

Degradation of Tertiary Butyl Alcohol
by a Pseudomonas sp. Isolated from Groundwater

by

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(ABSTRACT)

A Pseudomonas sp. capable of degrading tertiary butyl alcohol (TBA) as a sole carbon source, was isolated from a groundwater aquifer (50 ft. deep) at a petroleum refinery. The most probable number (MPN) of TBA degrading microorganisms was 4.9×10^3 organisms/g (dry wt) of subsurface soil. Pristine subsurface soils, which did not have a history of petroleum contamination, had MPNs of < 2 TBA degrading organisms/g (dry wt) indicating a natural enrichment process at the refinery site. The Q_{O_2} of Pseudomonas sp. was 4.2 ml O_2 /mg dry wt/h when TBA was the substrate. The optimum pH for growth was 7.0. The organism grew faster in continuous culture when TBA was the sole carbon source with a doubling time 33.6 h. The doubling time in batch culture was 112.3 h. When yeast extract was added to a mineral salts + TBA medium to concentrations greater than 1 mg/ml, TBA degradation was inhibited. When the yeast extract concentration was 0.1 mg/ml, a diauxy effect was seen in the growth rate. This suggested that TBA degradation by Pseudomonas sp. was subject to a regulatory mechanism.

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INTRODUCTION

The chemical contamination of groundwater induced by certain human activities is an important environmental issue because groundwater produces nearly 95% of the United States' drinking water supply (2). According to one hypothesis, the elimination of toxic recalcitrant organic compounds from groundwater habitats may require an extended period of time because the environmental conditions are not favorable for biodegradation. For example, petroleum products from many of the 3.5 million underground gasoline storage tanks in the US may be leaking into subsurface soil (4). Once an organic contaminant enters subsurface soil, either by percolation or direct introduction into the aquifer, it can be affected by adsorption, volatilization, chemical alteration and microbial activity (26). The degree to which the first three affect the contaminant can be predicted by the chemical and physical properties of the compound and the soil. The extent which microorganisms can metabolize organic contaminants in subsurface habitats is not known, although the potential has been demonstrated for some compounds and soils (3,9,18,26).

Until recently, it was assumed that aquifers did not have a functional population of microorganisms because the population was low and environmental conditions were unfavorable for microbial growth. Subsequently it has been demonstrated that subsurface soils contain a significant bacterial population (3,7,9,18). Epifluorescent direct microscopic counts of subsurface soils have yielded estimates of 10^6 - 10^8 bacterial cells/g dry wt soil, whereas, bacterial plate count procedures on subsamples of subsurface material have produced viable bacterial population ranging from 100 to 10^6 cfu/g dry wt soil

(3,7,8,16,18). White et al. (25) used a phospholipid technique to demonstrate that a population of 10^7 bacterial cells/ g dry wt soil was present in aquifer material.

We have measured the bacterial population of subsurface soil at 4 different locations in three different states. The subsurface bacterial population was measured using the epifluorescent microscope and plate count (0.1% yeast extract-0.1% peptone and 10% soil extract media) procedures, and populations in the range of $10^7 - 10^9$ and $10^5 - 10^7$ were detected by these respective techniques. (R. Benoit, J. Novak, C. Goldsmith and J. Chaddock. 1985. Abstracts of the Annual Meeting for Microbiology, p. 258)(3). The subsurface soil used in this study was taken from a refinery site in Philadelphia, PA. This soil had been contaminated for over ten years with petroleum products including oxinol (50:50 mixture of methanol and tertiary butyl alcohol).

Tertiary butyl alcohol (TBA) is one of the alcohols used to enhance octane ratings in gasoline. TBA is totally miscible with water, therefore, if there is a leak of a gasoline storage tank, TBA could diffuse freely into an aquifer. Tertiary butyl alcohol is a four carbon alcohol which has a molecular weight of 74.12, a melting point of 25°C and a boiling point of $82 - 83^{\circ}\text{C}$. It has an LD_{50} orally in rats of 3.5 g/kg (23).

Horn et al (11) demonstrated that TBA could be degraded under aerobic conditions by an activated sludge culture. We have shown that TBA can be degraded in groundwater microcosms incubated under laboratory conditions at a maximum rate of 2 mg TBA/liter/day. In

this work we report the first isolation of a pure culture of a bacterium which can utilize TBA as an energy source.

MATERIALS AND METHODS

Site Description and Soil Sampling Procedures

Subsurface soil and groundwater were collected from a gasoline refinery in Philadelphia, Pennsylvania. This site had over a ten year history of gasoline spills. Subsurface samples were collected to depths of 50 feet. The soil used in this study was from the 50 foot depth and it had a fine sandy texture. The water table was at 17 feet. The chemical/physical characteristics of the subsurface soil are given in table 1. When the soil sample was processed, a gasoline odor was detected.

Conventional drilling equipment was used and the samples were taken by an Osterburg hydraulic sampler, a Denison sampler, or a Pitcher Barrel sampler depending upon soil conditions. Shelby tubes were placed into the samplers for soil extraction. Bentonite drilling mud was used to stabilize the hole. An immediate concern was that the drilling mud might introduce microorganisms into the subsurface soil sample. Therefore, to ensure aseptic collection of samples, a lithium chloride tracer was added to the drilling mud. Each sample was analyzed for the presence of lithium by atomic adsorption spectrophotometry and was also inspected visually for the presence of drilling mud. Subsurface soil was removed from the Shelby tubes by a soil extrusion device which aseptically pared away the outer 2 to 3 centimeters of the soil core. In addition, the ends of the soil core were pared away by a flame sterilized spatula. The samples were placed directly into sterile Mason jars with teflon lids and were stored at 4°C for transport back to the laboratory (9,26).

Media and Reagents

Aerobic and anaerobic enrichment cultures for the growth of TBA decomposers were initiated using a mineral salts medium with TBA as the major, or sole, carbon source. The medium used for aerobic enrichments was a mineral salts medium of the following composition (per liter distilled deionized water): $(\text{NH}_4)_2\text{SO}_4$, 1.2 g; $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.1 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.1 g; ferric citrate, 0.002 g. The phosphate buffer added after autoclaving consisted of (per 200 ml of distilled deionized water): K_2HPO_4 , 0.2 g and KH_2PO_4 , 0.1 g. The final pH of the medium was adjusted to 7.5 with 0.1 N NaOH. This mineral salts base was described as basal inorganic medium A (14). Stock TBA solutions, which were designed to yield TBA concentrations which ranged from 100 to 1500 mg/liter final concentration of culture medium, were filter sterilized and added aseptically to the autoclaved medium described above. A trace element solution (1.2 ml/l of medium) described by Krieg (14) was also filter sterilized and added to the medium. This medium, which will be referred to as BMA+TBA, was used for all aerobic cultures in this study unless otherwise indicated.

The medium used for anaerobic enrichments had the following composition (per liter of distilled deionized water): NaEDTA, 20 mg; FeSO_4 , 12 mg; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 200 mg; $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 75 mg; NaCl, 1.0 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; thiamine HCl, 1.0 mg; and biotin, 1 mg. Two ml of sterile stock phosphate buffer was added aseptically after autoclaving. The phosphate buffer stock consisted of (per 200 ml distilled deionized water): KH_2PO_4 , 1.6 g and K_2PO_4 , 2.4 g (1). This anaerobic mineral salts basal solution and the phosphate buffer stock solution were prepared according to the V.P.I. and S.U. Anaerobic

Laboratory Manual (10). One ml of a filter sterilized trace element solution (1) was added aseptically and anaerobically to 1 liter of the above after autoclaving and mixing. This complete basal solution will be referred to as BMB. TBA was added to BMB to a concentration of 500 mg/l of TBA in the medium. The complete medium was used for the growth of all anaerobic bacteria tested in this study and will be referred to as BMB+TBA medium.

The following plate count medium was used in growth experiments of Pseudomonas sp. strain VT100 : 1.0 g Na succinate, 1.0 g Na acetate, 1.0 g Difco yeast extract, 0.5 g Difco peptone, 15 g agar, and 1000 ml of BMA.

The TBA used throughout this study as well as all organic solvents and other reagents were obtained from Fisher Scientific Products (Raleigh, NC). All media and agar were obtained from Difco Laboratories (Detroit, Michigan). All glassware used for culture work or media preparation was acid washed with chromic acid and rinsed six times with tap water and ten times with distilled deionized water.

TBA Analysis

A gas chromatographic procedure was used to measure the degradation of TBA in culture media. Sterile controls were used for each interval in all experiments. A two ul sample was injected into an HP-5880A gas chromatograph (Hewlett-Packard, Corvallis, Oregon) equipped with a stainless steel column packed with 0.2% Carbowax 1500 on 80/100 mesh Carbopak C. A Flame-Ionization detector (FID) was used to detect the TBA. Other instrument parameters were as follows: injection port temp 125°C; FID temp 250°C; carrier flowrate (N₂) 24cc/min; and oven temp 120°C. The detection limits for TBA on this

column were found to be approximately 0.1 mg/liter (26).

Bacterial Enumeration of Subsurface Soil.

Immediately after the groundwater material arrived at the laboratory we enumerated a small sample of the soil using three techniques. Epifluorescent microscopy (24a), viable counts using 0.1 % yeast extract and peptone plates and 10 % soil extract plates, and a most probable number (MPN) technique were used to enumerate the total, viable, and the functionally viable population of each sample. In the MPN, BMA+TBA was used for aerobic incubation and BMB+TBA was used for anaerobic incubation. Five replicate tubes for each sample dilution were used for statistical reasons. An MPN culture was considered positive if it became turbid and if an observable decrease in TBA concentration compared to an uninoculated control. The number of organisms per gram dry soil wgt. which could degrade TBA anaerobically and aerobically were calculated using the MPN table in Franson (ed.)(6a).

Culture Isolation from Enrichment Cultures

Aerobic enrichments were initiated by placing 10 g (wet wt) of subsurface soil into 500 ml Erlenmeyer flasks containing 200 ml of BMA+TBA medium (approximately 500 mg TBA/liter). The enrichments were allowed to incubate at 25°C for 3 months which was the time required for the medium to become turbid. Ten ml of the enrichment culture was transferred to sterile BMA-TBA medium and incubated until the medium became turbid. After three serial transfers, culture isolation procedures were used to obtain pure cultures of bacteria which degrade TBA.

Anaerobic enrichments were inoculated, incubated and transferred in the anaerobic glove box (Coy Laboratory Products, Inc, Ann Arbor, Michigan). Strict anaerobic conditions were maintained throughout the experiment. All enrichment cultures were tested for loss of TBA by gas chromatographic analysis. Cultures were selected for further analysis based upon culture turbidity and TBA utilization.

