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CONTINUOUS BIODEGRADATION OF WASTE XYLENE

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ABSTRACT

Use of selected microorganisms for the degradation and/or detoxification of hazardous organic compounds is gaining wide acceptance as an alternative waste treatment technology. The INEL Biotechnology Unit is developing the technology for the in-plant treatment of waste industrial solvents. The work centers around the use of microorganisms specially selected for their ability to degrade common industrial solvents such as benzene, toluene, xylene, etc. Because these waste solvents are often contaminated with other materials (heavy metals, water, detergents, etc.) they are difficult and expensive to dispose and many times are not economical to recover through recycling. Even if the disposal option is used, the generator is still faced with continued liability in the event of mishandling or improper disposal of the waste. Biological treatment offers the option of reducing these solvents into harmless by-products provided that both the requisite microorganisms and the proper processing technology are successfully brought together. Work on the optimization of a bioreactor process for the degradation of xylene will be discussed.

INTRODUCTION

Common organic solvents are utilized for a variety of manufacturing, research, educational and health purposes and are produced in large quantities[1] e.g. toluene and benzene each of which are produced at a level of 10^9 lbs. per year. A large quantity of these solvents are not chemically altered during the process and become hazardous waste effluents. In addition to these wastes, another category of wastes was created by the regulatory agencies in 1985 called mixed hazardous waste. These wastes as defined by the regulation 40 CFR 261 contain both hazardous chemicals and radioactive substances. For instance, the valuable detection method employing liquid scintillation counting produces a liquid that contains an aromatic fraction usually consisting of toluene, xylene, or pseudocumene. Both the private sector and government facilities produce quantities of this material with an annual production of 200-300 thousand gallons. Although recently, biodegradable cocktails have been introduced into the marketplace, a large percentage of these wastes still contain the hazardous aromatic compounds. Other organic laden mixed hazardous wastes are also produced in this country. Some of these include waste oils/hydraulic fluids from the nuclear industry as well as organic solvents with radioactive materials as a result of nuclear material processing and manufacturing. It is estimated that the generation of these materials annually exceeds 12000 cu ft.

Well documented in the literature[2] is the fact that benzene and its methyl substituted derivatives are metabolized by microorganisms. Both the pathways involved in the individual enzymatic steps have been elucidated as well as many, if not all the genes coding for the degradative enzymes are known. However, very little information exists in the literature detailing what levels of xylene and toluene can be tolerated by organisms which can metabolize these compounds.

Due to the impact of these solvents on the environment and man, many policy and regulatory changes are forcing generators to consider drastic alternatives to their existing practices. Some of these alternatives involve solvent substitution, waste minimization and alternate technologies. Very few alternate technology approaches have dealt with the fact that in-line or end-of-pipe processing utilizing bioprocesses could allow the continuance of existing manufacturing practices. This study involved isolating microorganisms with the capability to use methylated benzene derivatives as the sole carbon source and to develop a bioprocess system capable of continuously degrading these compounds.

METHODS AND MATERIALS

Microbial conditions: All studies were carried out using a basal salts medium containing the following per liter: 0.7 g KH_2PO_4, 0.3g $MgSO_4$, 0.5g $(NH_4)_2SO_4$ and traces of the compounds, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot H_2O$ and $NaMoO_4 \cdot 7H_2O$. The final pH was adjusted to 6.5. All agar used was Difco Bacto Agar. All chemicals were of analytical grade or better. All cell viability studies were done on spread plates with a basal salts agar and placed in a desiccator at room temperature with an atmosphere of xylene or toluene.

*Work supported by U.S. DOE under Contract No. DE-AC07-76ID01570.

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Analytical conditions: Toluene and p-xylene were determined using a Perkin Elmer G.C. fitted with a 4 ft column packed with 3% SP-1500 on Carbopack, Supelco. The injector temperature was 275°C, column temperature 220°C, and the F.I.D. detector was maintained at 275°C. Helium was used as the carrier gas at a flow rate of 33 ml/minute. Toluene and p-xylene had retention time of 1.05 minutes and 2.47 minutes respectively.

Chemostat conditions: A New Brunswick MultiGen fermentor was used as Bioreactor 1. The flask was modified with glass ports at the one liter level and one port below that level. The air stream exiting this reactor was routed directly through a water cooled condenser and then via glass lines to the bottom of the second reactor which was fitted with a medium fritted sintered glass plate. This column reactor was 3.5 cm in diameter by 135 cm in height. All pumping was done through solvent resistant Viton tubing using Ismatec pumps.

