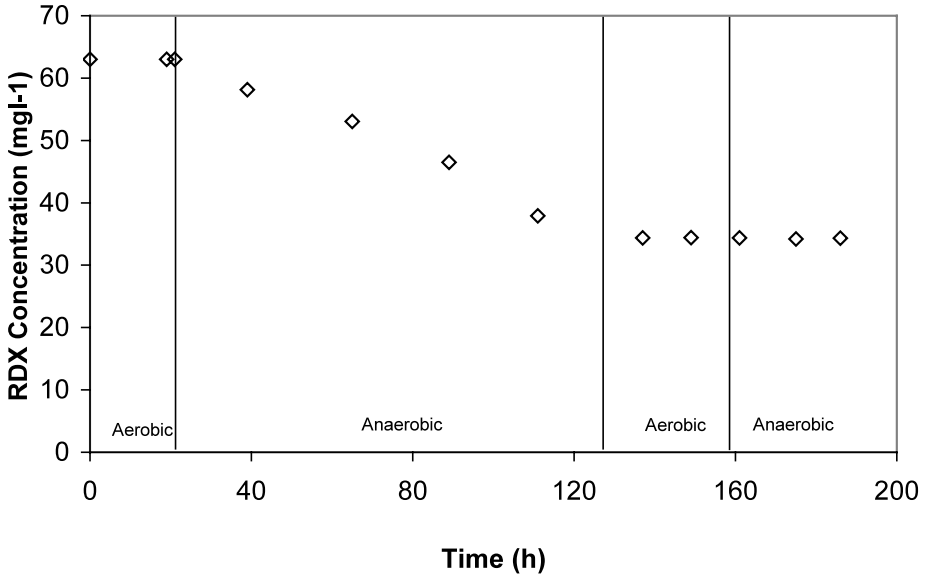

Fig. 1. Effect of aerobic/anaerobic cycling on RDX degradation.

3.6. Final solvent selection

Gibbs and Popolato (1980) have suggested that RDX (and other nitroamines) are relatively soluble in ketone solvents. As a result, solvent selection was further restricted to ketones and limonene (limonene was selected due to previous work with it as a delivery solvent). As expected, the ketones were capable of solubilizing three to four times more RDX than limonene (Fig. 3). It is important to note that all solvents tested were capable of dissolving more than ten times the amount of RDX than is soluble in water (the aqueous solubility of RDX is 65 mg l^{-1}), suggesting that any of these solvents are possible candidates for use in the two-phase system.

The deciding factor for solvent selection was cost (Fig. 3). Limonene proved to be the cheapest

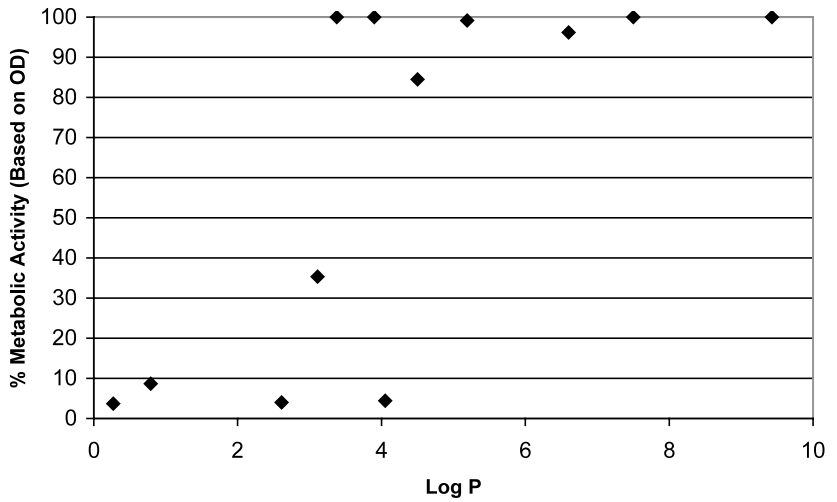

Fig. 2. Determination of the critical log P of *E. cloacae* ATCC 43560, through the use of solvents possessing various log P values.