The following methods were used to isolate bacteria from aerobic enrichments: streak culture on solid medium, serial dilution to extinction and growth in a continuous culture chemostat. The following media were used for pure culture isolation: T-soy agar, Plate Count Agar, Pseudomonas agar, Actinomycete agar, 0.1% yeast extract-0.1% peptone agar, and 0.1% yeast extract-1% TBA agar (Difco). None of the isolates which grew on these media could degrade TBA when reintroduced into BMA+TBA medium. Therefore, solid BMA which incorporated simple single carbon sources (0.1% wt to vol), such as Na succinate, Na pyruvate and methyl amine, were utilized. Stock cultures were maintained in BMA+TBA broth under refrigeration (4°C) or on Na acetate plates sealed with Parafilm in the refrigerator.

Ten ml of an enrichment culture which rapidly degraded TBA was serially diluted to 10^{-10} . One ml of each dilution was inoculated into 9 replicate culture tubes (16 mm) containing BMA+TBA broth with 0.1% Na acetate. These cultures were incubated at 25°C for 3 weeks. The highest dilutions of the medium which were turbid after incubation were streaked on BMA+0.1% Na acetate agar plate. Pure cultures obtained from these plates were stored as indicated above.

Culture Identification

Identification was based on the classification scheme in Bergey's

Manual of Determinative Bacteriology (20). Biochemical tests were performed as described in the manual or by the methods of Smibert and Krieg (22). The mole percent guanine + cytosine of the Pseudomonas sp. strain VT100 high molecular weight DNA was measured by the methods described by Johnson (12,13).

Effect of pH on TBA Degradation and Growth

A 1 liter solution of BMA+TBA medium was prepared and divided into 8 portions. The pH was adjusted to pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using 0.1 or 1.0 N HCl or NaOH. These media were filter sterilized with 0.2 μ M Gelman filters and a Millipore filter apparatus. Thirty-two 16 mm screw cap tubes were sterilized and 9.5 ml aliquotes of media of the appropriate pH were added to 4 replicate culture tubes. Three tubes of media at each pH were inoculated with 0.5 ml of a washed Pseudomonas sp. VT100 suspension and 0.5 ml of sterile medium was added to a tube of sterile control medium. Growth was measured as increased optical density (O.D.) on a Nephelometer. TBA degradation was measured by removing 0.2 ml aliquots from each tube of medium at different times and analyzing the spent medium on a gas chromatograph.

The Effect of Yeast Extract on Degradation and Growth

BMA+TBA medium was prepared, filter sterilized and dispensed (8.5 ml) into each of thirty-five sterile 16 mm screw cap test tubes. The concentrations of yeast extract in the media were as follows: 10 mg/ml, 1 mg/ml, 1.0 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml, and 0.0001 mg/ml. One ml of the appropriate dilution of the yeast extract stock solution was added to each of 5 replicate broth tubes of BMA+TBA medium. There were 5 replicates of BMA+TBA set up as controls. One

tube in each replicate set of media served as an uninoculated control. One half an ml of a washed cell suspension of the Pseudomonas sp. VT100 was added to the remaining 4 tubes of each medium. One half an ml of BMA was added to each uninoculated control so the final volume in each tube was 10 ml. The viable count of the Pseudomonas sp. VT100 in each medium at the start of the experiment was 1.9×10^6 cfu/ml. The media tubes were tightly capped to limit the loss of TBA by volatilization, however, every other day the caps were loosened and each tube vortexed for 20 seconds to ensure aeration of the media and to limit clumping. The optical density of the cultures was measured with a Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Co, Inc. New York). Aliquots (0.2 ml) were removed from each tube of medium approximately every 7 days and were analyzed on the gas chromatograph for disappearance of TBA over time.

Chemostats

Customized 1 liter and 2 liter Erlenmeyer flasks (figure 1) were used as culture vessels. Twenty liter polypropylene nalgene carboys served as sterile medium and waste reservoirs. Fisher brand thick wall amber tubing was used for all connections. The flow rate was controlled by a Wheaton MP-3 microtubing peristaltic pump (Wheaton Instruments, Millville, New Jersey). The medium in the culture vessels was stirred by a Belco Multi-Stir magnetic stir plate. The medium was also aerated using aquarium pumps. Air was sterilized by passage through two serial filters, and bubbled into the medium. The plastic tubing in the medium had very small holes to decrease the air bubble size and increase the efficacy of the aeration.

A 10 ml aliquot was removed from each chemostat daily. The pH,

turbidity, and concentration of TBA were measured for each aliquot by techniques described above. Since TBA was stripped from the medium rapidly by the aeration process, a sterile control flask was used to correct for TBA volatilization.

Oxygen Uptake

Eighty ml of a cell suspension grown in continuous culture was harvested by centrifugation at 10,000 rpm for 10 minutes and the supernatant was discarded. The cell pellet was resuspended in distilled water and reharvested as above. The cells were resuspended in 15 ml of BMA (pH 7.0). Three replicate 1 ml aliquots were used for a dry weight analysis. The total protein of the cell suspension was determined using the method of Lowry et al (15) on a 1.5 ml sample. The remaining 10.5 ml cell suspension was incubated at 25°C for 24 h to lower the endogenous level of O₂ consumption. Oxygen uptake was measured using a Clark cell oxygen electrode in a jacketed cuvette and a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Spring, Ohio). The temperature was maintained at 25°C with a Haake Circulating Water Bath (Haake Co., Berlin). The oxygen electrode was calibrated according to the method of Robinson and Cooper (21). The volume of the Clark cell was 1.7 ml. Fifty ul of the TBA stock solution (3.4 g/l) was added to the Clark cell with a gas tight Hamilton syringe through an entry port in the ground glass stopper so that the final concentration of TBA in the cell at time zero was 100 ug/ml. Oxygen uptake was calculated in terms of Q_{O₂}. The units used were ul O₂/mg dry wt/h and ul O₂/mg total protein/h. All values represented were corrected by subtracting the endogenous rate of O₂ consumption. TBA was the sole carbon source.

Table 1.* Chemical and physical parameters of groundwater from Philadelphia Pennsylvania.

| Parameter | Amount (mg/l) |
|-------------------------------|---------------|
| Cl ⁻ | 9.52 |
| Br ⁻ | 0.114 |
| NO ₃ ⁻ | Trace |
| SO ₄ ²⁻ | 50.12 |
| Fe ⁺⁺ | 29.1 |
| Ca ⁺⁺ | 57.5 |
| Mg ⁺⁺ | 120 |
| Na ⁺ | 15.6 |
| K ⁺ | 4.8 |
| Dissolved O ₂ | 0.5 |
| Temperature | 13° C |
| pH | 6.5 |

* Reference C. D. Goldsmith, 1985. VPI & SU Dissertation. (8)

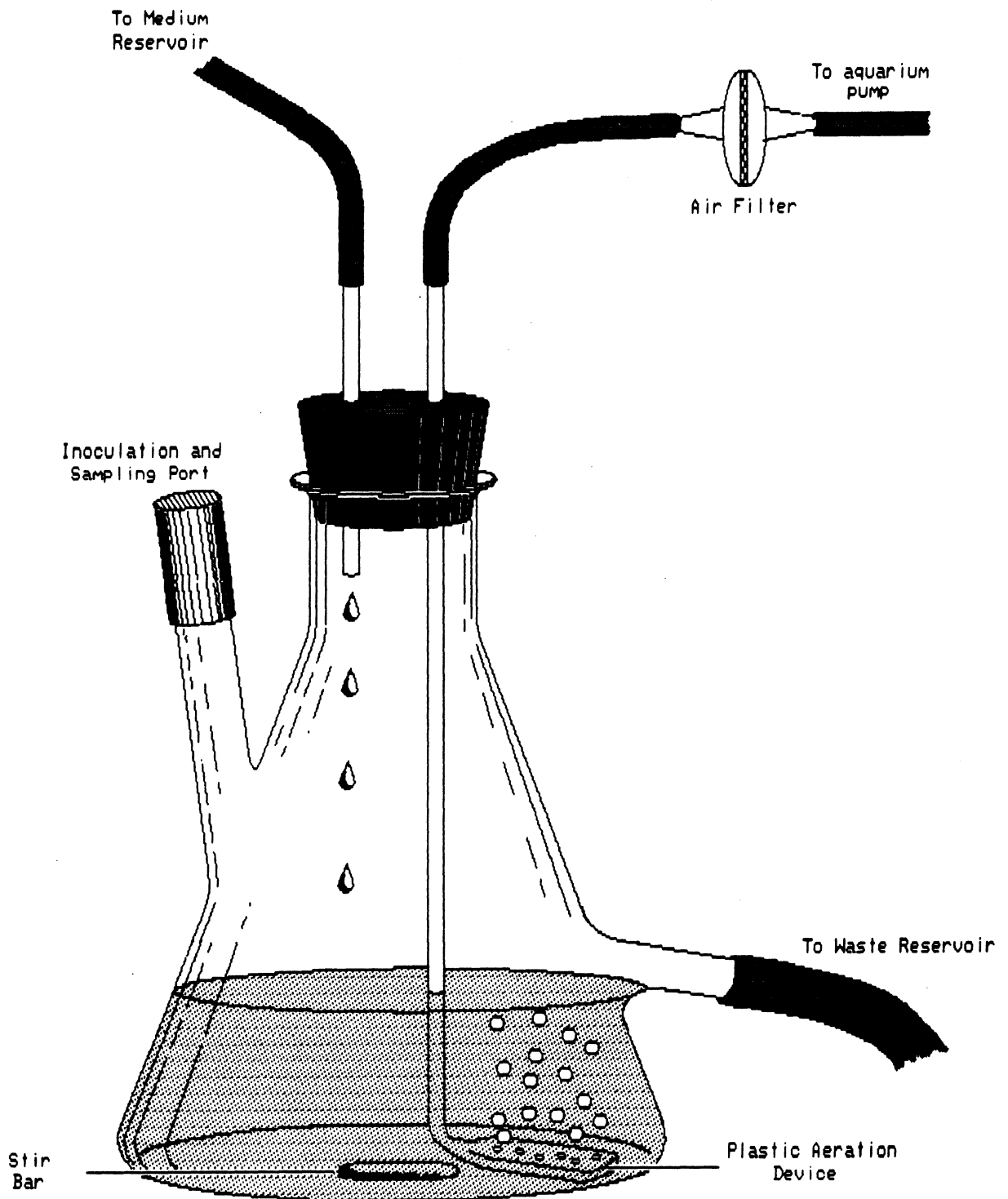


Figure 1. Illustration of the culture vessel used in the continuous culture experiment. (Illustration provided by N. R. Krieg).

RESULTS

Enumeration of Soil Samples.