Sampling procedures: Mass balance data were gathered from both reactors. Liquid effluents samples were taken directly from both reactors with sterile syringes. The air samples were collected on charcoal traps over a ten minute time period. The charcoal was then placed in small scintillation vials and extracted with carbon disulfide. Recovery values ranged from 88 to 100% depending on the charcoal used.

RESULTS

Many industrial waste samples were collected and screened for organisms that could degrade toluene, xylenes and pseudocumene. Several isolates were chosen from flasks that had one gram of sample added to a mineral salts media, pH 6.5 with 1-5 ppm of the desired aromatic. After several transfers on basal agar medium incubated in a desiccator with an open beaker of p-xylene or toluene, the pure colonies were introduced into liquid broth with 1-10 ppm of the aromatic. These isolates would grow in the media as determined by turbidity. However, after several days the cells would die and clump. In order to control the essential parameters of aeration and pH, a bioreactor was set up and inoculated. Initially toluene or p-xylene was introduced via vaporization and the cells were maintained in a mineral salts media in a pH range of 5.2-5.9. The cells grew to a density of 10^6 to 10^7 cell/ml and have survived under a continuous feed condition for 3 years.

Growth Studies

Selected isolates were transferred from plates incubated in a desiccator with an atmosphere of toluene or p-xylene into liquid broth tubes containing mineral salts media with 1-10 ppm of either aromatic. These tubes became the stock cultures for growth and utilization tests. In Figure 1 and 2, three isolates were followed for their ability to grow in and utilize p-xylene or toluene. Growth was determined by an increase in turbidity and by the disappearance of the compound as analyzed by gas chromatography. These tests were done under controlled conditions by adding 0.2 ml of the stock solution to sealed 100 ml serum bottles that contained 20 ml of sterile media saturated with the aromatic compound. The bottles were agitated by placing them inverted on a shaker table. Samples were taken with a microliter syringe and immediately injected into the G.C.

A single isolate from these tests was chosen for further studies and run in triplicate with 2.0 ml of inoculum, see Figure 3.

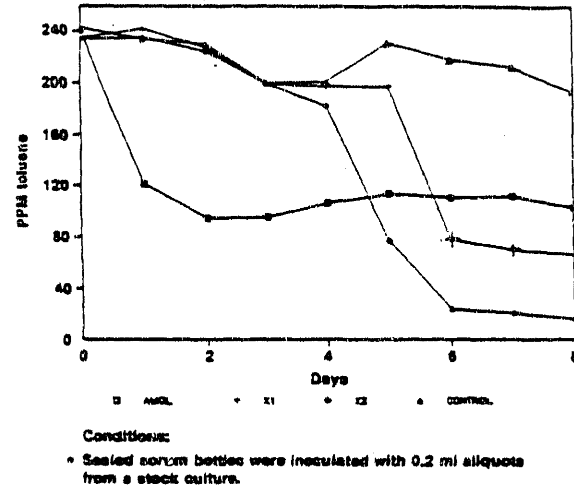

Figure 1. Isolates tested for toluene degradation.

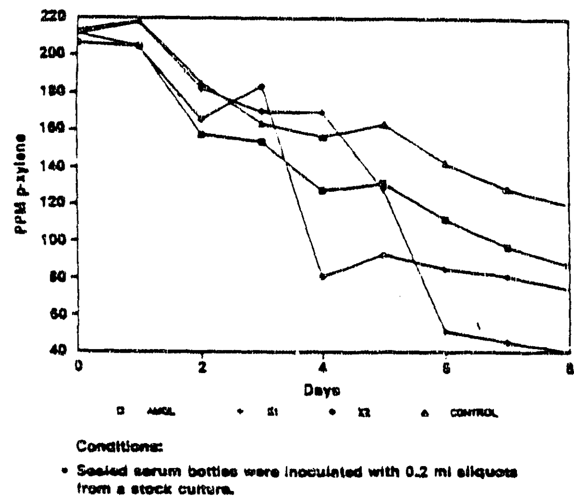

Figure 2. Isolates tested for xylene degradation.

