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AEROBIC BIODEGRADATION STUDY OF [redacted] AND AMMONIUM PERFLUORO-OCTANOATE (C8)

E.I. DuPont De Nemours and Company
Fluoroproducts SBU

Submitted To:

Vinci M. Felix
FLPR/Chambers Work

James M. Odom
CR&D/Glasgow

SUBMITTED BY:

ENVIROGEN, INC.
Princeton Research Center
4100 Quakerbridge Road
Lawrenceville, N.J. 08648

Written By:

Mary F. DeFlaun, Ph.D.
Applications Manager, Bioremediation Technologies

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Executive Summary

The purpose of this study was to evaluate the ability of aerobic bacteria, previously shown to have a broad range of degradative capabilities, to degrade [redacted] and ammonium perfluoro-octanoate (C8). Nine different bacterial strains, known to metabolize compounds similar to [redacted] and C8, were tested in bottle assays for their effectiveness against these compounds. Experiments conducted with both [redacted] for dehalogenation analysis, and [redacted] to test for production of $^{14}CO_2$, failed to show degradation of [redacted] by all nine bacterial strains. Analysis for C8 by existing methods lacked the sensitivity needed to analyze degradation of this compound. Due to the high degree of fluorination of both [redacted] and C8, however, it is more likely that these compounds would be degraded by anaerobic dehalogenation pathways. It is our recommendation that a more sensitive method for C8 analysis be developed, and that anaerobic consortia be tested for their ability to degrade both [redacted] and C8.

Introduction

ENVIROGEN has experience in the biodegradation of [redacted] compounds. With a grant from the National Science Foundation, ENVIROGEN has investigated the degradation of [redacted] It was hypothesized that the bacterial strains that were competent in degrading these compounds would also be able to degrade [redacted] and ammonium perfluoro-octanoate (C8). This research was conducted under a joint development agreement between DuPont and ENVIROGEN (see attached agreement).

Scope of Work

ENVIROGEN proposed to investigate the degradation of two different fluorinated or perfluorinated compounds using three different bacterial strains previously shown to degrade [redacted]

Materials and Methods

Strains and Media. A total of nine different strains were tested for their ability to degrade [redacted] *Pseudomonas mendocina* KR1 (T4MO; Whited and Gibson, 1991) and *Pseudomonas putida* F1 (TDO; Wackett and Gibson, 1988) were both grown on a combination of toluene vapors and 0.04% glutamic acid in basal salts media (BSM; Hareland et al., 1975). The methanotroph *Methylosinus trichosporium* OB3b (OB3b; Stirling and Dalton, 1979) was grown on BSM with methane as the sole carbon and energy source, and the propanotrophs *Mycobacterium vaccae* JOB5 (JOB5; Wackett et al., 1989), ENV2C, ENV2D, ENV2R, ENV2W and ENVOB were grown on propane. [redacted] and C8 assays were run in parallel groups, using aliquots of the same cultures. For each assay, the cells were collected by centrifugation, washed and suspended in an equal volume of fresh BSM medium to an OD_{550} of 2.0. Cultures prepared for ion chromatography analysis were washed and

suspended in phosphate buffer and those for HPLC analysis were prepared in MOPS buffer.

Activity Assays. Oxygenase activity in certain strains (*P. mendocina, P. putida, M. trichosporium)* was monitored by performing a [redacted] degradation assay to insure production of the degradative enzymes. The cultures were harvested by centrifugation and suspended in BSM to an optical density of 2, as measured at 550 nm. Five milliliters of the cultures were then aliquoted into 15-ml serum vials, in triplicate, and sealed with Teflon lined septa. [redacted] that was diluted in methanol was then injected through the septa to a final concentration of 20 µM. The vials were then allowed to incubate at 25-30°C, shaking at 100 rpm in a horizontal position. A standard curve was generated by making [redacted] additions to serum vials that contained only BSM. The concentration of [redacted] was then determined by injecting 10 µl of headspace gas from the sample vials onto a GC that was equipped with an ECD.