The viable number of bacteria/g (dry wt) in the 50 ft sample of Philadelphia soil was 4.0×10^7 and 6.0×10^7 CFU/g using 10% soil extract agar and 0.1% yeast extract-peptone agar, respectively. Epifluorescent microscopy techniques yielded bacterial counts of approximately 3.8×10^8 bacteria / g. Aerobic MPN calculations resulted in estimates of 4.9×10^3 TBA degrading bacteria / g (confidence intervals: lower 1.7×10^3 ; upper 1.3×10^4). No dilutions in the anaerobic MPN were positive after 6 months incubation at 25°C (< 2 anaerobic TBA degraders/g).

Isolation and Identification.

Degradation of TBA was detected in aerobic enrichments inoculated with Philadelphia soil. Transfers of the enrichment cultures were made if TBA degradation was observed (approximately every 3 months). The degradation rate of the enrichment culture at a maximum was 9.0 mg TBA/liter/day (Fig. 2). Several attempts were made to isolate bacteria responsible for the degradation by streaking onto complex media like T-soy agar, nutrient agar, 10% soil extract, and agar plate count agar but none of the isolated colonies were able to degrade TBA when reinoculated into a defined medium with TBA as the sole carbon source. Using simple solid media like 0.1% acetate or 0.1% pyruvate or 0.1% succinate and 1.5% agar per liter of BMA resulted in small isolated colonies after two weeks incubation at 20° C. One of the colonies was small, round, convex, white and when reinoculated into BMA+TBA degraded significant amounts of TBA. Culture purity was

checked using conventional microbiological techniques including exhaustive streaking on differential media and microscopic observations. The isolate was gram negative, rod shaped, motile, oxidase positive, catalase positive, and nonfermentative. It oxidized glucose, xylose, and mannitol but did not oxidize lactose or maltose after 21 days of incubation at 20° C. It did not produce indole, hydrogen sulfide, or any pigments (visible or fluorescent). It did not utilize acetamide, esculin, or ONPG, and did not grow at 42° C. The mole % Guanine + Cytosine was 64. On the basis of these characteristics, the isolate was tentatively identified as a Pseudomonas species and was designated strain # VT100.

No degradation was observed in any of the anaerobic enrichments attempted.

Growth versus degradation of TBA by Pseudomonas sp. VT100.

Degradation rates were slower in pure culture than in the enrichment culture, however, Pseudomonas sp. VT100 was able to degrade significant amounts of TBA as a sole carbon and energy source. When grown on an alternate carbon source and reintroduced into BMA + TBA a lag period of 30 days for growth and degradation of TBA was observed. After the 30 day lag period linear growth and degradation resumed (Figure 3). The generation time was calculated using the formula $g = 0.693/a$. The most rapid doubling time measured for batch cultures was 112.25 hours (4.7 days). The maximum rate of degradation observed was approximately 3.5 mg TBA/liter/day.

Effects of pH.

The effects of pH on growth of Pseudomonas sp. VT100 and on the rate of degradation of TBA as a sole carbon source are shown in

figures 6 and 7. The optimum pH for growth and degradation was approximately 7.0. The maximum degradation rate observed was 3.5 mg/liter/day. The results of a qualitative study of the final pH of the medium after 60 days growth of Pseudomonas sp. VT100 can be seen in table 2.

Oxygen Uptake.

Oxygen was utilized by the isolate when given TBA as a sole carbon source. The maximum rate of oxygen uptake was observed in cells which were grown in chemostat with TBA as sole carbon source. The Q_{O_2} was 4.2 ul O_2 /mg dry wt/hour after correcting for endogenous respiration. The Q_{O_2} using mg protein was 18.26 ul O_2 /mg protein/hour. When potassium cyanide was added to the Clark cell respiration stopped (figure 7). Cultures grown on an alternate carbon source (0.1% acetate, succinate, pyruvate, or yeast extract) demonstrated no significant uptake of oxygen above the endogenous rate. The maximum observation period was 2 hours.

Continuous Culture.

The rate of volatilization of TBA due to the aeration of the chemostat was not linear (figure 8). An uninoculated control chemostat with an equivalent dilution rate was tested concomitantly with the inoculated chemostat (table 3). After reaching equilibrium the chemostats were allowed to incubate for several weeks. Measurements of TBA concentration, pH, turbidity, and viable counts were made on each chemostat. Due to slight variations in aeration rate, dilution rate, barometric pressure, and initial concentration of TBA in the sterile medium reservoir an accurate estimate of TBA

degradation rate could not be determined. The growth rate for Pseudomonas sp. VT100 was calculated using the formula $g = 0.693/a$. Where $a = d$ the dilution rate. The doubling time was 33.64 hours (1.4 days) (Table 4).

Effect of Yeast Extract.

Growth was observed for all cultures at all concentrations of yeast extract. The highest growth rate was observed in the 10 mg/ml treatment. The lowest growth rate was observed in the 0.01, 0.001, 0.0001, and 0.0 mg/ml yeast extract treatments (table 5). A graphical representation of some of these data can be seen in figure 9. Degradation of TBA was observed in all of the treatments except the 10 mg/ml yeast extract treatment (table 6). The highest degradation rate after 46 days of incubation was observed in the 0.001 mg yeast extract/ml treatment (figure 10). The average rate of degradation for that treatment was 4.35 mg/liter/day.

Table 2. Effect of pH on growth and degradation of TBA by Pseudomonas sp. VT100 in a basal salts medium with TBA as the sole carbon source after 60 days of incubation at 25° C.

| Initial pH | Final pH | TBA degraded | Turbid |
|------------|----------|--------------|--------|
| 2.5 | 2.5 | - | - |
| 3.0 | 3.1 | - | - |
| 4.0 | 4.0 | + | + |
| 5.0 | 4.0 | ++ | ++ |
| 6.0 | 4.0 | ++ | ++ |
| 7.0 | 4.2 | ++ | ++ |
| 8.0 | 6.5 | ++ | ++ |
| 9.0 | 9.0 | - | - |
| 10.0 | 10.0 | - | - |

- negative
+ slightly positive
++ positive

Table 3. Chemostat characteristics for continuous culture of Pseudomonas sp. VT100 using TBA as the sole carbon and energy source. Data are for chemostat at equilibrium.

| Parameters | Uninoculated control | <u>Pseudomonas</u> sp. |
|--|-------------------------|-------------------------|
| pH | | |
| Reservoir | 7.0 | 7.0 |
| Culture vessel | 6.74 ± 0.1 | 6.2 ± 0.2 |
| Turbidity (Klett units) | 0 | 44.0 ± 2.8 |
| Flow rate (ml/hour) | 13.4 ± 0.5 | 13.4 ± 0.5 |
| Culture vessel vol. (ml) | 630 | 650 |
| Dilution rate (hour ⁻¹) | 2.13 X 10 ⁻² | 2.06 X 10 ⁻² |

Table 4. Comparison of the growth of Pseudomonas sp. VT100 in batch and continuous culture in a basal salts medium with TBA as the sole carbon source.

| Parameter | Continuous Culture | Batch Culture |
|-----------------------------|--------------------|---------------|
| g (doubling time) (hour) | 33.6 | 112.3 |
| Initial pH | 7.0 | 7.0 ± 0.2 |
| Final pH (60 days) | 6.2 ± 0.2 | 4.2 ± 0.1 |

Table 5. Growth of Pseudomonas sp. VT100 in response to different yeast extract concentrations. The number of replicates is four (n = 4) for all treatments. The values represent the mean \pm the standard deviation for each time period. Growth is shown in Klett units.

| TIME days | Milligrams Yeast Extract / ml | | | | | | |
|--------------|-------------------------------|------------|------------|------------|------------|------------|-------------|
| | 10.0 | 1.0 | 0.1 | 0.01 | 0.001 | 0.0001 | 0 (no y.e.) |
| | KLETT UNITS | | | | | | |
| 0 | 1 \pm 1 | 2 \pm 1 | 0 \pm 1 | 4 \pm 3 | 6 \pm 1 | 5 \pm 2 | 3 \pm 1 |
| 2 | 54 \pm 4 | 22 \pm 1 | 18 \pm 1 | 9 \pm 1 | 7 \pm 1 | 7 \pm 1 | 9 \pm 2 |
| 6 | 90 \pm 5 | 55 \pm 2 | 30 \pm 1 | 13 \pm 1 | 11 \pm 2 | 12 \pm 1 | 13 \pm 2 |
| 10 | 112 \pm 5 | 71 \pm 1 | 33 \pm 2 | 15 \pm 2 | 14 \pm 2 | 14 \pm 1 | 16 \pm 3 |
| 14 | 105 \pm 10 | 79 \pm 3 | 35 \pm 1 | 19 \pm 2 | 19 \pm 2 | 19 \pm 3 | 21 \pm 2 |
| 28 | ND | ND | 41 \pm 1 | 35 \pm 7 | 38 \pm 5 | 36 \pm 5 | 37 \pm 5 |
| 35 | ND | ND | 47 \pm 1 | 44 \pm 9 | 51 \pm 4 | 46 \pm 8 | 51 \pm 7 |
| 40 | 103 \pm 9 | 91 \pm 1 | 59 \pm 8 | 51 \pm 8 | 60 \pm 4 | 55 \pm 8 | 60 \pm 7 |
| 45 | 102 \pm 10 | 93 \pm 1 | 65 \pm 6 | 53 \pm 5 | 65 \pm 4 | 60 \pm 9 | 68 \pm 8 |

ND not determined

Table 6. Degradation rates of TBA in response to different concentrations of yeast extract. The initial concentration of TBA for each replicate was 500 mg/liter. Concentrations which were slightly different from the target values in that set were adjusted to 500 mg/liter for comparative purposes. The number of replicates is four (n = 4) for all treatments. The values represent the mean \pm the standard deviation for each time period.

| TIME days | Milligrams Yeast Extract / ml | | | | | | |
|--------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 10.0 | 1.0 | 0.1 | 0.01 | 0.001 | 0.0001 | 0 (no y.e.) |
| | [TBA] mg / liter | | | | | | |
| 0 | 500 \pm 5 | 500 \pm 5 | 500 \pm 5 | 500 \pm 5 | 500 \pm 5 | 500 \pm 5 | 500 \pm 5 |
| 6 | 498 \pm 5 | 499 \pm 4 | 501 \pm 6 | 496 \pm 3 | 486 \pm 11 | 489 \pm 8 | 492 \pm 5 |
| 15 | 494 \pm 13 | 499 \pm 6 | 458 \pm 13 | 490 \pm 2 | 470 \pm 2 | 472 \pm 15 | 456 \pm 10 |
| 30 | 494 \pm 12 | 492 \pm 7 | 427 \pm 6 | 444 \pm 9 | 394 \pm 8 | 439 \pm 7 | 423 \pm 6 |
| 37 | 482 \pm 15 | 461 \pm 51 | 419 \pm 11 | 462 \pm 12 | 377 \pm 6 | 422 \pm 8 | 355 \pm 18 |
| 46 | 494 \pm 14 | 471 \pm 18 | 374 \pm 32 | 375 \pm 25 | 300 \pm 26 | 382 \pm 7 | 334 \pm 23 |

