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Biodegradation of aromatic compounds in the presence of secondary substrates

Vogel, Catherine Mary, M.S.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

BIODEGRADATION OF AROMATIC COMPOUNDS IN THE PRESENCE OF SECONDARY SUBSTRATES

by

Catherine Vogel

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APPROVAL BY THE THESIS DIRECTOR

This thesis has been approved on the date shown below:

B. E. Logan

B.E. Logan
Assistant Professor of
Civil Engineering and
Engineering Mechanics

4-24-91
Date

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ABSTRACT

Experiments were conducted to examine the biodegradability of three aromatic compounds typically found at groundwater contamination sites; benzene, toluene, and chlorobenzene. A pure *Pseudomonas* species, JS6, was used in all batch experiments. JS6 was grown on benzene, chlorobenzene, toluene, yeast extract, or glucose as sole source of carbon and energy. These cultures were in turn used to test the biodegradability of the three aromatics of interest both in the presence and absence of the chemical used for acclimation. The results indicated that the presence of a non-aromatic substrate (yeast extract or glucose) enhanced the biodegradation of the aromatic compound of interest. Results also indicated JS6 has the ability to simultaneously degrade two aromatic compounds when the following mixtures were tested; toluene and benzene, toluene and chlorobenzene, and chlorobenzene and benzene. Enzyme induction experiments confirmed that the enzymes required for the initial breakdown of the aromatic compounds tested are inducible, not constitutive.

Chapter 1

Introduction

1.1 Motivation

Petroleum hydrocarbon contamination of soil and groundwater is a widespread problem in the United States. Nearly fifty percent of the contamination sites on Air Force installations in the United States can be attributed to jet fuel spills or leaks. This percentage is increasing as new contamination sites are discovered through the inspection of underground oil and gas storage systems (Downey and Elliot, 1989).

JP-4 is the primary aircraft fuel used by the Air Force (Bishop et al., 1983). Approximately 3.2 billion gallons of JP-4 are used per year. At any given time, there can be up to 20.1 billion gallons of JP-4 stored on U.S. Air Force installations (Downey, 1989). It is not surprising that a JP-4 contamination problem exists with this volume of fuel continuously being transported and used.

JP-4 is a complex blend of up to 300 different components ranging from C4 to C16 hydrocarbon compounds; JP-4 can contain up to 25% aromatics by volume (Bishop et al., 1983). The aromatic constituents are the main concern due to their severe health effects and the strict environmental regulations placed upon them. Benzene, which

is the most hazardous component of JP-4 due to its toxicity, has been classified as a human carcinogen by the International Agency for Research on Cancer (Arthur D. Little, Inc., 1985).

The Environmental Protection Agency (EPA) has assigned a Maximum Contaminant Level Goal of zero for benzene (Code of Federal Regulations 40, 1987). The Safe Drinking Water Act, as amended in 1986, requires the EPA to regulate 83 new drinking water contaminants (Federal Register, 1988). This group of chemicals includes benzene, toluene, and xylene which are aromatic constituents of JP-4. Table 1.1 is a list of the most frequently seen chemicals at groundwater contamination sites identified by the Air Force's Installation Restoration Program (IRP). The high concentrations found for the aromatic components of JP-4 illustrate the severity of the groundwater contamination problem.

The restoration techniques most commonly used to remediate jet fuel contamination sites include physical containment and removal of the contaminated material for subsequent physical treatment. A contamination plume may be isolated by installing various barriers to groundwater flow such as slurry trench walls, infiltration controls and grout curtains. The plume may also be controlled through the use of passive groundwater interception systems or well pumping