Activity in the propanotroph JOB5 was monitored by degradation of BrMe in a bottle assay similar to the [redacted] assay. The rest of the propanotrophs were grown on propane which induces the appropriate enzyme system, however, these strains were not monitored for activity prior to testing their activity against [redacted]

[redacted] *Degradation Assays.* Culture preparation was performed as above and 5 µl of ^{14}C-[redacted] [45 µCi] was added with a micro-pipette prior to sealing the vials with Teflon-lined crimp caps. The cultures were then allowed to incubate at room temperature on a 100 rpm shaker until they were sacrificed at 0, 2, 7, 24, and 48 hours. Longer incubations with the propanotrophs JOB5 and ENVOB (68 hours, 5 days, and 13 days) were performed with a constant propane feed. The incubation was terminated by the addition of 1.0 ml of 1 N HCl. Acid addition kills the cells to prevent any further activity, and forces carbon dioxide out of solution and into the headspace of the serum vials. The vials were then returned to the shaker until all of the incubations were terminated. The vials were subsequently purged with nitrogen gas to strip the CO_2 into an NaOH trap. For analysis, 0.1 to 1.0 ml of the NaOH solution was transferred to a scintillation vial containing 5 ml of scintillation cocktail. The assay vial was then centrifuged to obtain the cell-free supernatant which was removed from the vial and retained. The cells were suspended in fresh BSM, washed and centrifuged. The supernatant from this wash was added to the original supernatant. An aliquot of the washed cells and the supernatant were both added to scintillation vials containing 5 ml of cocktail. The amount of radioactivity present was determined by scintillation counting.

For analysis of degradation by ion chromatography, cells were washed twice and suspended in phosphate buffer to an OD_{550} of ~ 2.0. Ten milliliters of washed cells in phosphate buffer were added to 50 ml serum vials. Autoclaved cells were used as killed controls for this assay. 10 µl of [redacted] stock (2 mg/ml) were added to each vial for a final concentration of 10 ppm. The vials were sealed and placed horizontally on a shaker at room temperature (JOB5) or 30°C (OB3b, ENV2C, ENV2R, and ENV2W). For analysis, the caps were removed from the vials and the contents were filtered (0.2 µm) into 10 ml

serum vials to remove the cells. The vials were placed at 4°C until analysis by ion chromatography.

[redacted] *Analysis by Ion Chromatography.* HPLC grade [redacted] was used to prepare a 254 ppm working standard in reagent grade deionized water. Combined with the [redacted] working standard, fluoride was also added to ensure validity in mixture and concentration upon analysis. Ion chromatographic parameters were developed using 1.0 mM sodium carbonate/1.7 mM sodium bicarbonate as the mobile phase and a Dionex AS4A anions column as the stationary phase. Flow and pressure were all set to normal parameters for standard anions applications. Ion chromatographic standards were prepared for [redacted] and analyzed at the following concentrations: 0.8, 1.5, 3.1, 7.7, and 15.4 mg/1. The chromatographic retention time fo[redacted]s 1.75 minutes and the fluoride check is at 0.87 minutes. Both compounds calibrated to a 0.995 or better correlation coefficient encompassing all calibration levels.
The quality control measures for a [redacted] analysis series is:

Reagent Blank
A continuing calibration check standard (1-2 check standards)
Matrix duplicates
Matrix Spikes
Final continuing calibration check standards

A typical number of samples would range from 20 to 50 samples and be analyzed within 48 hours of receipt (usually upon receipt). Spike recoveries averaged 85 to 115% recovery fo[redacted] spikes as well as additional anions spiked into the matrix. For each set, all samples were pre-filtered then placed directly into auto sampler vials. Each sequence was reviewed and presented in a simple data summary format as requested by the submitter.

No significant peculiarities or matrix interference were noted on the analysis of [redacted] samples. [redacted] standards were also prepared and analyzed by ion chromatography. These standards were compared to the 'cold' [redacted] standards with a 113% recovery of the [redacted] material. No abnormalities were noted in the ion chromatographs.

[redacted] *Analysis.* A commercially purchased stock of [redacted] was used to prepare a 5000 ppm working standard in reagent grade deionized water. Ion chromatographic parameters were developed using 1.8 mM sodium carbonate/1.7 mm sodium bicarbonate as the mobile phase and a Dionex AS4A anions column as the stationary phase. Flow and pressure were all set to normal parameters for standard anions applications. Ion chromatographic standards were prepared fo[redacted]nd analyzed at the following concentrations: 1.0, 2.0, 5.0, and 10 mg/1. The chromatographic retention time fo[redacted]s 0.97 minutes.

C8 Degradation Assay. Culture preparation was performed as above except that C8 was added to a final concentration of 5 ppm prior to incubation, and the cultures were

suspended in MOPS buffer rather that BSM in an attempt to eliminate any potential interference with the analysis. Following a 12 hour incubation the vials were centrifuged and the supernatant was analyzed.

