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**DEGRADATION OF HAZARDOUS CHEMICALS IN LIQUID RADIOACTIVE
WASTES FROM BIOMEDICAL RESEARCH USING A MIXED MICROBIAL
POPULATION**

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As the costs associated with treatment of mixed wastes by conventional methods increase, new technologies will be investigated as alternatives. This study examines the potential of using a selected mixed population of microorganisms to treat hazardous chemical compounds in liquid low level radioactive wastes from biomedical research procedures. Microorganisms were isolated from various waste samples and enriched against compounds known to occur in the wastes. Individual isolates were tested for their ability to degrade methanol, ethanol, phenol, toluene, phthalates, acetonitrile, chloroform, and trichloroacetic acid. Following these tests, the organisms were combined in a media with a mixture of the different compounds. Three compounds: methanol, acetonitrile, and pseudocumene, were combined at 500 $\mu\text{L/L}$ each. Degradation of each compound was shown to occur (~75% or greater) under batch conditions with the mixed population. Actual wastes were tested by adding an aliquot to the media, determining the biomass increase, and monitoring the disappearance of the compounds. The compounds in actual waste were degraded, but at different rates than the batch cultures that did not have waste added. The potential of using bioprocessing methods for treating mixed wastes from biomedical research is discussed.

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Introduction

In 1995, Wolfram et al. and Attalla et al. reported their findings on the success of bioprocessing stored liquid mixed waste. Although these investigations were successful, most of the methylated benzene solvent-based liquid scintillation cocktail formulations have been replaced by the so-called biodegradable non-hazardous formulations. Therefore, the waste generated by this powerful analytical technique is no longer considered a mixed waste. This waste now can be incinerated or disposed of via the sanitary sewer if its radionuclide content meets the discharge limits of the permit.

The successful demonstration of a bioprocess containing a pure strain of a selective and adapted natural occurring organisms, which was able to process the waste, is encouraging. There are over 50,000 hospitals in the United States. Most of these hospitals perform some diagnostic services or are contracted to independent laboratories for this service. In the histological diagnostic area, many procedures require that a tissue sample be processed through a series of organic chemical solvent steps in order to complete the staining phase. These solvents become waste and require handling, shipping, and incineration. In addition, laboratory operations and medical research facilities generate many small amounts of organic solvent waste per year. In some of the larger medical research facilities [e.g., National Institute of Health (NIH) facility in Bethesda, Maryland] these quantities of liquid wastes become quite large and, in some instances, are costly to dispose (Kirner 1994).

There is a large impetus today to use bioremediation to assist in the remedial action of removing contaminants from soil and ground water. Both in situ and ex situ processes are now being implemented (Alleman and Leeson 1997). The market value of this relatively new treatment technology is large. Most of the applications are utilizing natural occurring microorganisms. However, fewer applications of bioprocessing for the treatment of stored or contained waste have been developed. This report describes an attempt to determine if a bioprocess concept could be an alternate treatment for some of these contained wastes.

Methods and Materials

Cell cultures used were Selma-acetonitrile, NIH-methanol, and *Psuedomonas putida* Idaho-pseudocumene. Cultures were first plated on nutrient agar (NA) or Tryptic Soy Agar (TSA) for isolation of pure colonies. Once colonies were established, they were maintained at 4°C until the time of the experiment. Inoculum for liquid cultures was produced by taking colonies from the plates, using a sterile inoculating loop, and inoculating 100 mL-sterile mineral salts media. Media consisted of $(\text{NH}_4)_2\text{SO}_4$ 0.5 g/L, MgSO_4 0.5 g/L, K_2HPO_4 0.6 g/L, KH_2PO_4 0.6 g/L and trace mineral solutions containing sodium molybdate, ferrous sulfate, and manganese chloride (1 mL/L of each). All cultures were grown using sterile 250 mL Erlenmyer flasks. Cultures were incubated at room temperature on a shaker until growth was evident. Once flasks were turbid, the cells were harvested by centrifugation for ten minutes at 7,000 rpm and then resuspended in 40 mL sterile minimal salts media (resuspended volume depended upon number of vials used in each experiment or the cell density desired).