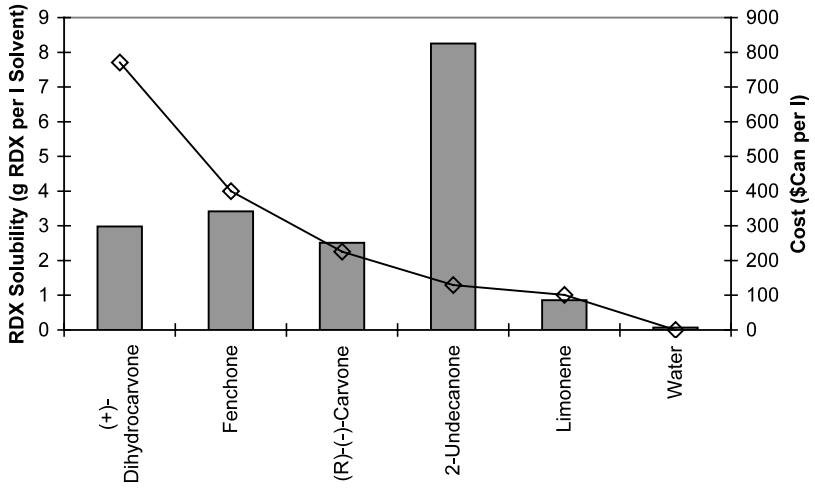

Fig. 3. Evaluation of solvents for the TPPB with *E. cloacae* ATCC 43560, based on RDX solubility in the solvent and the unit cost of the solvent. (◇) unit cost of solvent ($Can l^{-1}), bar graphs indicate RDX solubility (g l^{-1} of RDX in solvent).

of the final solvents, but an increase in price of $28.10 per l to 2-undecanone led to nearly a 10-fold increase in RDX solubility. 2-Undecanone, capable of solubilizing 8.25 g l^{-1} of RDX at a cost of $129.10 per l was selected as the solvent for use in the two-phase system.

3.7. TPPB in batch operation

The first demonstration of concept of the two-phase system was undertaken in batch mode. The switch from aerobic cell growth to anaerobic conditions was imposed after 30 h, after the cells had reached their maximum cell density. As expected, the aqueous phase RDX concentration remained constant for the majority of the cultivation (Fig. 4) at 42 mg l^{-1} (the equilibrium concentration under experimental conditions), whereas the organic phase concentration decreased at a fairly consistent rate (ca. 280 mg l^{-1} per day of RDX based on solvent volume, equivalent to 23 mg l^{-1} per day of RDX based on aqueous volume). Within 213 h, both the aqueous and the organic phase concentrations of RDX were undetectable (below 1 mg l^{-1}), indicating the elimination of RDX from the system. This corresponds to a volumetric degradation rate of 22 mg l^{-1} per day and a specific utilization rate of 0.126 mg g^{-1} h^{-1}.

3.8. TPPB in fed-batch operation

Only the aqueous phase concentration of RDX was monitored, as the volume of 2-undecanone added initially (50 ml) and in the spikes did not provide a sufficient reservoir of solvent from which to accurately sample. Once the aqueous phase concentration dropped below the equilibrium concentration of 42 mg l^{-1}, another bolus of 100 ml of solvent (containing RDX) was added. Again, once the aqueous phase concentration dropped below 42 mg l^{-1}, a 50 ml bolus of solvent and RDX was added.

A time–course plot of RDX concentration in the aqueous phase is shown in Fig. 5. The switch from aerobic cell growth to anaerobic conditions was imposed after 30 h, after the cells had reached their maximum cell density The 'theoretical' RDX concentration shown on this figure is the concentration that would be present in the aqueous phase if all the RDX in the system could be solubilized in the aqueous phase (calculated by dividing the total amount of RDX added to the system by the total aqueous phase volume). The theoretical change in this concentration is based on the volumetric

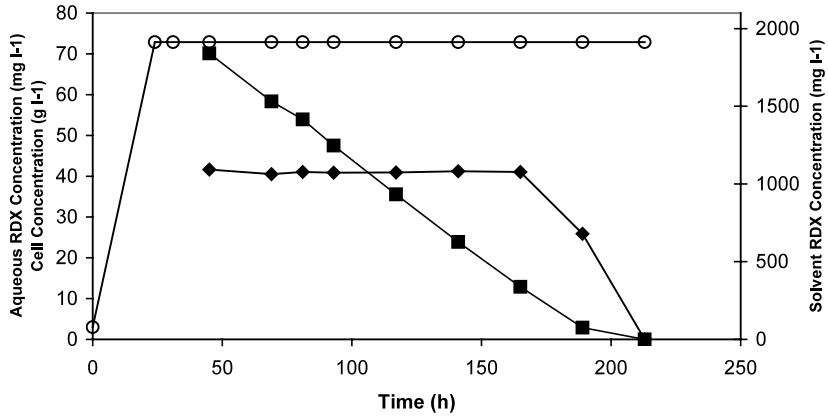

Fig. 4. Time course plot for the TPPB operating in batch mode. (○) Cell concentration × 10 (g l^{-1}), (■) organic phase RDX concentration (mg l^{-1}), (◆) aqueous phase RDX concentration (mg l^{-1}). Switch from aerobic to anaerobic conditions imposed at 30 h.

degradation rate of 22 mg l^{-1} per day determined in the previous batch experiment.