C8 Analysis. Three different methods were attempted for derivitization and analysis of the compound C8 on various GC-detector configurations:

I. DuPont Method

1. 1 gram (1 ml) C8 solution into a 60 ml serum vial. *
2. Add 20 ml 20% phosphoric acid solution and 20 ml methylene chloride.
3. Crimp seal vial and shake 1 hr.
4. Remove methylene chloride and dry over sodium sulfate anhydrous**.
5. Evaporate methylene chloride with a nitrogen stream at 40°C.
6. To dried sample add 3N methanolic HCl (10 ml).
7. Seal crimp vial.
8. Heat solution at 50 to for 2 hr. Cool, insert 25 gauge needle to vent.
9. Add 10 ml of hexane with a syringe and mix.
10. Add 20 ml NaCl solution via syringe - remove vent needle and shake.
11. Analyze on GC.

* this solution was prepared with 40 µl of C8 solution (provided by DuPont) in 79.96 ml methanol. Concentration of this solution is 500 ppm.
* *this step was also performed using dry ice and a vacuum.

II. A. BF_3 - Methanol Esterfication Method (Metcalfe and Schmitz, 1961)

1. 25 µl of 20% solution (provided by DuPont) diluted in 5 ml ether in a 10ml serum vial.
2. 2 ml of ether solution placed in 10 ml serum vial and 2 ml of BF_3 reagent (Supelco cat# 3-3021) added.
3. Bottle placed in a beaker of water and boiled for 3-5 minutes.
4. 1 ml of water is added to stop reaction. Allow layers to separate.
5. Remove top layer and analyze.

II. B. BF_3 - Methanol Esterfication Method (SW 846; Method 6640 EPA)

1. Stock Solution prepared in ethyl ether. Final concentration of 1 mg/mL.
2. A serial dilution of stock prepared
   a. 1/100 (10 µL in 1 mL) 10 ng/µL
   b. 1/1000 (1 µL in 1 mL) (1ng/µL)
   c. 1/10000 (10 µL of 1/100 in 1 mL)

3. Esterify 0.5 mL of each solution and a 0.5 mL hexane blank
   a. add 0.5 mL of solution to serum vial.
   b. add 0.5 mL BF_3
   c. heat at 50°C in a sealed serum vial for 30 min in a water bath.
   d. cool, add $NaSO_4$ solution, shake 1 minute
   e. quantitatively transfer to flask and concentrate to 0.5 mL.
   f. analyze via GC/MS

III. Pentafluorobenzyl Bromide Derivitization Method (Flanagan and May, 1993)

1. Extract 25 ml of 5000 ppm C8 solution with 25 ml ether.
2. Remove 1 ml of ether extract and place in 25 ml acetone
3. Add 0.5 gram of K_2CO_3 (dispersing agent).
4. Add 250 µl pentaflurobenzyl bromide
5. Reflux for 3 hours
6. Analyze GC/MS

High Performance Liquid Chromatography (HPLC) Method

1. Standards were prepared in accordance with method guidelines provided by DuPont.
2. Methods were reviewed:
   The following was steps were taken:

   a. Standards were prepared in deionized water in sterile plastic containers.
   b. C8 stock was provided by DuPont
   c. Zorbax column was purchased
   d. Standards were analyzed via HPLC
   e. Standards were analyzed via Mass Spec
   f. Standards were analyzed via GC-ECD
   g. Standards were prepared a second time and analyzed again using the methods listed above.

Results

Degradation. The results of activity assays on the bacterial strains tested indicated that the appropriate enzyme systems had been induced (Figure 1). Nine strains were tested for their ability to degrade [redacted] Assays were conducted with ^{14}C labeled [redacted] to determine if these strains would produce $^{14}CO_2$ and with HPLC grade [redacted] for dehalogenation analysis.

[redacted] degradation or [redacted] dehalogenation were not detected in incubations with any of the strains tested, even with [redacted] incubations of up to thirteen days (JOB5 and ENVOB with propane feed), or two days incubation with [redacted] for fluorine analysis (Figure 2). Both JOB5 and OB3b were assayed at several different densities (OD=1, 2, and 3) with negative [redacted] results (Figure 3). The levels of ^{14}C carbon dioxide detected in the traps from the incubations with [redacted] failed to exceed the levels that were observed in the controls which contained no bacteria, and/or no labeled [redacted] In some experiments, the vials containing live cells produced slightly higher levels of ^{14}C trapped in the NaOH, however, the total was never more than 0.2% higher than the controls. Soluble and total non-volatile (including cells) ^{14}C was never significantly different in the experimental and killed controls (Figures 4 and 5).

[redacted] *Analysis.* The objective of the [redacted] analysis was to determine whether it co-eluted with any currently calibrated compound (F, Cl, NO_2, NO_3, PO_4, [redacted] and SO_4); and whether the [redacted] stock was contaminated with [redacted] The analysis of [redacted] demonstrated that the elution time did not interfere or co-elute with any other compounds, and that the [redacted] was not contaminated with this compound. The closest eluting compound was fluoride which was 0.87 min. Both [redacted] and fluoride were easily distinguished and quantified. No contamination with [redacted] was observed in samples or standards.