CHEMICAL	CONCENTRATION RANGE (ug/L) LOW	HIGH
Chloroethane		2.4
Di-n-butyl phthalate	4.6	5.9
N-Nitrosodimethylamine		6
Bis(2-ethylhexyl) phthalate	3	8
Naphthalene	6	8
Trichlorofluoromethane	2.8	10.6
2,6-Dinitrotoluene		13
N-nitrosodiphenylamine		25
1,2,4-Trichlorobenzene	7	41
1,1,-Dichloroethylene	1.2	42
1,1,2,2-Tetrachloroethane		48.8
Diethyl Phthalate	2	52
1,2-Dichloroethane	1.8	58
Tetrachloroethylene	2.5	67
Carbon Tetrachloride	18	78
Bis(2-chloroethyl) ether	13	200
1,4-Dichlorobenzene	7.5	290
Xylene	250	310
1,2-Dichloropropane	2	460
2,4-Dimethylphenol	2	580
Chloroform	.4	810
Vinyl Chloride	40.7	1190
1,1-Dichloroethane	1.8	1900
Chlorobenzene	1.7	4200
Ethylbenzene	3	7400
1,3-Dichlorobenzene	6.6	7900
1,1,1-Trichloroethane	1	12000
1,2-Dichlorobenzene	5	12700
1,2-Trans-dichloroethylene	0.5	15000
Benzene	62	25000
Toluene	5	80000
Trichloroethylene	5	130000
Methylene chloride	10	170000

Table 1.1: Identification of Groundwater Contaminants Through the Installation Restoration Program (IRP), Phase II Efforts (As of March 1984) (Reference: Installation Restoration Program, Information Management System (IRPIMS), Volume 2, Appendices. Prepared for the USAF OEHL, Brooks AFB TX by Weston. 1985.)

systems. Once the plume is contained, the contaminated soil can be excavated and transported to a hazardous waste landfill. Extremely high costs and site specific physical constraints usually prohibit the use of this disposal option (Lee et al., 1987).

The contaminated material can also be treated in situ or above ground by various physical treatment technologies. In situ soil venting and radio frequency thermal soil decontamination are two methods used for removing the more volatile contaminants from the soil. Soil venting involves the use of blowers to pull large volumes of air through the contaminated soil. The air flow causes volatilization of the contaminants and the resultant air stream is vented to the atmosphere. Radio frequency thermal soil decontamination involves the use of electromagnetic energy to heat the soil in situ and thereby volatilize the contaminants. Electrodes placed into the contaminated zone provide the energy to heat the soil, while vacuum pumps provide negative pressure on the treatment zone to pull the contaminated vapors above ground. Both of these remediation technologies have seen success in field applications (Downey and Elliot, 1989). A major drawback to these methods is that they are not destructive technologies. The contaminant is only being transferred from one phase to another. The

contaminated air stream that is generated may require further treatment depending on local air emission regulations.

Above ground treatment methods, such as air stripping and carbon sorption, are effective for removing contaminants from groundwater, but because these methods are not destructive techniques, they also create secondary hazardous waste streams. In the case of air stripping, the gas phase may require additional treatment, while the spent carbon from the carbon sorption process will require regeneration or disposal as a hazardous waste.

The development of a biological treatment technology for the treatment of JP-4 contamination would offer a potential solution to the drawbacks of the more frequently used physical treatment methods. Recent literature shows accumulating evidence that naturally occurring biota can transform xenobiotic compounds in the environment. Over 200 species of bacteria, yeasts and filamentous fungi able to degrade hydrocarbons compounds have been isolated. Bacteria are considered to be the primary degraders of hydrocarbons although yeasts and fungi can also contribute to their degradation. The most common bacterial hydrocarbon degraders include Pseudomonas, Achromobacter, Arthrobacter,Micrococcus, Nocardia, Vibrio, Acinetobacter,

Brevibacterium, Corynebacterium, and Flavobacterium (Carlson, 1981).

In a 1981 study conducted to determine the biodegradability of 96 Environmental Protection Agency (EPA) Priority Pollutants, nearly all of the compounds were degraded to some degree by a wastewater microbial inoculum (Tabak et al., 1981). Kobayashi and Rittmann (1982) provide a good summary of microbial species capable of degrading almost every class of man-made compounds.