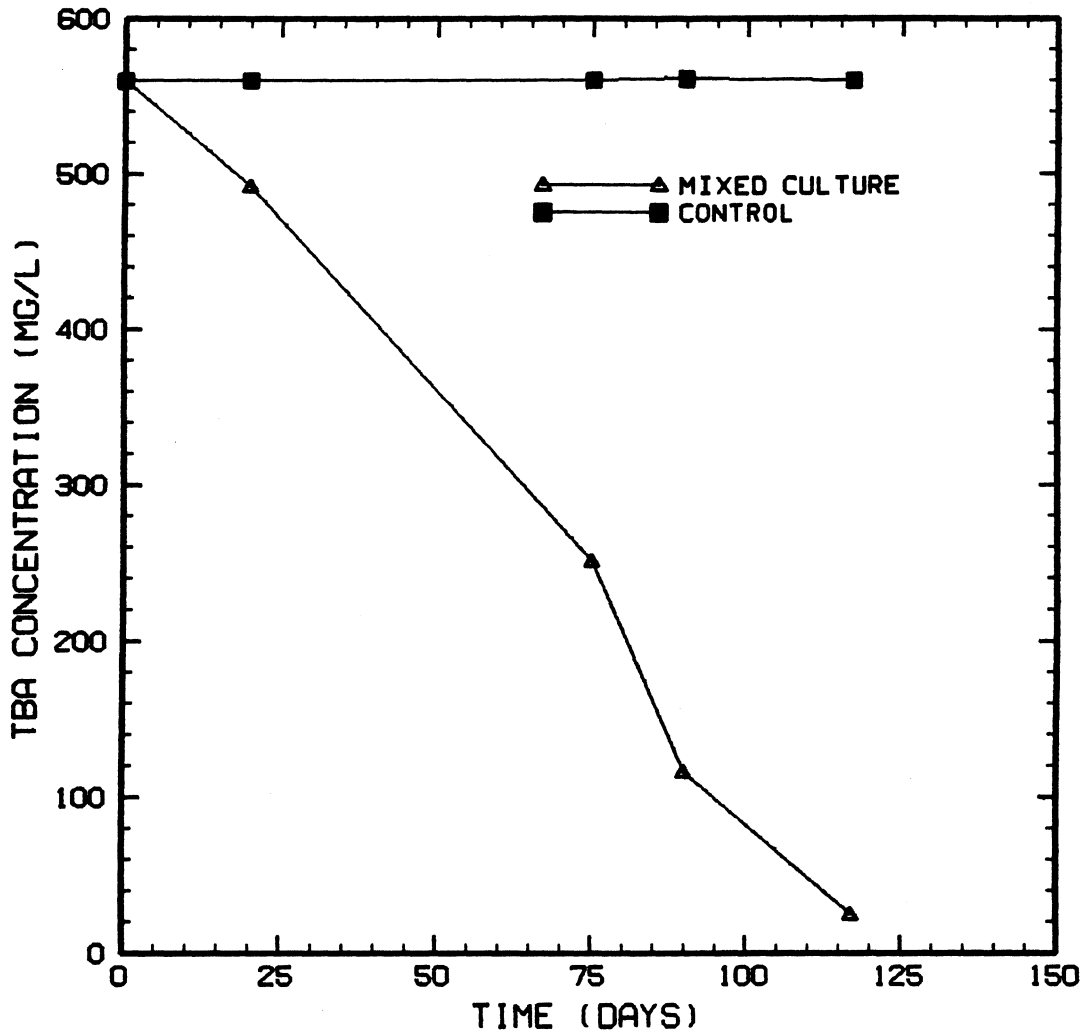


Figure 2. Aerobic degradation of TBA by an enrichment culture in a basal salts medium with TBA as the sole carbon source. Philadelphia soil was the inoculum. The maximum degradation rate observed was 9.0 mg TBA/liter/day. The control is an uninoculated broth treated identically to the enrichment culture.

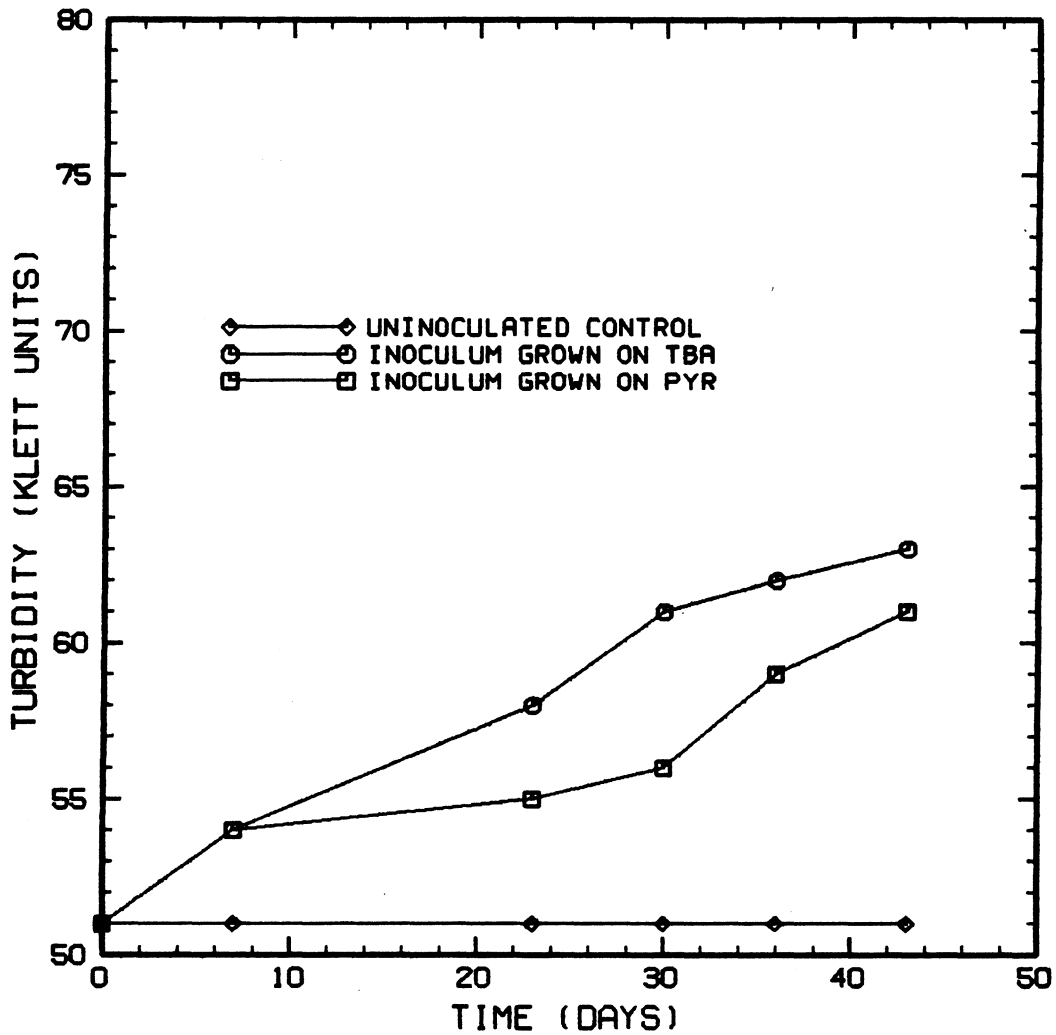


Figure 3. Growth of *Pseudomonas* sp. VT100 in a basal salts medium using TBA as sole carbon source measured on a Klett-Summerson colorimeter using a blue filter. The inoculum of one treatment was grown on TBA medium and the inoculum of the other treatment was grown on basal salts medium with Na pyruvate (PYR) as the carbon source. The initial viable count for each of these experiments was 5.2×10^5 CFU/ml and the final viable count was 2.7×10^7 CFU/ml for the circles and 1.9×10^7 CFU/ml for the squares.

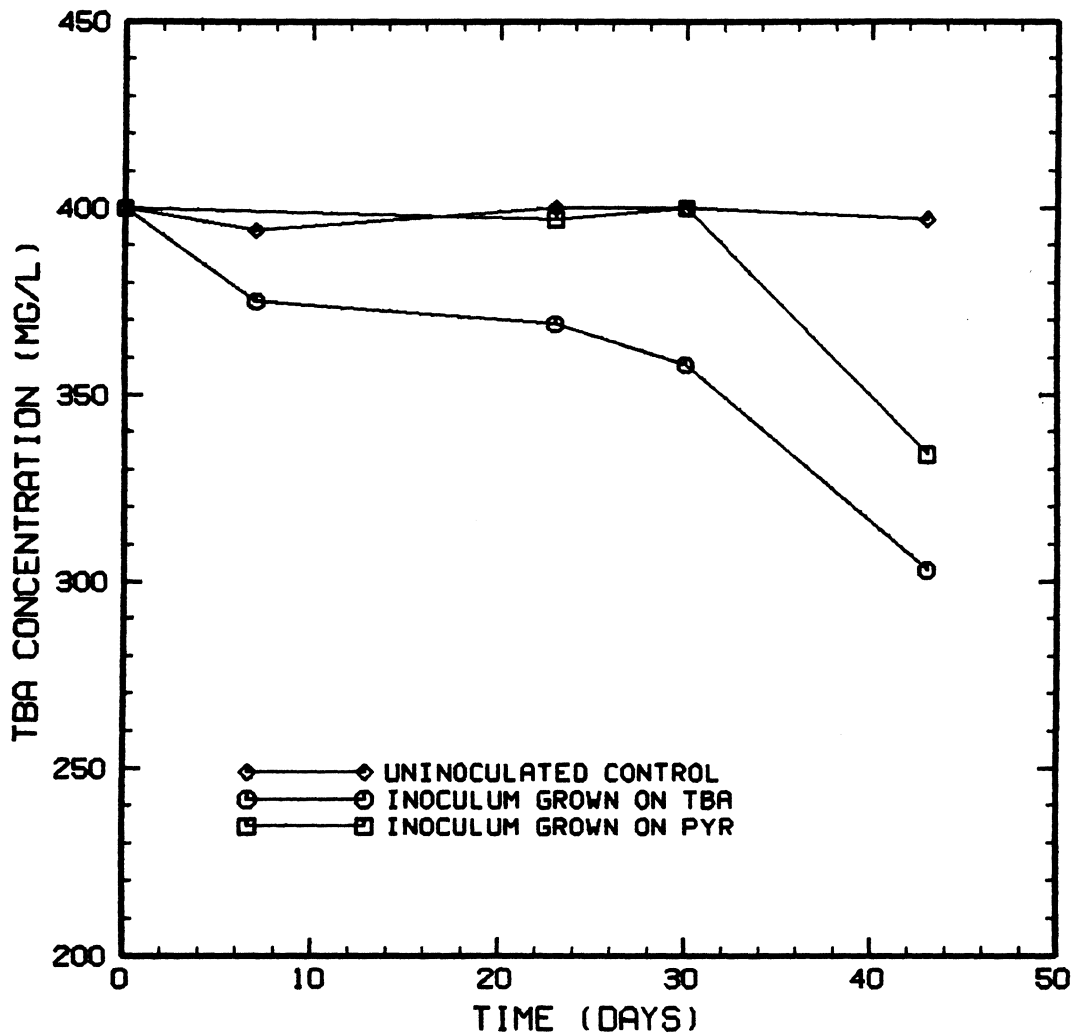


Figure 4. Degradation of TBA by Pseudomonas sp. VT100 when inoculum was grown on a basal salts TBA medium or basal salts pyruvate medium (PYR). This figure corresponds to figure 3. A thirty day lag period can be noted in growth (fig 3) and degradation (above) when VT100 is grown on an alternate carbon source and then reintroduced into Basal salts medium + TBA.