C8 Degradation. JOB5 was incubated with C8 and analyzed by HPLC analysis and a variety of GC methods. Failure to detect C8 at sufficiently low concentrations by any of these methods terminated this work.

C8 Analysis. A standard curve analysis of C8 by HPLC showed a good response at 50 ppm, but no quantification at 10 ppm. The lack of sensitivity of this method combined with the interference with the buffers in which the bacteria were suspended made it inappropriate for this application.

The derivatization reactions for GC detection appeared to randomly fragment the C8 into smaller compounds. Repeated analysis of these compounds had a negative impact on both the instrument and the column. C8 was not detected by any of the methods described above.

Discussion

A suitable method for the detection of C8 at low concentrations that is compatible with the buffer solutions necessary for bacterial degradation experiments needs to be developed before further work on the degradation of this compound can proceed. Due to the high degree of fluorination of this compound, however, it is unlikely that the oxygenase enzymes of aerobic bacteria will be effective in the degradation of this compound. In the work that ENVIROGEN did on the degradation of HCFCs and HFCs, compounds with a high degree of fluorination were generally not degraded. It is more likely that this compound would be degraded by an anaerobic dehalogenation pathway.

None of the cultures, representing a range of bacterial oxygenase systems was able to degrade [redacted] As noted above, in our previous work with fluorinated compounds, the higher the degree of fluorination, the less likely it was to be degraded. This compound is also more likely to be degraded by anaerobic bacteria.

Recommendations for Future Research

C8 Degradation. It seems unlikely that an HPLC method will be sensitive enough to measure degradation at low concentrations, therefore, a suitable method for detection by GC/MS should be developed. Methyl perfluorooctonoate is available commercially as a standard. This standard could be used to develop the method for detection of the derivatized compound. Once this standard can be detected reproducibly at a range of concentrations, a reliable method for the derivatization of the compound can be developed. Once a suitable analysis system has been established it is our recommendation that anaerobic consortia be tested for their ability to degrade this compound.

[redacted] *Degradation.* Although a considerable amount of work has already been done, it is ENVIROGEN's recommendation that the anaerobic work on [redacted] degradation be continued. This work is more likely to be productive than the further investigation of aerobic pathways for this degradation.

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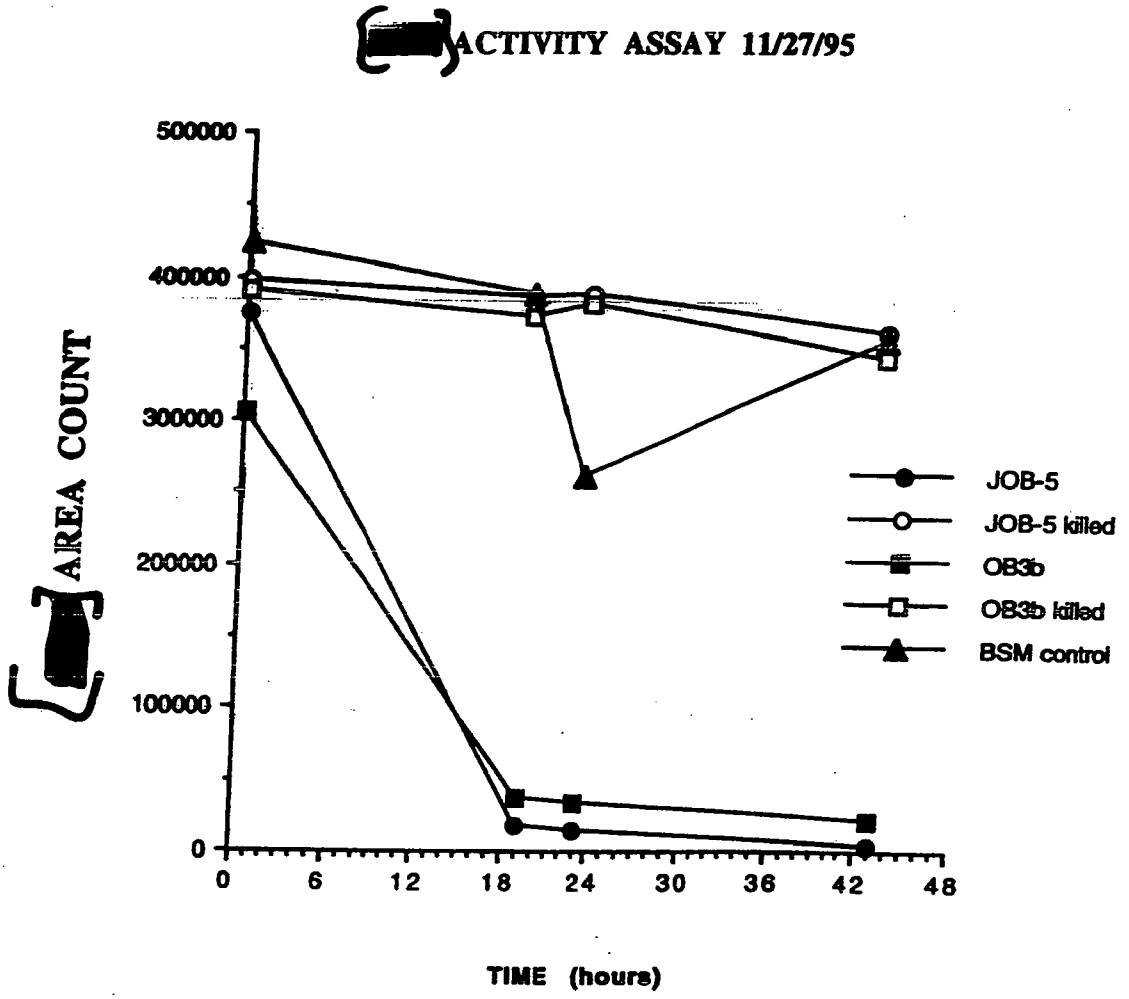