In each experiment, 100 mL sterile serum vials were used. The vials were autoclaved for 20 minutes prior to use. Under sterile conditions, 50 mL of minimal salts media was dispensed into each vial. The vials were closed with a rubber stopper and capped using an aluminum seal. The three compounds were added to the vials using a sterile gas chromatography syringe. Cell inoculum was added using a sterile 1 mL syringe with a 23 gauge needle. Vials were placed upside down on a shaker at room temperature and allowed to incubate.

Gas chromatography (GC) analysis, cell counts, and total organic carbon (TOC) were done once growth was observed in the vials. Samples were withdrawn from the serum bottles in a sterile 1 mL syringe with a 23 gauge needle. The GC column was a 30 m capillary with a liquid phase of DB-624 from J&W Scientific. Carrier gas was Helium. The parameters were set as follows: Injector temp. 230°C, Detector temp. 260°C, with cell counts obtained by first performing a 1:10 dilution series in sterile water out to 10^8 . Each dilution was then plated onto NA or TSA plates and incubated at 30°C until colonies formed and could be counted.

In a previous study, diagnostic wastes from a local hospital [Eastern Idaho Regional Medical Center (EIRMC)] and an independent laboratory were treated using a pure strain of a pseudomonad (Wolfram and Rogers 1996). The xylene content as well as some other unidentified organic compounds (e.g., alcohols and acetic acid) were removed. The meta- and para-xylene isomers in this waste were removed below the discharge limits as shown in Fig. 1. In the same investigation, an outdated source of para-xylene was being sent to a waste stream for incineration from a university laboratory. This waste was also processed with the removal of the p-xylene (Fig. 2). The bioprocess system was a sequential continuous flow bioreactor series, as shown in the schematic in Fig. 3. Following a short start-up period, a four week run was made for the processing of this waste during which the xylene was degraded to 1 $\mu\text{L/L}$ at a feed rate of 1.25 mL of neat xylene per liter, per day (Fig. 4). This reactor series consisted of two continuously stirred tank reactor (CSTR) units, and one biofilter. The waste was metered into the first CSTR unit along with nutrient solution (ammonium and micro nutrients) and oxygen. The contents flowed from the first reactor to the second CSTR unit and finally, the gaseous stream was plumbed to the biofilter. The biofilter system consisted of a column filled with inert support material which had been colonized with the pure strain of *Pseudomonas putida* Idaho. This same strain was inoculated in both of the CSTR units as well.

The waste handling facility at the Bethesda NIH medical center has developed an elaborate waste separation system which serves the entire complex (Kirner et al. 1994). The objective of this system was to determine if the vast quantity of different mixed and hazardous wastes compositions generated from the biomedical research could be grouped to assist in the management of the waste, and to minimize the quantity of the more difficult to dispose of liquid wastes. Five waste groups: gel fixing/washing, HPLC scintillation, filter washing, unknown, and cell culture labeling made up the majority of the total waste volume generated per annum (Fig. 5). In addition to these wastes, there are other aqueous waste which have very low levels of organics and short-lived radionuclides.

This facility has disposal contracts for all of these wastes. However, at times, some wastes enter these streams, making them more difficult to dispose, which can increase the costs

for disposal dramatically. For these reasons, NIH Waste Management Group decided to look for alternatives for disposal including performing treatments of some of the waste on site (NLLWMP 1995). This study focused on a number of compounds and the feasibility to biodegrade these compounds as mixtures of the compounds. The compounds of interest were the following: trichloroacetic acid, chloroform, methanol, acetonitrile, pseudocumene, and di-n-butyl phthalate.

Results

Microbial Isolation

Samples were collected from several locations at their facility to determine if any organisms had adapted to the waste solutions in which they were in contact. The locations included filter pads used in separating waste solids, inside of waste feed lines, sludges from the barrels and aqueous waste storage tanks, and from some of the liquid waste itself. Organisms were found in most of these locations even though, in some cases, a biphasic solution was present and germicides were also present (Table 1). The isolates were then tested to determine if their metabolism could indeed degrade any of the selected compounds. The results are shown in Figs. 6 to 9. Some of the isolates did not perform well when the concentrations of the organic compound was increased [e.g., acetonitrile (Fig. 10)]. In these cases, organisms were used from previous isolations that had been performed on industrial waste samples and that had been tested on the selected compounds. The organisms finally selected for the degradation of 10 mL/L acetonitrile and pseudocumene have been shown to tolerate and grow in the presence of the compounds. In the case of pseudocumene, that concentration causes a biphasic solution unless a surfactant is added.