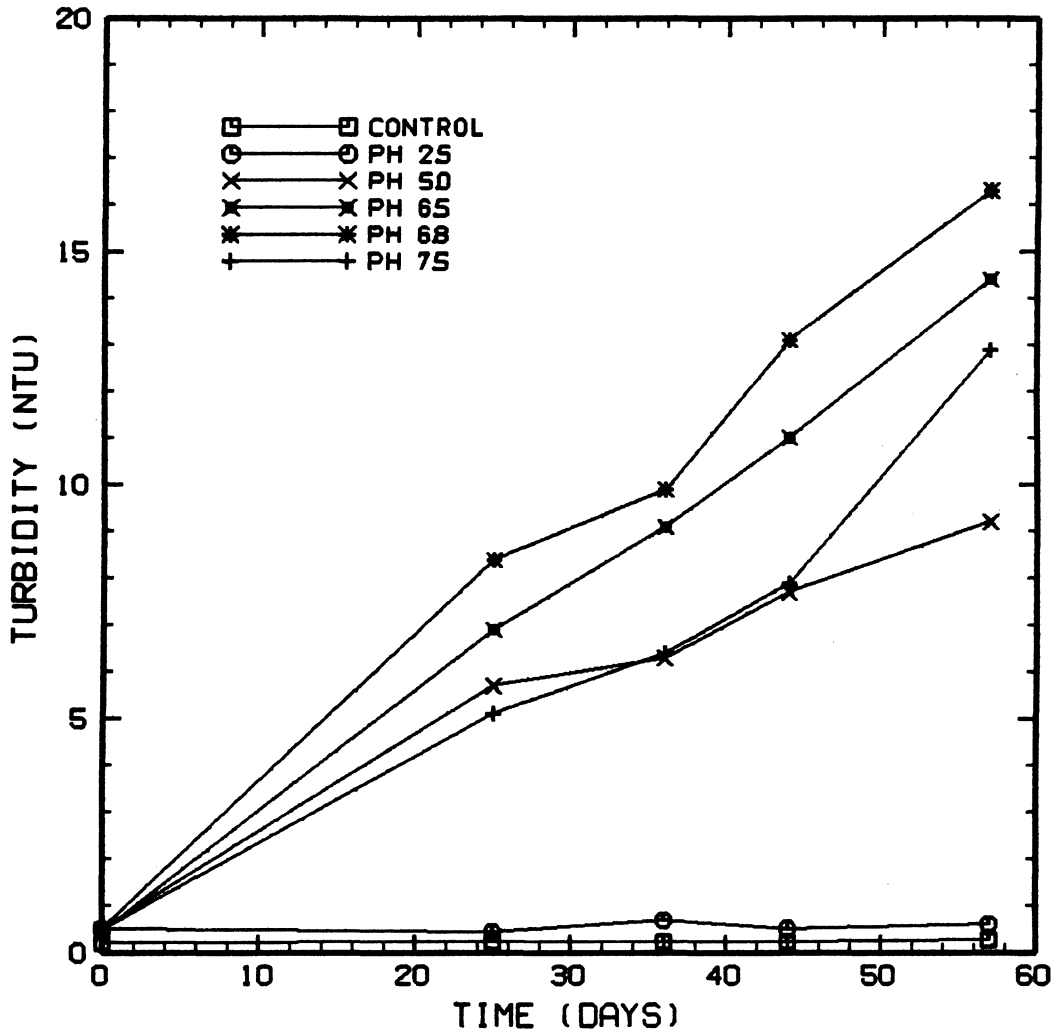


Figure 5. Effects of pH on the growth of *Pseudomonas* sp. VT100 in a basal salts medium with TBA as the sole carbon source. Growth is measured in Nephelometric Turbidity Units (NTU).

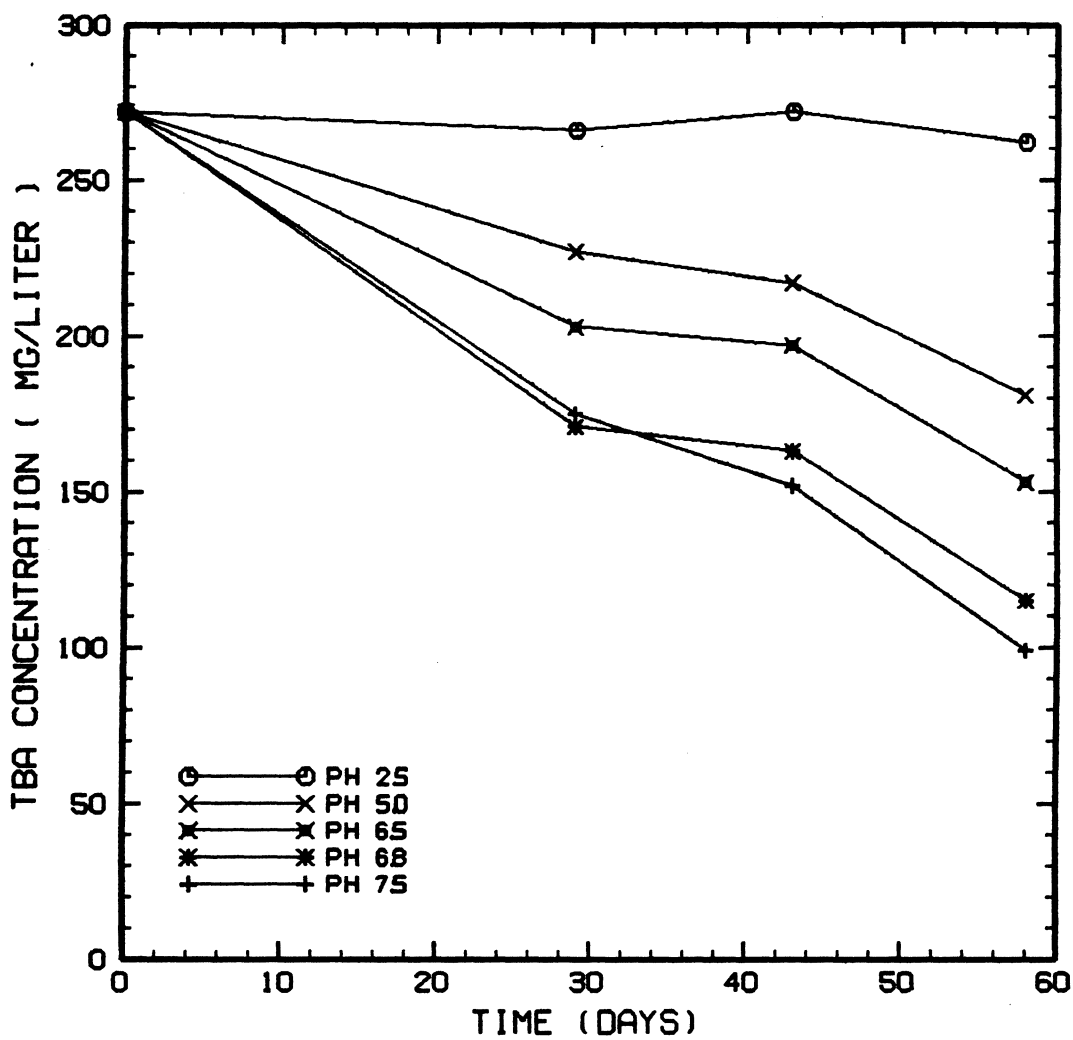


Figure 6. Effect of pH on the degradation of TBA by Pseudomonas sp. VT100 in a basal salts medium with TBA as the sole carbon source. This figure corresponds to figure 5.

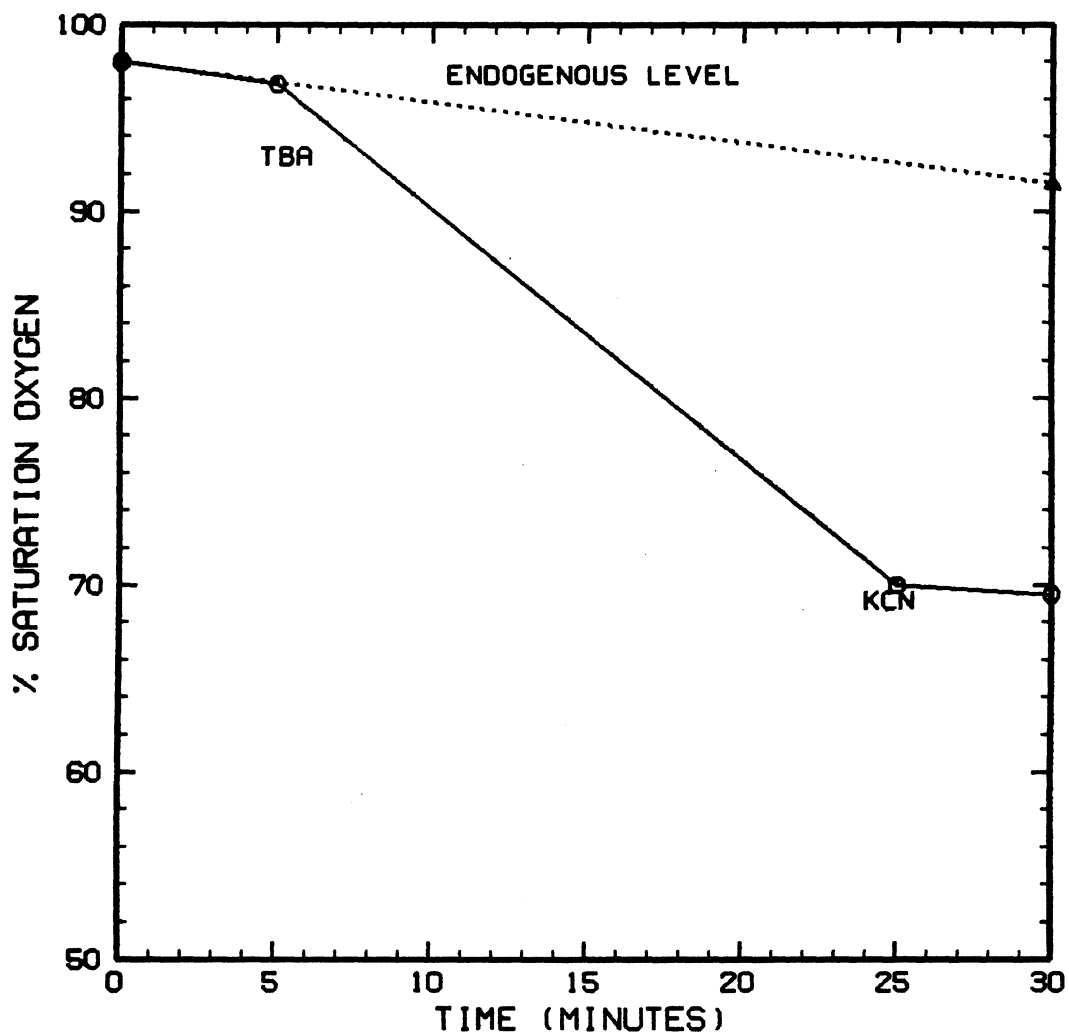


Figure 7. Rates of oxygen uptake by *Pseudomonas* sp. VT100 in a basal salts medium with TBA as the sole carbon source. TBA was added at 5 minutes and KCN was added at 25 minutes. Superimposed at the top (dashed line) is the endogenous rate of O₂ uptake.