Figure 1
Example of results from an activity assay for M. vaccae JOB5 and M. trichosporium OB3b.

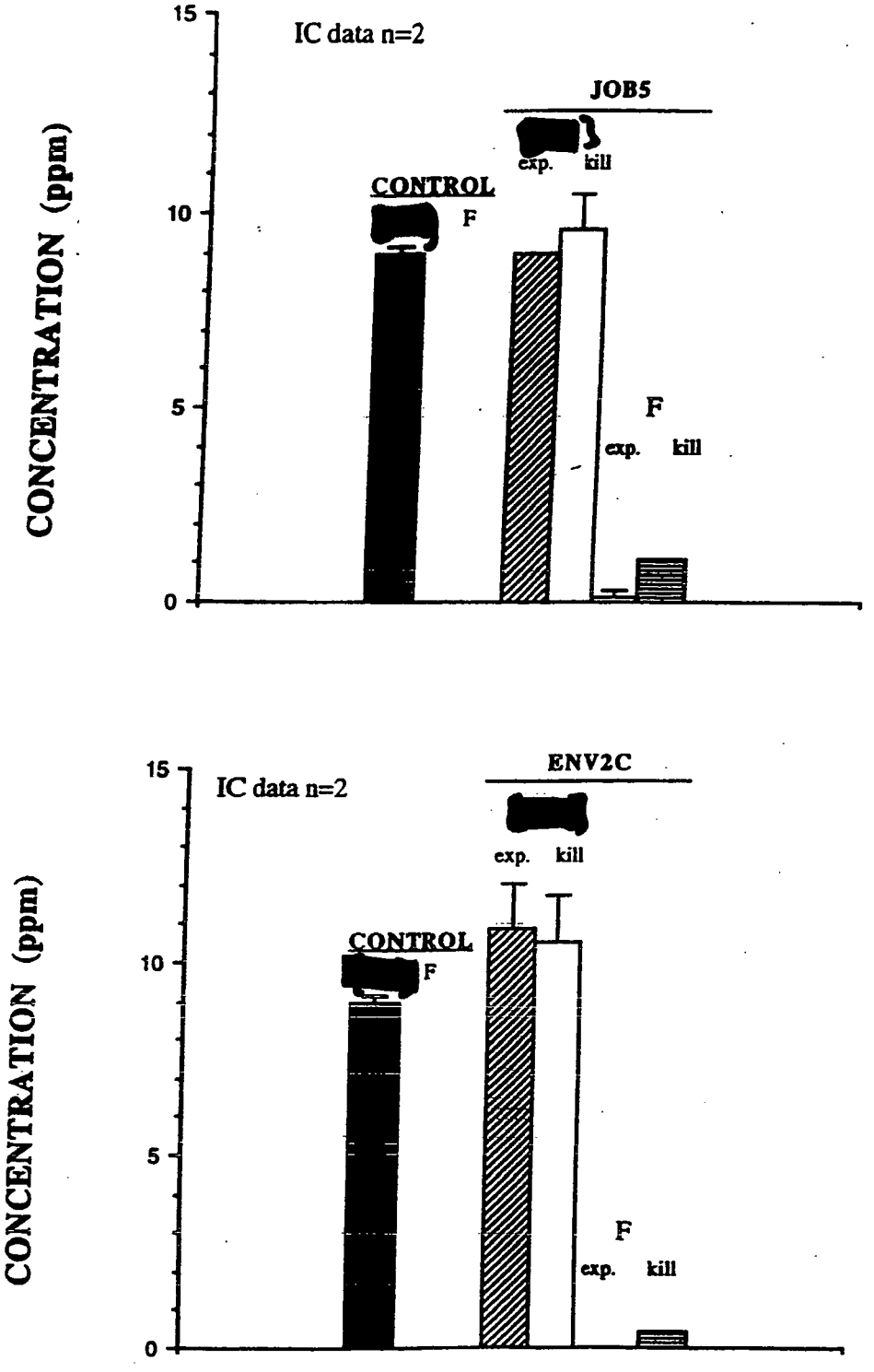
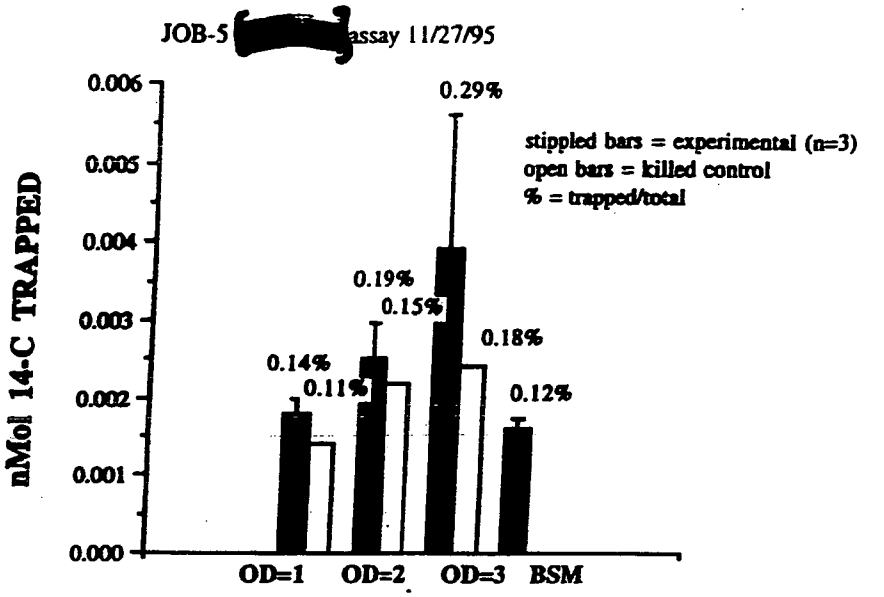