Hydrocarbon mixtures, such as in gasoline or JP-4 have also proven to be readily degradable by indigenous microbial communities. Following an accidental gasoline spill in Ambler PA, the contaminated groundwater was supplemented with organic nutrients and oxygen. The major components of the gasoline were readily biodegradable. Similar results were obtained when gasoline-contaminated sediment from the Ohio River was studied. The adapted microbial community quickly biodegraded the petroleum hydrocarbons (Spain et al., 1983). The key to the biodegradation of even the most recalcitrant compounds is to provide the optimum environmental conditions to the microorganisms having the appropriate metabolic capabilities to accomplish the required degradation process.

The transition of current lab-scale biodegradation technology to pilot and full-scale field efforts will

require much additional information. The chemical structure of the contaminant, the environmental conditions and the physiology of the microorganisms must be addressed when evaluating the biodegradation of xenobiotic compounds. Often at field sites, a contamination plume will contain a wide variety of chemicals in varying concentrations. However, most current lab-scale research has concentrated on the degradation of a single substrate by a pure bacterial culture. Such work yields information about the catabolic pathways, pathway regulation and the genetics of the microorganisms but these results cannot easily be applied to field situations. Little information is available concerning how bacteria perform when faced with a diverse mixed waste stream. This research effort will specifically explore the effect of the presence of a secondary substrate on the degradation of aromatic hydrocarbons.

1.2 Research Objectives

The objective of this research was to examine the biodegradability of three aromatic compounds. Benzene, chlorobenzene, and toluene were studied due to their toxicity, frequent contribution to groundwater contamination problems and degree and type of halogenation. Benzene and toluene are constituents of JP-4 while the chlorinated

benzenes have been used extensively as solvents and degreasers by the Air Force.

A pure *Pseudomonas* species, desiginated JS6 (Spain and Nishino, 1987), was used in all the batch experiments. JS6 was grown on the following substrates as sole source of carbon and energy; benzene, chlorobenzene, toluene, glucose, and yeast extract. These cultures were in turn used to test the biodegradability of the three aromatics of interest both in the presence and absence of the chemical used for acclimation.

Chapter 2

Literature Review

2.1 Biodegradation of Non-Polar Aromatic Hydrocarbons

2.1.1 Source of Aromatic Hydrocarbons in the Environment

Aromatic hydrocarbons are among the most frequently found groundwater contaminants (Reinhard et al, 1984; Sawhney and Kozloski, 1984). Their presence in the environment can be traced back to both natural and man-made sources. Aromatic hydrocarbons have been found in lignin and its breakdown products, algae and plant extracts (Young, 1984). However, their occurance in the environment due to industrial operations is of much greater concern.

From 1940 to 1970, the production of synthetic organic chemicals increased from 2.5 million tons per year to 90 million tons per year. Over 200,000 new organic chemicals are being created per year in the world, and over a thousand of these will eventually be put to some commercial use. Although not all of these chemicals are aromatic compounds, the aromatic compounds are of the greatest concern (Young, 1984).

Over 70% of the supply of aromatic hydrocarbons originates from petroleum with the remainder coming from

coal and imports. The numerous industrial uses of aromatic hydrocarbons include being used as the starting material for the synthesis of plastics, paints, pesticides, dyes, and resins, its many uses as a solvent, and its use in aviation and automobile fuels (Young, 1984).

2.1.2 General Considerations for Anaerobic Biodegradation

Anaerobic conditions in the subsurface environment can commonly exist. When biological activity deplets the environment of oxygen, microbes will use other electron acceptors such as nitrate, sulfate or carbonate. Thermodynamics suggests that nitrate is the favored electron acceptor over sulfate or carbonate, and sulfate is favored over carbonate (Young, 1984).