As in the case of batch operation, the aqueous phase concentration was maintained at 42 mg l^{-1} until sufficient RDX had been consumed from the organic phase to affect the equilibrium partitioning. At the completion of the cultivation (216 h), the levels of RDX in both the aqueous and the organic phases were undetectable, showing the complete disappearance of RDX from the system.

The rates of degradation appear to be approximately the same after each of the three 'spikes', showing that the system is unaffected by the addition of more solvent and RDX. The overall volumetric rate of degradation for the three-spike system was 21 mg l^{-1} per day, with a specific

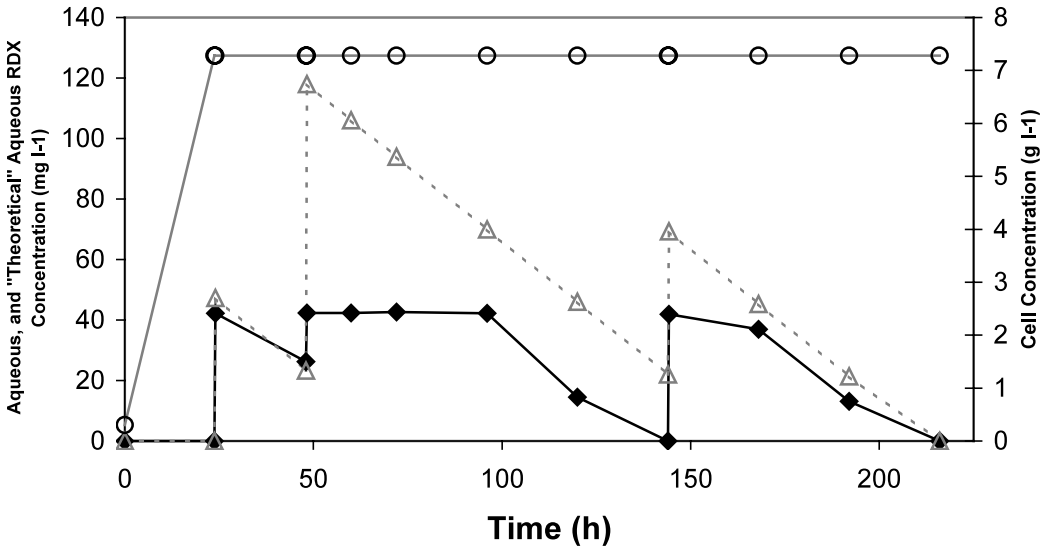

Fig. 5. Time course plot for TPPB operating in fed-batch mode. (○) Cell concentration (g l^{-1}), (◆) aqueous phase RDX concentration (mg l^{-1}), (△) theoretical RDX concentration based on total RDX added to system, total volume of system, and rate of degradation from batch study. Switch from aerobic to anaerobic conditions imposed at 30 h.

degradation rate of 0.120 mg g^{-1} h^{-1}. This corresponded to a degradation of 568 mg of RDX in 216 h. These rates are very similar to the batch system, showing that the system was not negatively affected by the imposed feeding strategy.

4. Discussion

The destruction of RDX from past-date munitions is a major concern, as the storage of these materials poses a great hazard (Shaw and Cullinane, 1998). Few pure species of organisms have been isolated that are capable of degrading nitroaromatic compounds, and degradation is further complicated by the limited solubility of RDX in medium, making high volumetric productivities or fed-batch operation difficult.

E. cloacae ATCC 43560 has shown the ability to degrade TNT and PETN (French et al., 1998), and subsequent enzymatic studies have shown the oxygen-insensitive nitroreductase enzyme capable of degrading RDX (Kitts et al., 2000). In the present work, *E. cloacae* has demonstrated the ability to degrade RDX utilizing methods used previously with other species of Enterobacteriaceae (Kitts et al., 1994). It should also be noted that, although no attempts to confirm RDX mineralization via radiolabelling were undertaken, no degradation intermediates were detected by HPLC even though theoretical breakdown products (such as 1-nitroso-3,5-dinitro-1,3,5-triazacyclo-hexane, and 1,3,5-trinitroso-1,3,5-triazacyclohexane) could be detected using standards.