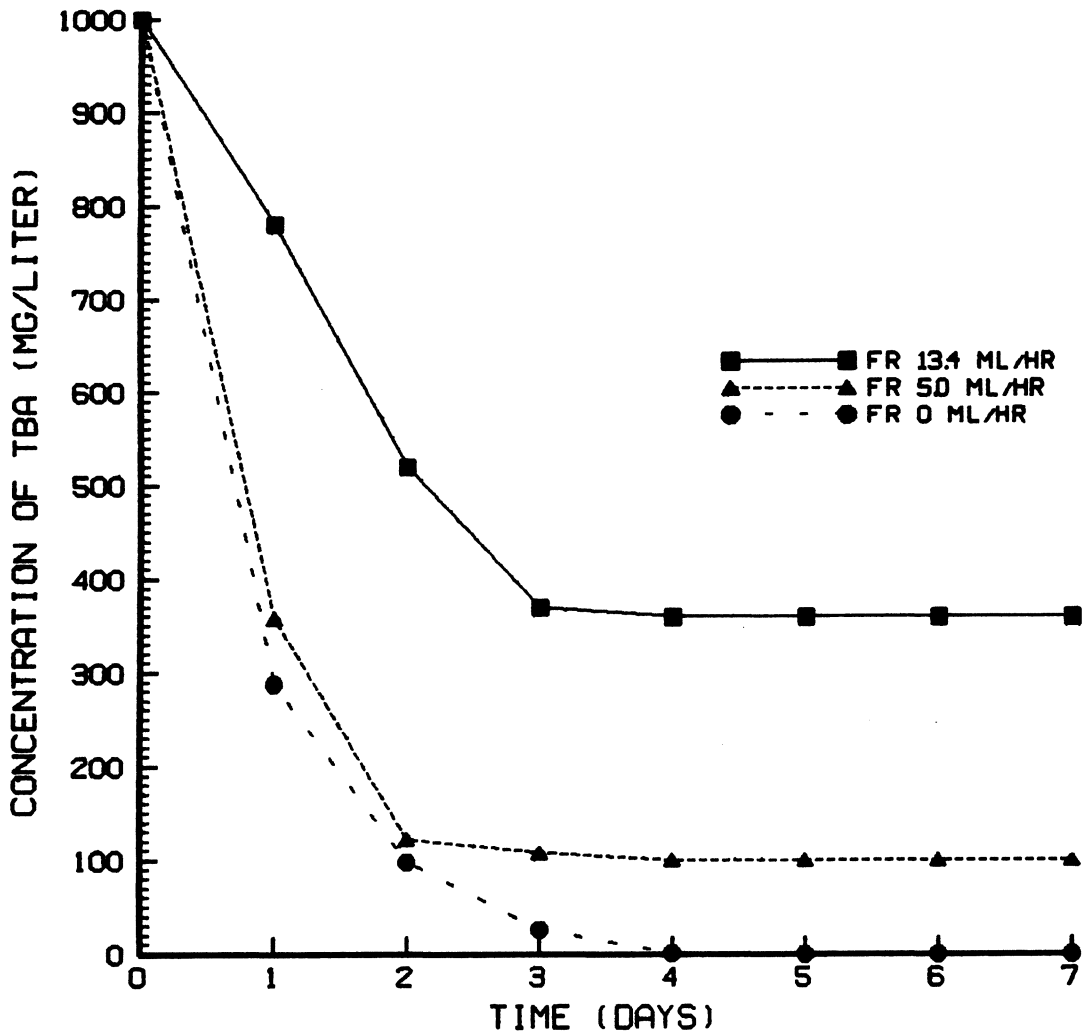


Figure 8. Diagram illustrating the nonlinear rate of volatilization of TBA due to the aeration of the control chemostat. The TBA concentration goes to zero if the flow rate (FR) is zero. When the flow rate is 5.0 ml/hr the TBA reaches equilibrium at a concentration of 100 mg/liter. When the flow rate is increased to 13.4 ml/hr the equilibrium shifts so that the concentration of TBA in the culture vessel is 380 mg/liter. Over long periods of time the equilibrium concentration becomes increasingly variable due to fluctuations in barometric pressure and aeration rate.

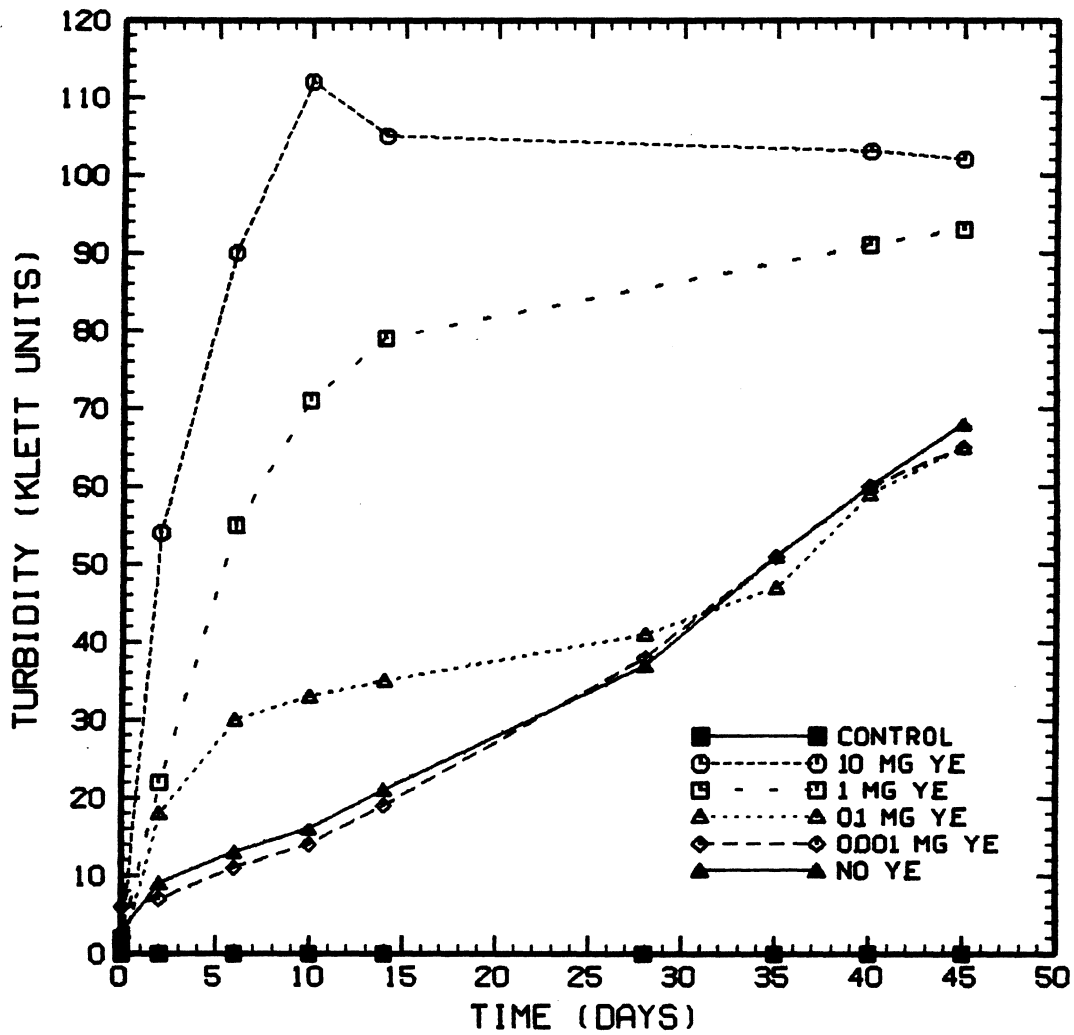


Figure 9. Growth of *Pseudomonas* sp. VT100 measured on a Klett-Summerson colorimeter. There was 500 mg of TBA in each treatment and varying concentrations of yeast extract (YE). Concentration of YE were in mg/ml.