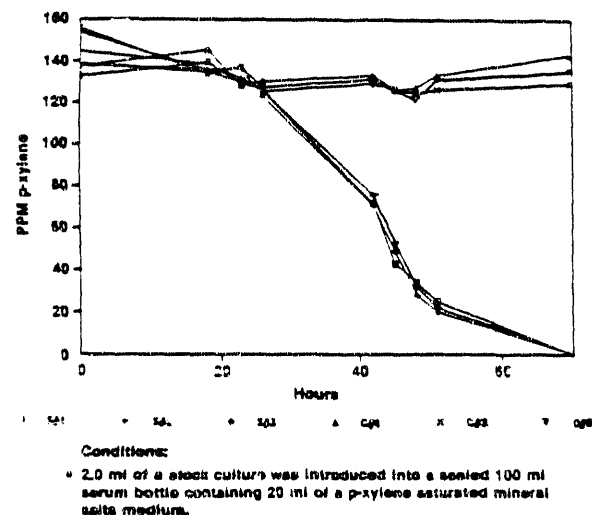

Figure 3. Degradation with a single isolate.

Batch Studies

Aliquots of 20-25 ml were taken from the chemostat and introduced into 100 ml serum bottles which had been flushed for five minutes with an atmosphere of p-xylene. Disappearance of xylene was monitored over time by removing samples and poisoning them with mercuric chloride in small 1.5 ml vials. These vials as well as the serum bottles were sealed with teflon-aluminum crimp caps. All sample vials were stored at 4°C until G.C. analysis was done.

The serum bottles were placed inverted on a shaker table. Control serum bottles produced by placing 1.0 ml of 1% mercuric chloride in the vial prior to addition of the sample. A typical degradation curve is shown in Figure 4 for p-xylene.

In order to determine if this isolate could maintain viability and still metabolize p-xylene in the presence of an emulsifying agent, similar studies were done with the addition of Triton X 100. Three levels of triton were used, 100, 1000 and 10,000 ppm. Following the initial rise of xylene after addition of the emulsifier to chemostat grown cells, degradation occurred rapidly, Figure 5. It was noted that the control did not show a corresponding increase in xylene concentration even though it also contained 1000 ppm of triton. This increase in xylene concentration is apparently due to an emulsifier produced increase in the dispersion of the organic in the aqueous solution. The viable cell count for the 1000 ppm bottle was taken at zero time and after 24 hours. The zero time sample had 80×10^8 cells while the 24 hour sample showed 6×10^8 cells. A triplicate test was run with a concentration of 1000 ppm triton using inoculum grown under xylene starvation conditions. In this case, each serum bottle received 6µl of p-xylene and the resulting degradation of xylene was followed (Figure 6).

In order to investigate the increase in xylene seen in Figure 5 a study was set up utilizing chemostat grown and starved inoculum. Results of this study are seen in Figure 7. In this study it was found that the xylene content of the control remained high while in the non-poisoned treatment xylene was degraded to the detection limits in four hours. Three other treatments were started with cells grown in the chemostat. No xylene was added to any of these three samples beyond what was already present in the media of the chemostat. As a control, one bottle did not receive triton and was poisoned at zero time. Another control received triton at zero time but was poisoned after 1.5 hours. The third treatment received triton at zero time and was not poisoned. All treatments which received triton at zero time were shaken for ten minutes following its addition and then sampled. Both treatments with triton showed elevated levels of xylene (340 and 375 ppm) as compared to the poison control without triton (115 ppm), see Figure 8. This does indicate that the inoculum carries xylene in an emulsified state and above saturated conditions.

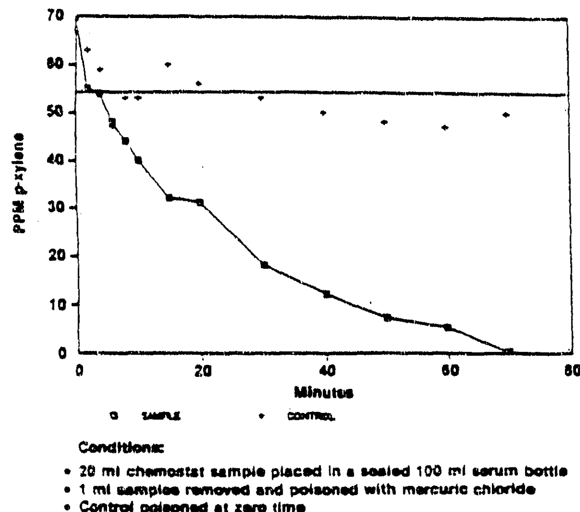

Figure 4. p-xylene degradation with cells grown in the chemostat.