Our work has also shown the apparent need of ATCC 43560 for one or more components present in yeast extract to enable RDX to be degraded, as a defined medium was unable to provide adequate nutrients. Alternatively, it is possible that residual yeast extract remaining in the medium after aerobic growth could serve as an electron donor for anaerobic RDX removal. In either case, an examination of the medium in terms of the minimum nutrients and/or electron donors required to promote RDX degradation warrants further work, as the practical implementation of a biological process to degrade RDX-containing munitions would be constrained by the relatively high cost of using the levels of yeast extract employed in this work.

It additionally appears as though aerobic conditions, although initially useful in encouraging the generation of high cell concentrations, has a negative impact on the cells' ability to degrade RDX (Fig. 1). It also appears as though the provision of aerobic conditions after an anaerobic RDX degradation period subsequently inhibits further RDX degradation once anaerobic conditions are re-introduced. Reports in the literature (Ronen et al., 1998; Freedman and Sutherland, 1998) have suggested, however, that aerobic/anaerobic cycling could enhance the degradation of nitroaromatic compounds, either through aerobic degradation of anaerobically-produced intermediates, or through the provision of a sole nitrogen source during aerobic operation.

Solvent selection is a key step in the development of a TPPB system, since the solvent must have properties which are compatible with both the organism and the xenobiotic. Through determination of the critical log *P* of *E. cloacae*, and the subsequent determination of the classes of solvents that are bioavailable to the organism, the solvent selection process was limited to ketone solvents with log *P* values greater than 3.2. The critical log *P* of *E. cloacae* is similar to the values found for other gram-negative bacteria (e.g. *Pseudomonas putida*—3.2 (Collins and Daugulis, 1996); *Pseudomonas* sp.—3.1 (Collins and Daugulis, 1999); *Sphingomonas aromaticivorans* B0695—3.8 (Janikowski et al., 2002)). This critical log *P* value is relatively low compared with many other organisms (Inoue and Horikoshi, 1991) and would normally expand the number of solvents that would be potentially suitable for use in a TPPB system using this organism. However, the biodegradability of alcohols by the cells, the difficulty in solubilizing significant quantities of RDX in many solvents, and the cost of many ketone candidates, significantly limited the solvent selection process. Overall, 2-undecanone, was clearly the best overall solvent for the present system, and it is interesting to note that this solvent was also the one of choice for a substan-

tially different TPPB application (Collins and Daugulis, 1996).

The demonstration of concept experiment (Fig. 4) clearly showed that very large amounts of RDX can be loaded into a bioreactor, via solubilization in the solvent phase, and delivered to the cells on demand through equilibrium partitioning. These results are, therefore, entirely consistent with prior work in our laboratory in which the partitioning and degradation of large masses of inhibitory (e.g. phenol, benzene, toluene, xylene) or poorly soluble substrates (e.g. polyaromatic hydrocarbons) has been achieved in partitioning bioreactors (Collins and Daugulis, 1996, 1999; Daugulis and Janikowski, 2002). The linear decline in RDX concentration in the solvent phase is consistent with the system having a constant cell concentration, and with the cells having a constant specific rate of substrate utilization (0.126 mg g^{-1} h^{-1}), which has now been determined for *E. cloacae* ATCC 43560 for the first time. The volumetric rate of RDX degradation (22 mg l^{-1} per day) appears to be the highest rate reported in the literature to date. Higher initial cell concentrations would improve this rate further, and the optimization of medium formulation (noted above) also holds promise for improving the process further.

The fed-batch strategy employed in this work, and shown in Fig. 5, has also demonstrated that the system is able to completely consume repeated feedings of RDX (via the solvent) at a volumetric and specific rate very similar to batch mode. Successful implementation of fed-batch feeding in partitioning bioreactors has been previously employed extensively in our laboratory for other recalcitrant substrates (Collins and Daugulis, 1997a,b). This method of feeding was intended to mimic a hypothetical situation in which RDX extracted from munitions could be cycled to the TPPB system. Although not shown here, the re-use of the solvent in a TPPB to capture additional xenobiotic materials is readily possible (Yeom et al., 2000), and we, therefore, envisage a closed loop of solvent extracting RDX from munitions, delivering it to the TPPB for degradation, and being re-used for additional RDX solubilization. Fed-batch processing of substrates cannot continue indefinitely, however, without the periodic provision of additional nutrients, as we have previously demonstrated (Collins and Daugulis, 1997b). Nevertheless, to our knowledge, this work represents the first demonstrated example of a continuous bioprocess to degrade stored energetic materials.

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