Biodegradation Investigations on Mixtures of Organic Liquids

The hazardous compounds in these wastes are usually there as mixtures. A bioprocess could only be considered successful if all the hazardous components were removed. Therefore, a test was done to find out the potential of combining several pure strains of organisms to

determine if the compounds could be degraded simultaneously. The three compounds chosen for these tests were methanol, pseudocumene, and acetonitrile. Batch conditions were used for these tests. Serum bottles were inoculated with resuspended cells from the individual strains. The test was done in triplicate. The three compounds decreased during the course of the experiment (Fig. 11). The production of biomass was followed by taking samples for absorbance measurements. An increase in cell number was observed during this test (Fig. 12).

Biodegradation of Actual Waste Samples

Waste samples provided, were used to determine if the microorganisms selected for the degradation of methanol, pseudocumene, and acetonitrile, were able to maintain viability and grow. Serum bottles with 50 mL of mineral salts media were inoculated with the three strains of organisms to give a 10^6 cells/mL. The results of one of these tests for waste #5286 is shown in Fig. 13. Solution turbidity slowly increased in the serum bottles and samples were withdrawn for analysis.

A second test was done using a larger inoculum (approximately 1×10^7 cells/mL as the initial cell concentration. Aliquots of the waste (0.1 and 1.0 mL) were then injected into two serum bottles. Both of the waste dilutions showed an increase of organisms to 5×10^8 cells/mL in seven days. In this same test, 0.1 mL of waste was injected into a third and fourth serum bottle followed by the addition of methanol and acetonitrile, and were inoculated. Pseudocumene was not added because of interfering compounds in the waste sample which prevent the analysis for this compound. Samples were withdrawn from these replicated bottles during the course of the experiment and the levels of methanol and acetonitrile were checked. In this test, the decrease in both methanol and acetonitrile occurred over a period of only seven days. Three different wastes were tested (Figs. 14 to 16). The greater cell density improved the rate at which the compounds were removed from the solution in the serum bottles.

Discussion

Most of the bioprocessing for remedial action of contaminants has been done on environmental matrices (i.e., groundwater and soil). These systems are open and the concentrations of the contaminants are usually very low. However, Wolfram et al. (1995) showed that a selected strain of microorganism could successfully treat a contained, stored mixed waste by removing the high concentrations of methylated benzene compounds. The effluent from this treatment was considered as an aqueous low-level waste which can be disposed. The selected organism used in the treatment of the concentrated organic mixed waste was isolated from an industrial contaminated soil sample. Therefore, in this study, the initial isolations were made from samples taken from the generator's waste site. Although organisms were found, very few could degrade the contaminants of interest. Probably two reasons can account for this: first, the wastes generated in biomedical research contain many non-hazardous compounds (i.e., sugars, organic acids, amino acids, proteins, carbohydrates, and lipids). These compounds can interfere with the metabolism of the hazardous compounds. Secondly, many of the sampled sites (e.g., drums, tanks and process lines) are thoroughly cleaned frequently or have different types of wastes come into contact with the surfaces from which the samples were taken. Therefore, the microbial adaptation has not had sufficient time to occur.

Several of the compounds of interest for treatment and removal were trichloroacetic acid, chloroform, and phthalates with saturated straight chain alkane groups. No organisms were found in the waste samples that could use these compounds as the sole carbon source. Yu et al. (1995) reported that an isolate capable of degrading TCA aerobically was found. This isolate could not utilize the mono- or di-chloroacetic acid compounds. Kim et al. (1997), reported that microorganisms from samples taken from the subsurface and grown on butane could biotransform chloroform. Butane served as a co-metabolite and complete dechlorination occurred with concentrations of chloroform as high as 1200 µg/L.