Figure 2
Examples of [redacted]ion chromatography data for two propanotrophs, M. vaccae JOB5 and ENV2C. Fluoride (F) was not observed after incubations of all the strains tested with [redacted]



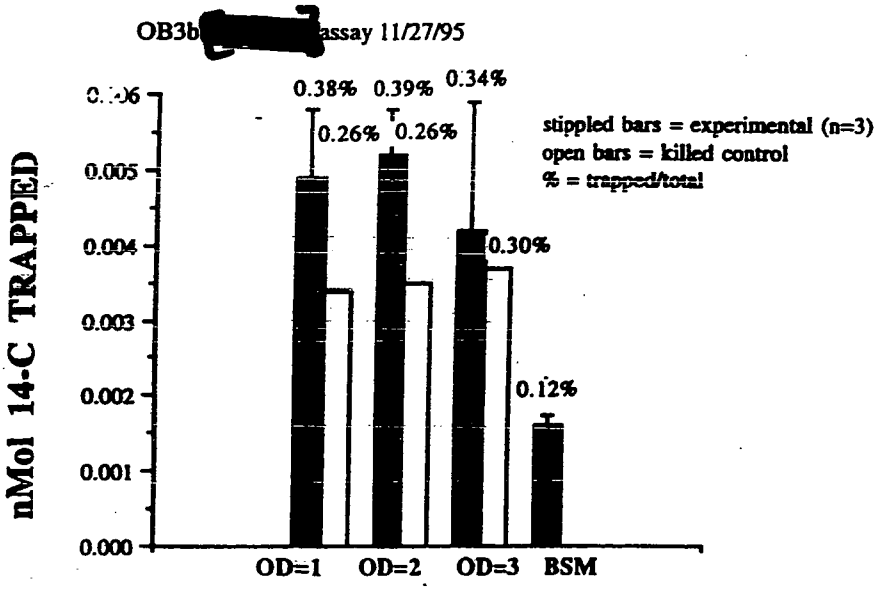
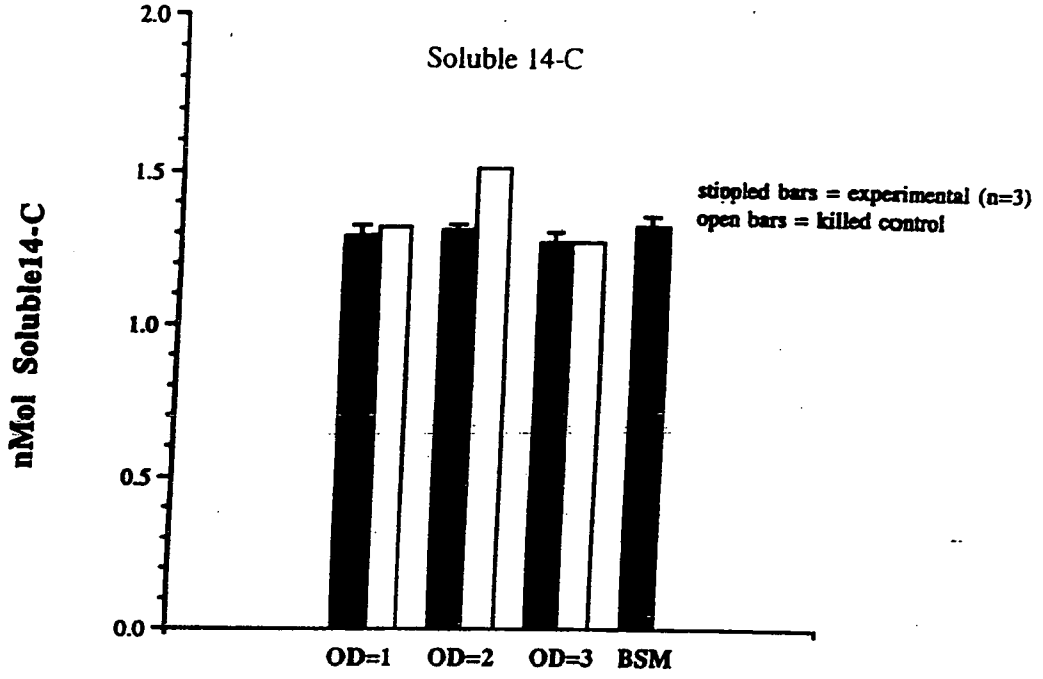


Figure 3
Two examples of representative results obtained by measuring ^{14}C in NaOH traps after incubation of [redacted] with three different cell densities. Results for JOB5 and OB3b are for both experimental and killed controls.