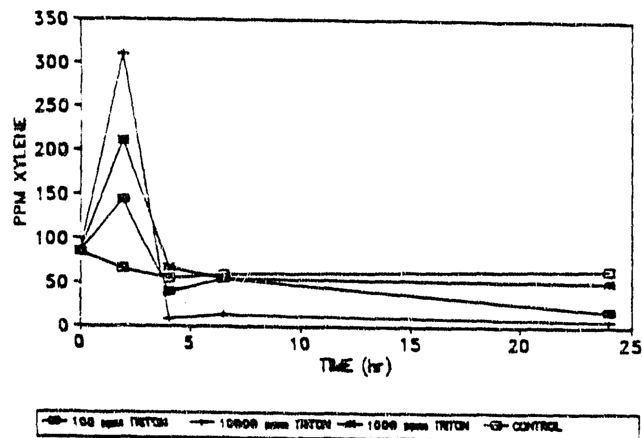

Figure 5. Xylene degradation. (Experiment 32790)

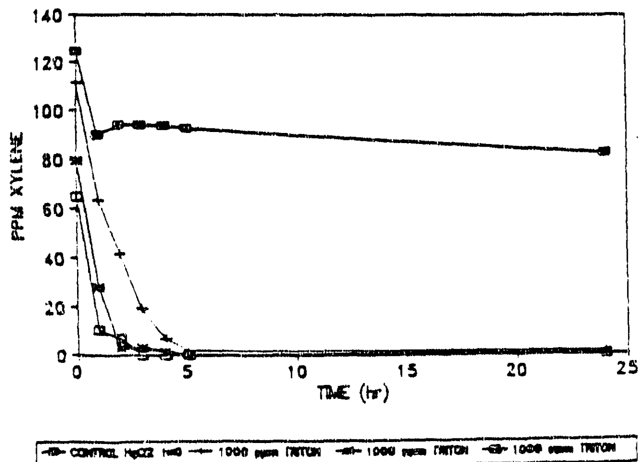

Figure 6. Xylene degradation.

Continuous Studies

The bioreactor was modified to provide a closed system from which mass balance studies could be obtained. All liquids, gaseous feeds, and effluents were pumped into and out of the bioreactors in glass tubing with joints being butted together with latex tubing. A secondary column bioreactor was added as a polishing stage. This column was 3.5 cm in diameter and 135 cm long. The column was fitted with a medium fritted disc near the bottom and two strands of Ring Lace (Dodwell and Co. Ltd, Japan) were inserted in the

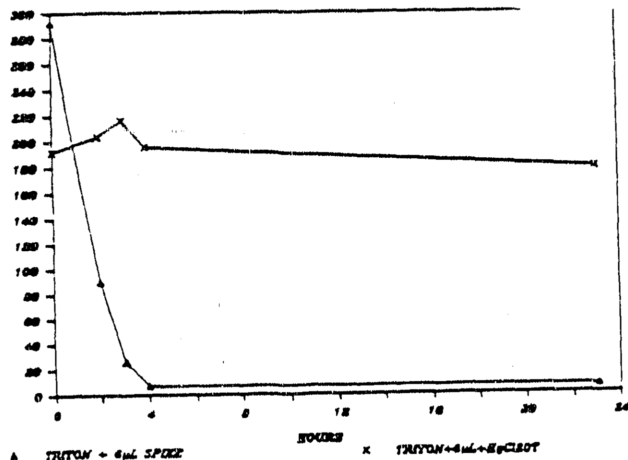

Figure 7. Xylene degradation.
(Experiment 42590 inoculum from flask)

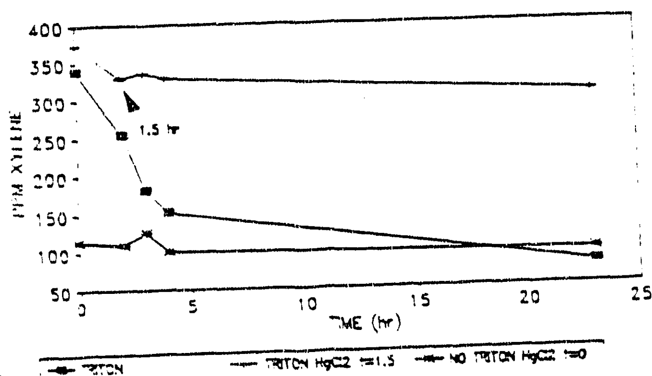

Figure 8. Xylene degradation--experiment 40290

column in hope of cell attachment. Waste effluent exited from the top of this up flow column. Studies have also been performed using a charcoal packed bed column in the secondary reactor.