The standard procedures used in performing biomedical assays as well as biomedical research produces liquid hazardous wastes. For instance, the regional hospital (200 bed facility)

from which a diagnostic staining waste was obtained, produces about one barrel per month. The drummed waste is presently sent to an incinerator 700 miles away. Assuming that this is an average for all of the 50,000 hospitals in the USA, there would be 50,000 barrels per month or 600,000 barrels of waste per annum to treat. In biomedical research, there are many additional waste streams, some of which contain traces of radionuclides. The NIH facility at Bethesda reports that there are 30 different groups of liquid wastes handled in their facility. The costs for disposal has steadily increased and, in some cases, the cost to remove and dispose of a single barrel can be \$50K. Therefore, alternate separation and treatment technologies that can reduce these disposal costs could be beneficial to the overall operation.

To verify the feasibility of using selected mix cultures of organisms to treat an aqueous solution that contains several hazardous components, several tests were run. Initially, three organisms were isolated that could tolerate and grow in 1000 $\mu\text{L/L}$ or greater of the compounds: methanol, acetonitrile, and pseudocumene. Although the waste samples provided contained only small amounts of these materials, additional amounts of these three organic compounds were added to the test solutions. Several of the wastes contained chlorinated solvents at a concentration approaching 10 $\mu\text{L/L}$ while other compounds (i.e., aromatic), were present at levels of 1 to 50 $\mu\text{L/L}$. The initial tests showing the removal of all three compounds simultaneously with the mixed culture of the three selected strains, proved that a strategy for bioprocessing mixtures could be accomplished. In Figs. 10 and 11, the biomass increased over time while the individual organic compounds decreased. The reduction of acetonitrile and pseudocumene was below the detection level of the instrument while methanol decreased to approximately 5 to 10 $\mu\text{L/L}$ and was still decreasing when the experiment was stopped. Other investigators have shown that bioremediation of contaminated soil and water with multiple components (e.g., petroleum products) is feasible.

In order to determine if the introduction of actual waste would inhibit the bioprocess, tests were performed which incorporated small amounts of the waste. The initial test combined the waste #5286 at 0.1 and 1.0 mL/50 mL. The test results (Fig. 12) showed that the compounds did decrease slowly over a 37 day period. The composition of this waste was complex in that it

contained many different organic solvents. However, the microbial population was still viable and did decrease the selected compounds to lower levels. It should also be remembered that under batch conditions, such as in this test, optimum degradation probably is not achieved. This is especially true if other components caused inhibition and remain in the presence of the inoculum for the period of the test. Continuous flow bioprocess systems usually achieve a better overall performance. These initial tests were to serve as an indicator of whether it is feasible to assume that a mixture could be degraded.

A second set of tests were done using a larger inoculum size. The removal rate of the compounds increased and were either at the detection limits or had leveled off, indicating further decreases were unlikely to occur. The three wastes tested did not completely inhibit the bioactivity of the introduced strains and cell growth was evident at the end of the seven day trial. These results indicate that bioprocessing of wastes can be expanded beyond those for open environmental systems (e.g., contaminated soil and water sites). This study showed that a mixture of hazardous compounds in a known waste can be bio-treated and degraded. The selection of adapted organisms capable of tolerating various organic liquids and possessing the ability to degrade one or more of the hazardous compounds could prove to be beneficial for the development of bioprocessing systems to treat many known wastes.

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Table 1. NIH Waste Collection Facility Sampling Locations

Sample Location	Type of Sample	Growth on Plates ^a
1. v 0007 drum bottle	smear	+
2. filter inlet aqueous radioactive waste	liquid	+
3. Composite from top layer	liquid	+
4. filter line	liquid	+
5. Filter bag 200 gal drum waste	sludge	+
6. filter from peroxide room	liquid	+
7. Gel washing drum	liquid	+
8. Drum liner #10019-01	smear	-
9. Drum #50195-02	smear	+
10. Drum #40195-05	smear	+
11. Filter from u.v. peroxide room	smear	+
a. "+" indicates that viable organisms were isolated from the sample collected from that location		

EIRMC Waste (610 g/L p-xylene)

Figure 1.

ISU Waste (670 g/L p-xylene)

Figure 2.

Figure 3.

Figure 4.

Feed Rate for ISU Waste
(pure *p*-xylene basis)

Figure 5. Waste generating processes.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 11.

Figure 12.

Figure 13.

Figure 14.

Waste 5286

Methanol/Acetonitrile

—■— Meth + 5286 -□- Meth + 5286C
—●— Aceto + 5286 -○- Aceto + 5286C

Figure 15.

Figure 16.

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