JOB-5 [redacted] assay 11/27/95



OB3b [redacted] assay 11/27/95

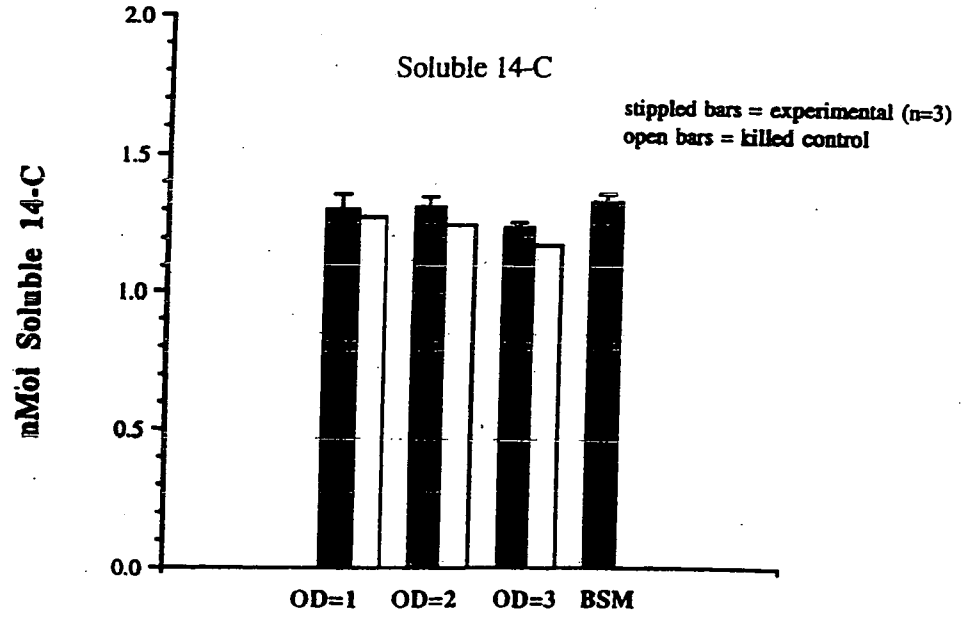
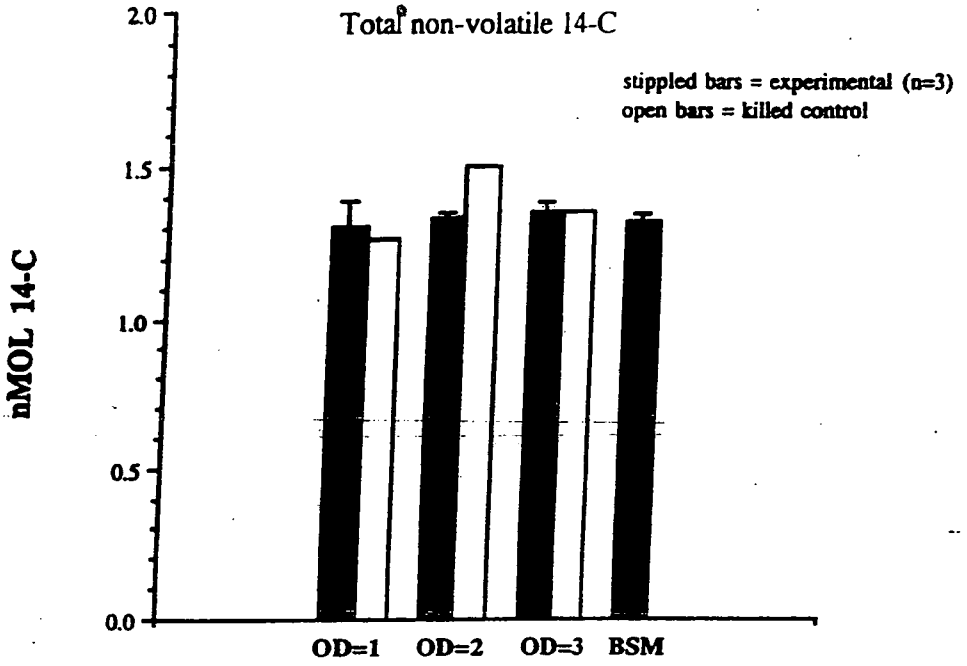


Figure 4
Soluble ^{14}C measured in the supernatant fraction of the [redacted] experiments with JOB5 and OB3b.