Initial testing without emulsifier was conducted by pumping neat p-xylene into a media filled bioreactor 1 (BR1). During the course of the study, gas and liquid samples were taken several times over an eight hour period each day. Gas samples were taken to determine how much of the p-xylene was being lost from the bioreactors due to volatilization. The quantity of xylene in the media was also determined. By knowing the rate of neat xylene feed as well as the content of xylene in exit air and liquid, total xylene degradation as well as a rate for xylene disappearance can be calculated. In Table 1, the results from three days of sampling are shown. The air flow was 100 ml/minute to bioreactor 1, p-xylene and media were added at a rate of 14-25 mg/minute and 0.72-0.80 ml respectively. During this study, an average of 88% was degraded overall for three consecutive days. After this work a series of studies with 1000 ppm of Triton X 100 in the basal salts media was conducted. The first study was again at 100 ml of air flow per minute. Table 2 contains the data obtained over the course of one day. The degradation level was maintained at 87%. Long term testing is now being performed to explore the effects of media flow and pure oxygen feed. In addition, the column was changed to a packed bed form with 103 grams of charcoal as the support matrix. This column was equilibrated with a xylene saturated sterile media until the inlet and outlet xylene concentrations were equal.

TABLE 1. DEGRADATION OF P-XYLENE UNDER CONTINUOUS FEED WITHOUT EMULSIFIER

Air Flow Ml/Min	Xylene Feed G/24 Hrs.	Percent Degraded	Rate Mg/Min
100	18.7	84	11.9
100	28.8	92	18.6
100	21.6	89	12.9

TABLE 2. DEGRADATION OF P-XYLENE UNDER CONTINUOUS FEED WITH EMULSIFIER

Air Flow Ml/Min	P-Xylene G/24 Hrs.	Percent Degraded	Rate Mg/Min
100	20.6	89.6	12.8
100	20.6	86.8	12.4
100	20.6	85.0	12.2
100	20.6	87.7	12.5

It was then placed in line with BR1 and run for two days before sampling occurred. An air flow of 100 ml/min was introduced into BR1 which had a working volume of one liter. The xylene and media flows were maintained at 11-13 mg/min and 0.5-0.6 ml/min respectively.

The results of BR1, Figure 9 indicate the cell density fell over the seven day run. The xylene content in the effluent increased. The efficiency of this reactor fell from 70% to 20% or less. The charcoal packed column (CBT) cell density was also high during the first part of the run; Figure 10. By the fourth day the cell population had decreased by more than an order of magnitude and the efficiency by the 5th day had dropped from approximately 80% to zero. The overall efficiency of this study is shown in Figure 11. In the first 4 days a dual reactor system with a total working volume of 1.3 liter degraded 65 grams of this toxic substance.

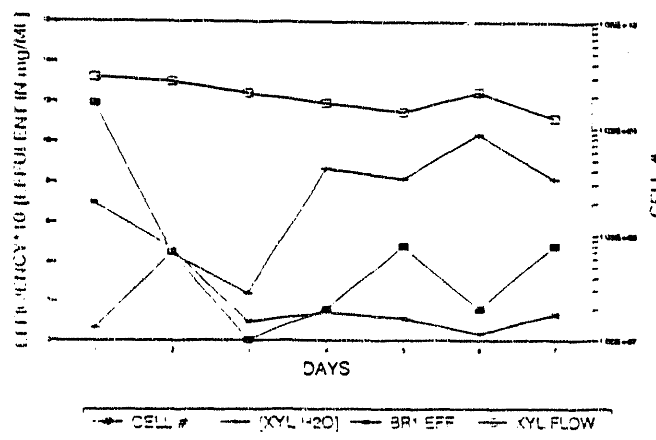

Figure 9. Cell density versus efficiency BR1
(Media flow 0.53 ml/min)