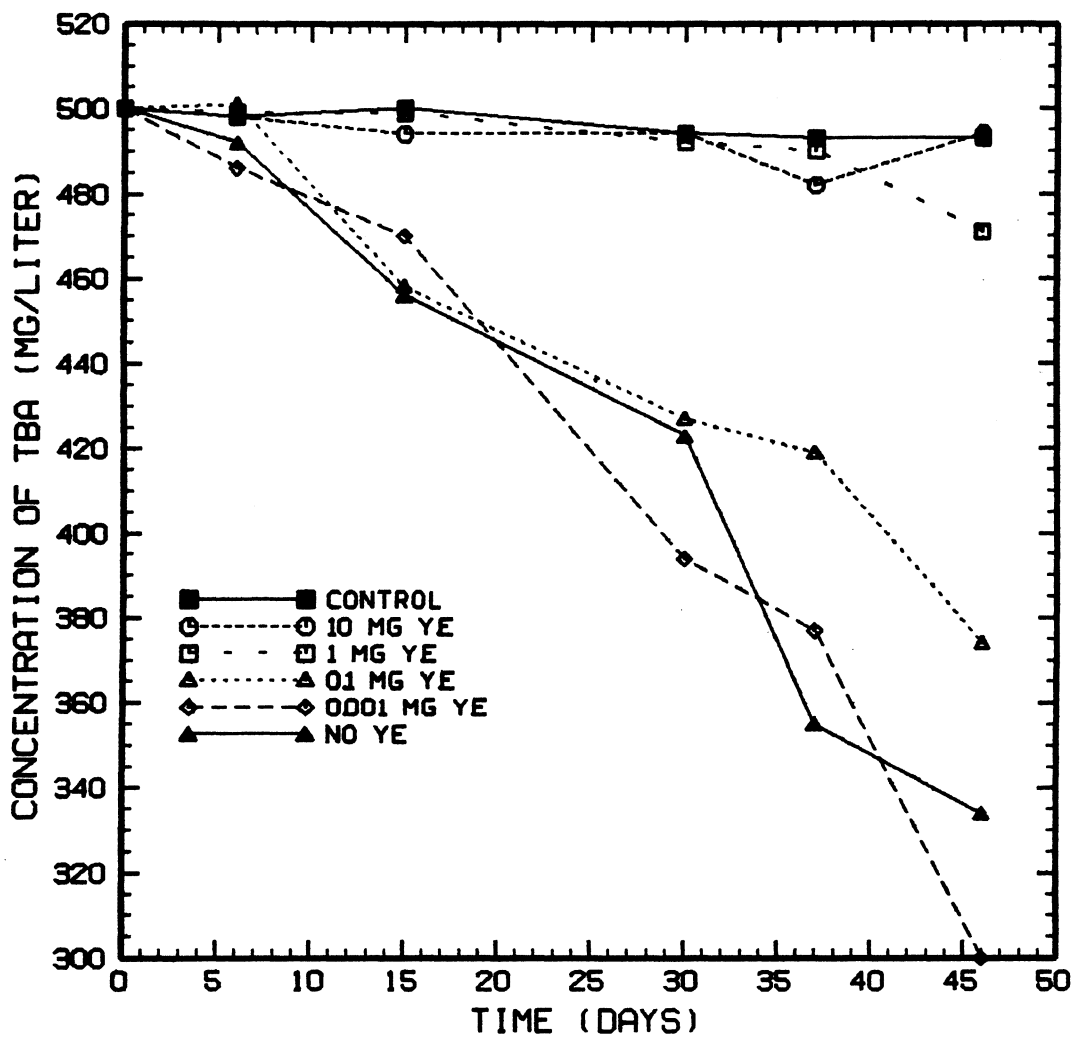


Figure 10. Degradation of TBA by *Pseudomonas* sp. VT100 in a basal salts medium with varying concentrations of yeast extract (YE) available as an alternate carbon source. The YE concentration is in mg/ml. This figure corresponds to figure 9.

DISCUSSION

Pseudomonas sp. VT100 was able to degrade tertiary butyl alcohol as a sole carbon and energy source. Although several investigators (3,9,11,18,26) have demonstrated biodegradation in mixed culture, this is the first report of a pure culture, to date, which degrades TBA. There is evidence for a natural enrichment culture in the refinery groundwater. A TBA degrading population of 4.9×10^3 organisms/g (dry wt) was measured in the original samples of the Philadelphia groundwater material. Other noncontaminated soils had less than two organisms/g (dry wt) using the MPN method. All enrichments and isolations were done using the 50 ft sample from Philadelphia. The VT100 strain utilized oxygen in a Clark cell when TBA was added as the sole carbon source. The Q_{O_2} was 4.2 ul O_2 /mg dry wt/hour (18.3 ul O_2 /mg protein/hour) and the bacteria had a doubling time of 33.6 hours at 25° C in continuous culture. Comparatively, this was a significant rate. The Q_{O_2} for many recalcitrant compounds have been calculated for pure cultures. Spain et al. (22a) calculated the rate of oxygen uptake for Pseudomonas sp. JS6 to be 3.78 ul O_2 /mg protein/hour when 1,2-Dichlorobenzene (1,2-DCB) was used as the sole carbon source. The doubling time (g) was 5.0 hours for JS6 when grown on 1,2-DCB at 30° C. The doubling time was 7 times faster for JS6 than for VT100, however, the rate of O_2 uptake was nearly 5 times greater for VT100 than for JS6.

The optimum pH for growth of VT100 and degradation of TBA was 7.0. The rate of degradation and growth decreased as the pH of the medium became more acidic. Media below pH 4.0 did not support the degradation of TBA by VT100. We have no evidence that the pH drop is

due to an intermediate acidic organic compound.

The effect of yeast extract on degradation of TBA and growth of VT100 varied with the concentration of yeast extract (YE). No degradation was observed in the 10 mg/ml treatment, although, the highest growth rate was observed in this treatment. VT100 preferred YE as a carbon source to TBA in the 10, 1, and 0.1 mg/ml YE treatments for the first thirty days of incubation. A diauxy effect was observed in the 0.1 mg/ml treatment (figure 9) suggesting TBA degradation may be subject to a regulatory mechanism. A slow basal level of degradation was observed in the 0.1 mg/ml YE treatment starting at time zero which may be decreasing the sharpness of the diauxy effect observed. The basal degradation may have occurred because the inoculum of VT100 used in this experiment was grown in continuous culture with TBA as the sole carbon source. We hypothesize that a high level of TBA degrading enzyme(s) existed in the cells so that an endogenous level of TBA degradation was observed even though YE was available. In addition, the concentration of YE (0.1 mg/ml) may not have been in sufficient excess to completely shut down synthesis of TBA degrading enzyme(s).

The growth rates of VT100 in the TBA + YE experiment decreased as concentrations of YE decreased except for YE concentrations below 0.1 mg/ml. Growth rates for these cultures were equal within the standard deviations. Degradation rates were slow or nonexistent for high concentrations of YE and equal within the standard deviations for cultures with concentrations below 0.1 mg/ml YE. The 0.001 mg/ml YE treatment culture showed a slight stimulation of TBA degradation but was not statistically significant.

The doubling time of VT100 in BMA+TBA was reduced by 3.5 times when grown in continuous culture compared to batch culture. Therefore, the chemostat was a superior method of culturing Pseudomonas sp. VT100 in a defined medium with TBA as the sole carbon source. Variations in aeration rate occurred throughout the long term study because inexpensive aquarium pumps were used which did not always deliver air at a constant rate. Changes in barometric pressure also caused increased variability in continuous culture TBA concentrations. Because of these variables, degradation rates obtained from continuous cultures could not be accurately compared to degradation rates observed in batch culture. However, a correlation between degradation and growth (turbidity) was observed throughout this study. Therefore, it is our hypothesis that the degradation rate of TBA by VT100 is greater in continuous culture than in batch culture.

Several factors probably contribute to the efficacy of the continuous culture method. First, the medium was constantly aerated so that oxygen was abundant for respiratory metabolism of TBA. In batch culture the medium was aerated intermittently so that oxygen could have been a limiting nutrient. Secondly, the chemostat had fresh medium flowing into the culture vessel which had a two fold effect; it permitted better buffering for the acidity produced during TBA degradation (the pH equilibrated at pH 6.2) and allowed for a constant concentration of TBA for biodegradation. The batch culture method does not permit these optimum conditions for culture growth and TBA degradation.

CONCLUSION

A Pseudomonas sp. VT100 was isolated from soil at the 50 ft depth of the subsurface of gasoline refinery which could use tertiary butyl alcohol (TBA) as an energy source. The Most Probable Number of TBA decomposers of this soil was 4.9×10^3 /g soil, and long term enrichment culture techniques were used for culture isolation. A defined mineral salts-TBA (500 mg/l) medium was used to grow the bacterium in batch culture at a maximum decomposition rate of 3.5 mg TBA/l/day at an optimum pH of 7.0. Yeast extract stimulates the growth of the bacterium in this medium, but it does not stimulate TBA decomposition at low concentrations and it inhibits the decomposition of TBA at high concentrations. When the culture inoculum was not grown in the TBA medium, the lag growth phase of the culture increased. The generation time of this Pseudomonas sp. VT100 was 112 h and 33.6 h when grown in batch and continuous culture, respectively. The chemostat is a superior method for growing bacteria on this substrate. This is the first report of a bacterium which can use TBA as an energy source in pure culture.

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APPENDIX 1

(Anaerobic TBA Enrichments)

Methods

The medium used for anaerobic enrichments had the following composition (per liter of distilled deionized water): NaEDTA, 20 mg; FeSO₄, 12 mg; MgSO₄·7 H₂O, 200 mg; CaCl₂·2 H₂O, 75 mg; NaCl, 1.0 g; (NH₄)₂SO₄, 1.0 g; thiamine HCl, 1.0 mg; and biotin, 1 mg. Two ml of sterile stock phosphate buffer was added aseptically after autoclaving. The phosphate buffer stock consisted of (per 200 ml distilled deionized water): KH₂O₄, 1.6 g and K₂PO₄, 2.4 g (1). This anaerobic mineral salts basal solution and the phosphate buffer stock solution were prepared according to the V.P.I. and S. U. Anaerobic Laboratory Manual (10). One ml of a filter sterilized trace element solution (1) was added aseptically and anaerobically to 1 liter of the above after autoclaving and mixing. This complete basal solution will be referred to as BMB. TBA was added to BMB to a concentration of 500 mg/l of TBA in the medium. The complete medium was used for the growth of all anaerobic bacteria tested in this study and will be referred to as BMB+TBA medium.

Anaerobic enrichments were inoculated, incubated and transferred in the anaerobic glove box (Coy Laboratory Products, Inc, Ann Arbor, Michigan). Ten g of subsurface soil was added to sterile 500 ml and 1000 ml sterile Erylenmeyer flasks with loose butyl rubber stoppers and placed in the glove box. Two hundred and 400 ml of sterile BMB+TBA medium was added to the respective flasks. The flask stoppers had butyl rubber septum ports to allow for aseptic and anaerobic transfer of cultures with sterile 10 ml syringes (Becton-Dickinson and Co., Rutherford, NJ). The enrichments were incubated at 25°C in the glove box for 2 years. An enrichment culture for denitrifying TBA degraders was initiated by substituting 1 g (NH₄)₂SO₄

in the BMB+TBA with 1 g KNO_3 . This enrichment was treated in the same manner as the other anaerobic enrichments. Strict anaerobic conditions were maintained throughout the experiment. All enrichment cultures were tested for loss of TBA by gas chromatographic analysis. Cultures were selected for further analysis based upon culture turbidity and TBA utilization.

Results

No degradation of TBA was observed in any anaerobic enrichments with either KNO_3 or $(\text{NH}_4)_2 \text{SO}_4$ even after two years of incubation under strict anaerobic conditions. See table below. All MPN enrichments yielded bacterial estimates of < 2 organisms/g soil (dry wt) capable of degrading TBA.