JOB-5 [redacted] assay 11/27/95



OB3b [redacted] assay 11/27/95

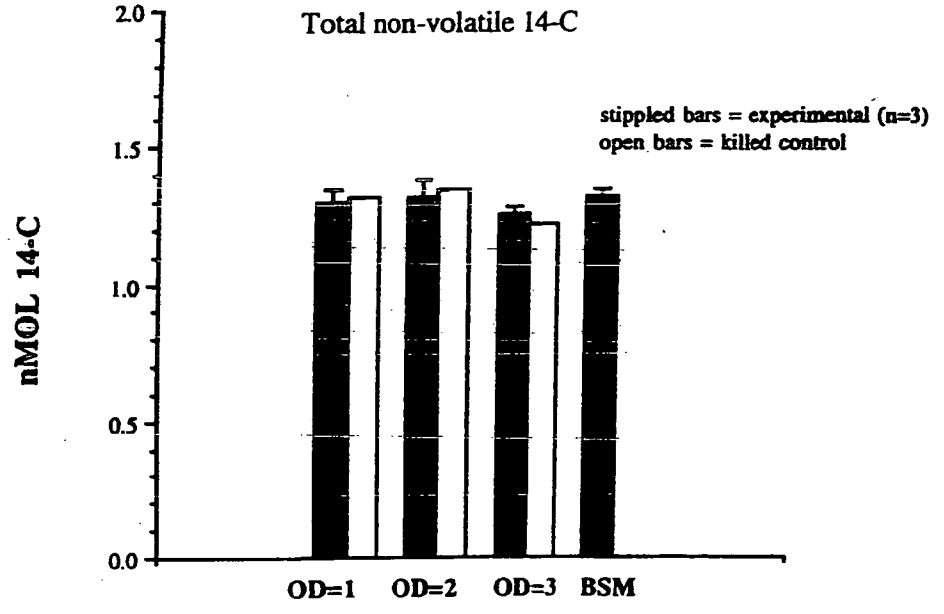


Figure 5
Total Non Volatile 14C measured in the supernatant and the washed cells in [redacted] experiments with JOB5 and OB3b.