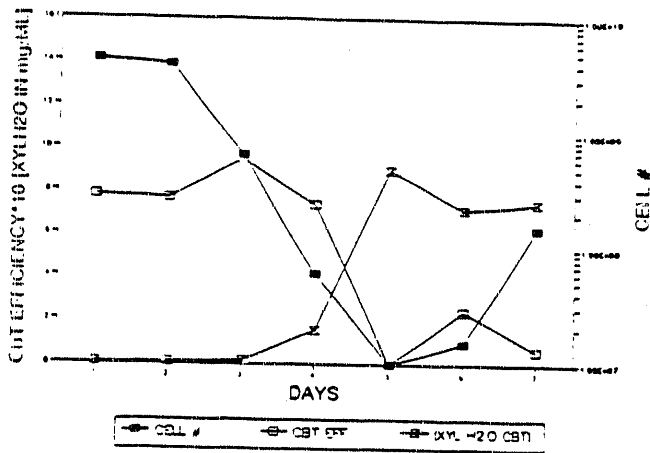

Figure 10. Cell density versus efficiency CBT (Media flow 0.53 ml/min)

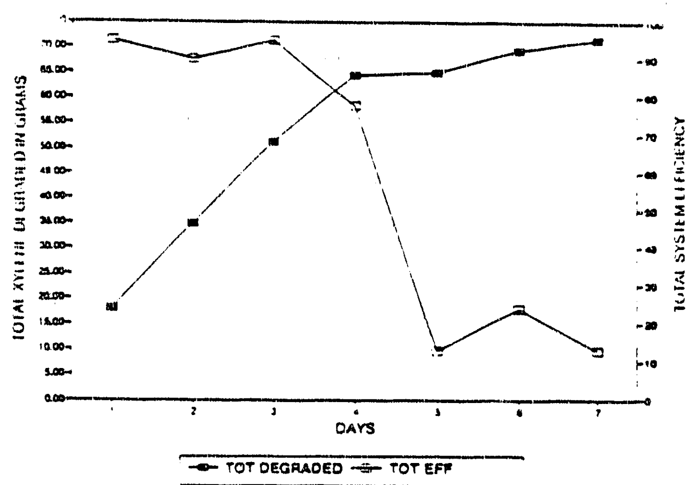

Figure 11. Total XYL degraded versus total efficiency. (Media flow 0.53)

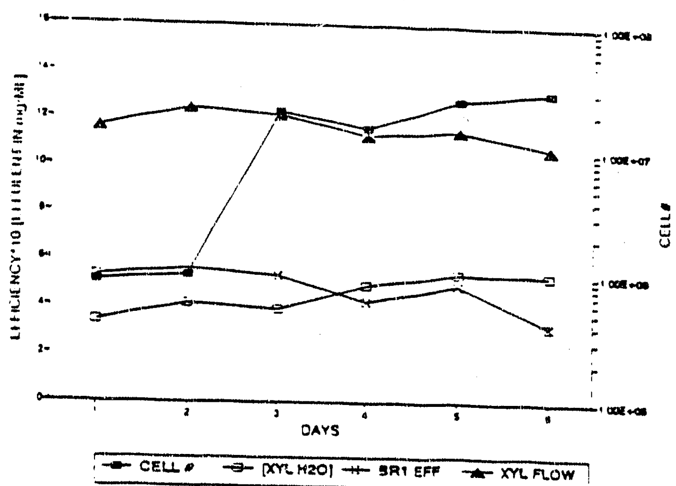

Figure 12. Cell density versus efficiency BR1. (Media flow 1.37 ml/min)