Table of anaerobic enrichment degradation of TBA as a sole carbon source.

| Time | Control | ELECTRON ACCEPTOR | |
|------|------------------|-------------------|-------------------------------|
| | | KNO_3 | $(\text{NH}_4)_2 \text{SO}_4$ |
| days | [TBA] mg / liter | | |
| 0 | 536 ± 16 | 491 ± 1 | 451 ± 23 |
| 14 | 536 ± 16 | 480 ± 2 | 490 ± 27 |
| 18 | 536 ± 16 | 472 ± 8 | 481 ± 5 |
| 35 | 536 ± 16 | 434 ± 35 | 471 ± 10 |
| 60 | 536 ± 16 | 467 ± 5 | 518 ± 31 |
| 105 | 536 ± 16 | 447 ± 21 | 500 ± 79 |
| 462 | 536 ± 17 | ND | 486 ± 12 |
| 644 | 536 ± 16 | 457 ± 22 | 482 ± 32 |

ND = not determined

APPENDIX 2

(Calculations)

Oxygen uptake

The oxygen electrode was calibrated according to the method of Robinson and Cooper (21). The volume of the Clark cell was 1.7 ml. Fifty μ l of the TBA stock solution (3.4 g/l) was added to the Clark cell with a gas tight Hamilton syringe through an entry port in the ground glass stopper so that the final concentration of TBA in the cell at time zero was 100 μ g/ml. Oxygen uptake was calculated as follows: (i) the decrease in percent saturation of O_2 over time was recorded for cells with and without substrate. These values were converted from percent O_2 to μ l O_2 consumed per minute to rate of oxygen uptake per hour by dividing the dry weight of the cells to give oxygen uptake/hr/mg dry cell weight Q_{O_2} . A value was also calculated using the total protein giving O_2 uptake/hr/mg protein; and (ii) the endogenous value was subtracted from the substrate to calculate the oxygen uptake/hr/mg dry cell weight with TBA as sole carbon source.

Growth rates

Batch culture. The generation time was calculated using the formula $g = 0.693 / a$; where $a = (\log N - \log N_0) 2.303 / t - t_0$; N = number of cells at time "t"; and N_0 = number of cells at time "t₀"; and $t-t_0$ = the change in time.

Continuous culture. The growth rate for Pseudomonas sp. VT100 was calculated using the formula $g = 0.693 / a$. Where $a = d$ the dilution rate. The doubling time was 33.64 hours (1.4 days).

APPENDIX 3

NaMoO₄ Experiment

Methods

An observation was made in the microcosm work that NaMoO_4 enhanced degradation of TBA (Novak, personal communication). From this observation several hypotheses were developed, one of which was that molybdate is needed for a "TBAase" enzyme to be active. To test the necessity of NaMoO_4 in pure culture, varying concentrations were added to BMA+TBA and growth and degradation rates were measured. BMA+TBA was prepared, filter sterilized, and 8.5 ml were dispensed into each of twenty-eight 16 mm screw cap test tubes. Serial dilutions of a 1 M NaMoO_4 solution were made in 9 ml BMA dilution blanks. The concentrations of NaMoO_4 in the dilutions were as follows: AM - 1 M, BM - 0.1 M, CM - 0.01 M, DM - 0.001 M, EM - 0.0001 M, and FM - 0.00001 M. One ml of each dilution was added to each of 4 replicate tubes of BMA and the series was designated by the dilution letter (i.e. 4 tubes labeled AM had 0.1 M NaMoO_4 , note that this is 0.1 times that of the dilution because it is effectively another 1:10 dilution). A final 4 tube series labeled GM had 1 ml BMA added to each tube. This was a control containing only the basal amount of NaMoO_4 from the trace element solution. Three tubes in each series were replicates, the fourth tube was an uninoculated control. One half an ml of a washed cell suspension of Pseudomonas sp. VT100 was added to the three replicates in each series. One half an ml of BMA was added to to the control in each series bringing the final volume in each tube to 10 ml. The tubes were capped tightly to limit the loss of TBA by volatilization. The caps were loosened and the tubes vortexed for aeration. The optical density and concentration of TBA in the medium were measured.

Results

The NaMoO_4 did not appear to affect the degradation of TBA in this experiment except at high concentrations. The 0.1 M treatment was inhibitory for growth and degradation.

Discussion

This experiment was filled with problems. The hypothesis was not tested at concentrations which would have been differential for a cofactor limiting system. In addition, BMA had a small amount of NaMoO_4 in it from the trace element solution which was added.

Recommendations

This experiment should be repeated using the proper (very low) used to enable the detection of very slight stimulatory or inhibitory effects within statistical significance. Finally, the experiment should be performed under anaerobic conditions as well to test an alternate hypothesis. In fact, the hypothesis which was really more appropriate for the original observation (which was made on anaerobic microcosms). The alternate hypothesis was that NaMoO_4 inhibited the reduction of sulfate by sulfate reducing bacteria and thus shunted their metabolism towards TBA metabolism.

APPENDIX 4

Data for Figures

DATA

Table 1a. Data for figure 2, aerobic degradation rate of TBA in mixed culture.

| Time (days) | control | mixed culture |
|-------------|---------|---------------|
| 0 | 560 | 560 |
| 20 | 560 | 492 |
| 75 | 560 | 251 |
| 90 | 561 | 116 |
| 117 | 560 | 25 |

Table 2a. Data for figure 3, growth of VT100 on BMA+TBA when inoculum grown on TBA or PYR.

| Time days | Control | TBA Grown | PYR Grown |
|-----------|---------|-----------|-----------|
| 0 | 51 | 51 | 51 |
| 7 | 51 | 54 | 54 |
| 23 | 51 | 58 | 55 |
| 30 | 51 | 61 | 56 |
| 36 | 51 | 62 | 59 |
| 43 | 51 | 63 | 61 |

Table 3a. Data for figure 4, Degradation rate of TBA by VT100 with inoculum grown on TBA and PYR.

| Time days | Control | TBA Grown | PYR Grown |
|-----------|---------|-----------|-----------|
| 0 | 400 | 400 | 400 |
| 7 | 394 | 375 | ND |
| 23 | 400 | 369 | 397 |
| 30 | 400 | 358 | 400 |
| 43 | 397 | 303 | 334 |

Table 4a. Data for figure 5, growth of VT100 at varying pH.

| pH of medium | 2.5 | 5.0 | 6.5 | 6.8 | 7.5 | |
|--------------|---------|-------------------------------|-----|------|------|------|
| time days | control | NEPHELOMETRIC TURBIDITY UNITS | | | | |
| 0 | 0.21 | 0.5 | 0.5 | 0.4 | 0.5 | 0.5 |
| 25 | 0.23 | 0.43 | 5.7 | 6.9 | 8.4 | 5.1 |
| 36 | 0.23 | 0.69 | 6.3 | 9.1 | 9.9 | 6.4 |
| 44 | 0.23 | 0.51 | 7.7 | 11.0 | 13.1 | 7.9 |
| 57 | 0.27 | 0.61 | 9.2 | 14.4 | 16.3 | 12.9 |

Table 5a. Data for figure 6, degradation of TBA by VT100 at varying pH.

| pH of medium | 2.5 | 5.0 | 6.5 | 6.8 | 7.5 |
|--------------|-----|-----|------------------|-----|-----|
| time control | | | | | |
| days | | | | | |
| | | | [TBA] mg / liter | | |
| 0 | 272 | 272 | 272 | 272 | 272 |
| 29 | 266 | 283 | 227 | 203 | 171 |
| 43 | 272 | 289 | 217 | 197 | 163 |
| 58 | 262 | 239 | 181 | 153 | 115 |

Table 6a. Data for figure 8, illustration of nonlinear volatilization rate of TBA in a chemostat.

| time control | days | FLOW RATE ml/h | | |
|--------------|------|------------------|------|------|
| | | 0 | 5 | 13.4 |
| reservoir | | [TBA] mg / liter | | |
| 0 | 1000 | 1000 | 1000 | 1000 |
| 1 | 1000 | 288 | 358 | 780 |
| 2 | 1002 | 96 | 122 | 520 |
| 3 | 998 | 26 | 108 | 370 |
| 4 | 1000 | 1 | 100 | 360 |
| 5 | 994 | 0 | 100 | 360 |
| 6 | 996 | 0 | 100 | 360 |
| 7 | 986 | 0 | 100 | 360 |

Table 7a. Data for figure 9, growth of VT100 in yeast extract experiment.

| TIME days | Milligrams Yeast Extract / ml | | | | | | |
|--------------|-------------------------------|------|------|------|-------|--------|-------------|
| | 10.0 | 1.0 | 0.1 | 0.01 | 0.001 | 0.0001 | 0 (no y.e.) |
| | KLETT UNITS | | | | | | |
| 0 | 1±1 | 2±1 | 0±1 | 4±3 | 6±1 | 5±2 | 3±1 |
| 2 | 54±4 | 22±1 | 18±1 | 9±1 | 7±1 | 7±1 | 9±2 |
| 6 | 90±5 | 55±2 | 30±1 | 13±1 | 11±2 | 12±1 | 13±2 |
| 10 | 112±5 | 71±1 | 33±2 | 15±2 | 14±2 | 14±1 | 16±3 |
| 14 | 105±10 | 79±3 | 35±1 | 19±2 | 19±2 | 19±3 | 21±2 |
| 28 | ND | ND | 41±1 | 35±7 | 38±5 | 36±5 | 37±5 |
| 35 | ND | ND | 47±1 | 44±9 | 51±4 | 46±8 | 51±7 |
| 40 | 103±9 | 91±1 | 59±8 | 51±8 | 60±4 | 55±8 | 60±7 |
| 45 | 102±10 | 93±1 | 65±6 | 53±5 | 65±4 | 60±9 | 68±8 |

ND not determined

Table 8a. Data for figure 10, degradation rates of TBA in response to different concentrations of yeast extract.

| TIME days | Milligrams Yeast Extract / ml | | | | | | |
|--------------|-------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | 10.0 | 1.0 | 0.1 | 0.01 | 0.001 | 0.0001 | 0 (no y.e.) |
| | [TBA] mg / liter | | | | | | |
| 0 | 500 ± 5 | 500 ± 5 | 500 ± 5 | 500 ± 5 | 500 ± 5 | 500 ± 5 | 500 ± 5 |
| 6 | 498 ± 5 | 499 ± 4 | 501 ± 6 | 496 ± 3 | 486 ± 11 | 489 ± 8 | 492 ± 5 |
| 15 | 494 ± 13 | 499 ± 6 | 458 ± 13 | 490 ± 2 | 470 ± 2 | 472 ± 15 | 456 ± 10 |
| 30 | 494 ± 12 | 492 ± 7 | 427 ± 6 | 444 ± 9 | 394 ± 8 | 439 ± 7 | 423 ± 6 |
| 37 | 482 ± 15 | 461 ± 51 | 419 ± 11 | 462 ± 12 | 377 ± 6 | 422 ± 8 | 355 ± 18 |
| 46 | 494 ± 14 | 471 ± 18 | 374 ± 32 | 375 ± 25 | 300 ± 26 | 382 ± 7 | 334 ± 23 |

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