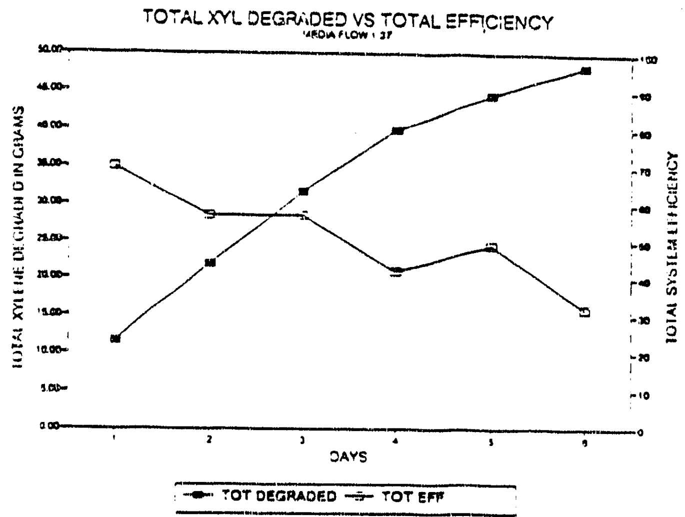

Figure 13. Total XYL degraded versus total efficiency (Media flow 1.37)

A study was initiated with a higher media flow to determine if either a nutrient or an inhibitory by-product might be causing the cell density decline. Following a two day period of no xylene addition the system was again run with a media flow of 1.3-1.4 ml/min versus the 0.5-0.6 ml/min of the previous run. In Figure 12, the cell density increased over an order of magnitude however, the efficiency remained about the same. Although this does not correlate with the cell density increase, the efficiency of BR1 was elevated in this run as compared to the efficiency of BR1 during the latter part of the previous run, 12% versus 45%. In Figure 13, the overall system efficiency and the total quantity of xylene are shown.

After the sixth day pure oxygen was added to BR1 at a rate of 100 ml/min to determine if this would stimulate additional activity. The efficiency quickly increased and stayed between 42-60% for the duration of the run in BR1, similar to the previous six days when air was added. The cell density was higher in this run as compared to the previous run that did not receive pure oxygen (Figure 14). The overall efficiency and total p-xylene degraded over this 5 day run are shown in Figure 15.

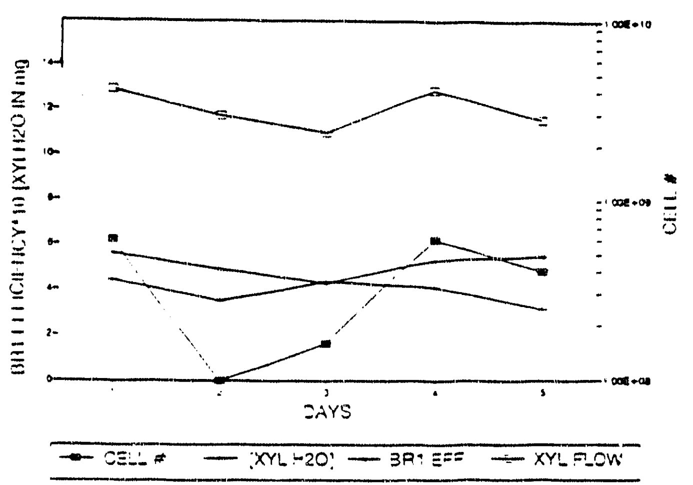

Figure 14. Cell density versus efficiency BR1. (O2 atmosphere media flow 1.37 ml/min)

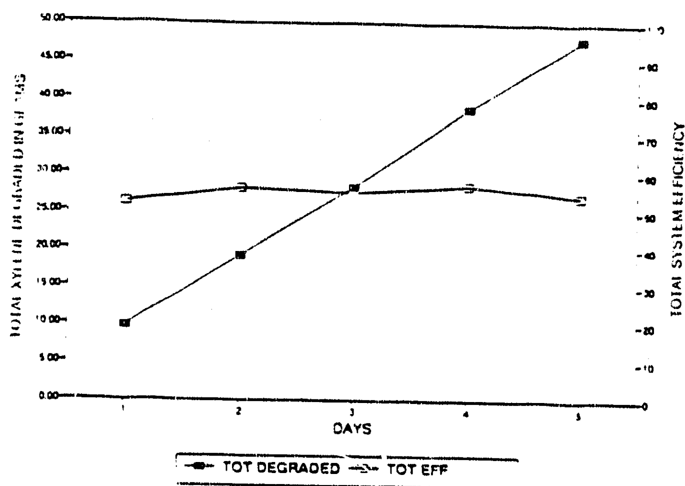

Figure 15. Total XYL degraded versus total efficiency. (Media flow 1.37 O2 atmosphere)

DISCUSSION

With the increasing attention being given to the quality of the environment and man's impact on it, and the need for our industrial base to maintain its competitiveness in the world marketplace, the development of in-line processing of hazardous by-products could be beneficial. Regulatory restrictions on all effluents, even air emissions is increasing.[3] Manufacturing and research endeavors producing these by-products are limited in their choices of handling these effluents once they are classified as wastes. The choices that are available are increasing in costs, and these costs are consuming profits. Our approach is an attempt to demonstrate that bioprocessing as an in-line system offers an alternative technology to these problems.

Contaminated soil and water samples where chosen as sources in which microbial adaptation may have occurred. Inoume and Horikoshi[4] recently reported in Nature, an organism was isolated from soil in Japan that could tolerate high concentrations of toluene but did not utilize it for growth. Their organism was identified as *Pseudomonas putida*. They also reported that it would grow on nutrient rich plates under a layer of toluene.

We have isolated a number of organisms from organic solvent contaminated soils. The isolate which is reported on here not only is tolerant to high concentrations of toluene and p-xylene but utilizes these compounds for its sole carbon source. Following the selection of the isolate, a series of batch tests were run to insure that growth and degradation were taking place. As indicated in the results, growth did occur along with xylene or toluene disappearance. Although the culture did lose viability under batch conditions after a number of days, propagation under chemostat conditions with pH and aeration control has sustained viability over a period of three years without reinoculation.

Rates obtained under batch conditions using initial rates from Figure 1 were in the range of 1 mg/minute/liter. Vecht et al[5] reported that an isolate of *Pseudomonas putida* grew well on toluene in batch and in chemostat conditions. Although rates were not reported cell mass and other growth conditions were given.

One of the applications thought to be promising for a bioprocess that could degrade xylene and toluene, would be the degradation of these compounds in a mixed hazardous waste known as liquid scintillation cocktail waste. This waste usually contains emulsifiers. Our data show that even with 10,000 ppm of Triton X 100 cell viability and metabolism of xylene can be maintained (Figure 5). The initial rate of metabolism was again about 1 mg/minute/liter.

Any process that might be of commercial importance should be operated on a semi or continuous basis. The chemostat was modified to allow mass balances to be taken and yet remain aerated. The initial runs were of short duration to enable us to see what parameters of air flow and emulsifier might be used. The air flows were varied between 100-600 ml/min. Flows over 100/ml/min appeared to vaporize very high quantities of xylene. From batch studies, 1000 ppm of triton was selected as the level of emulsifier. Thus far the longer runs have not yielded high efficiencies, 80% or greater but the hardiness of the organism has been established and in a one liter CSTR system routinely 50% of the xylene is degraded. Rates of degradation under chemostat conditions are usually greater then in batch varying from 6-24 mg/min/liter. Ehrhardt and Rehm[6] using a strain of *P. putida* for the degradation of phenol obtained rates of 10 mg/liter/minute. However, in a later report by Zache and Rehm[7] complete degradation of phenol took 80 hours with a rate of 0.25 mg/minute/liter for free cells and for immobilized cells, the rate was 1.8 mg/minute/liter. In our work it has been found that on a daily basis over 17 grams of xylene are being degraded per liter in BR1 with an average of 50% efficiency.

The configuration of the second bioreactor has and is still being altered. The initial column with very little support material yielded very low efficiencies (2-5%). It is felt that the small volume of the column (300 ml versus 1000 ml for BR1) as well as the unrestricted flow of vaporized xylene contribute to these low efficiencies. The partition coefficient of xylene between vapor and liquid phase in an aqueous system are poor and therefore, the retention time in the column for the vapor is too short to achieve liquid solubilization necessary for microbial degradation.

In summary, an isolate has been isolated which appears to have unusual capabilities for living in high concentrations of aromatic solvents and also utilizes these compounds as an energy and carbon source. This isolate, also determined to be a strain of *P. Putida* and has been patented, grows under a layer of p-xylene on basal salts agar media. We believe this is the first demonstration of growth of cells under a layer of xylene that also can utilize it as a carbon source. In order to develop the capabilities of this organism into an economical bioprocess, a series of studies is underway. We believe a simple bioprocess system incorporating this organism can serve as an in-line process to handle air emissions or liquids by-product effluents from industrial or laboratory processes. At present this process development is attempting to demonstrate that even a mixed hazardous waste can be treated and hopefully allowing it to be disposed of more easily than present regulations permit.

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