In the absence of molecular oxygen, microorganisms have evolved different mechanisms for the breakdown of aromatic compounds. These mechanisms have only begun to be explored. However, recent research has shown that toluene and 1,3-xylene are rapidly mineralized by a *Pseudomonas* sp. under denitrifying conditions when nitrate or nitrous oxide is provided as the sole electron acceptor (Zeyer et al., 1990). Toluene and benzene were found to be mineralized anaerobically by mixed methanogenic cultures (Vogel and Grbic-Galic, 1986). Based on the intermediates detected, toluene and benzene degradation proceeded via an initial

anaerobic oxidation by ring hydroxylation or methyl oxidation which resulted in the production of phenol, cresols, or aromatic alcohols (Grbic-Galic and Vogel, 1987). Toluene has also been shown to be completely oxidized by the iron-reducing organism, GS-15. This is the first example of a microorganism coupling the oxidation of an aromatic compound to the reduction of Fe(III) and of a pure culture being capable of anaerobically oxidizing toluene (Lovley and Lonergan, 1990).

Recent research has proven that aromatic compounds can be anaerobically biodegraded. However, biodegradation occurs at a much slower rate under anaerobic conditions vs aerobic conditions. The anaerobic biodegradative process also often depends on an assemblage of different microorganisms to completely mineralize the aromatic parent compound. This is typically true when considering methanogenic cultures (Young, 1984).

2.1.3 General Considerations for Aerobic Biodegradaiton

The first demonstration of a bacterium capable of using aromatic hydrocarbons for growth occured in 1908 with the isolation of a *Bacillus hexacarbovorum* that could grow on toluene and xylene. In the 1920's, researchers examined 245 soil samples and found that over 60% of the samples contained bacteria capable of oxidizing aromatic compounds.

Since these first reports of aromatic hydrocarbon degradation, similarities have been seen in the breakdown pathways in all the cases examined (Gibson and Subramanian, 1984).

It is generally accepted that bacteria initiate the oxidation of an unsubstituted aromatic hydrocarbon by inserting one molecule of oxygen into the aromatic nucleus via a dioxygenase multienzyme system. This step is termed dihydroxylation and is a prerequisite for the fission of the benzene ring. Dihydroxylation produces a dihydrodiol in which the hydroxyl groups have a cis relative stereochemistry. The dioxygenase enzymes appear to be unique to prokaryotic organisms. They have not yet been found in any higher forms of life. (Gibson and Subramanian, 1984 and Gibson, 1968).

The cis-dihydrodiol is then oxidized via a pyridine nucleotide-linked dehydrogenase which leads to the formation of a catechol. Catechol has been shown to be the central intermediate in the degradation of many aromatic compounds including phenol, naphthalene, phenanthrene, anthracene, o-cresol and benzene (Gibson, 1968). These first two reactions caused by the dioxygenase and dehydrogenase enzymes have been observed in aromatic compounds ranging in size from benzene to benzo[a]pyrene (Gibson and Subramanian, 1984). The initial reactions involved in the metabolic

pathway for an unsubstituted aromatic hydrocarbon is shown in Figure 2.1.

Once the two hydroxyl groups are incorporated into the aromatic ring, there are two methods by which the aromatic nucleus can be cleaved via dioxygenase enzyme systems. Ring cleavage can occur between adjacent carbon atoms that carry hydroxyl groups. This is termed ortho or "intradiol" cleavage. The ring cleavage product is then metabolized by the ortho or "β-ketoadipate" pathway (Bayly and Barbour, 1984).

Ring cleavage can also occur between two carbon atoms, only one of which carries a hydroxyl group. The other carbon atom can either be unsubstituted or substituted with something other than a hydroxyl group. This type of ring cleavage is termed meta cleavage. The two hydroxyl groups on the aromatic ring can either be ortho or para to each other. If the two hydroxyl groups are ortho to each other, the ring cleavage product is metabolized by the meta pathway. If the two hydroxyl groups are para to each other, the ring cleavage product is metabolized by the gentisate pathway (Bayly and Barbour, 1984).

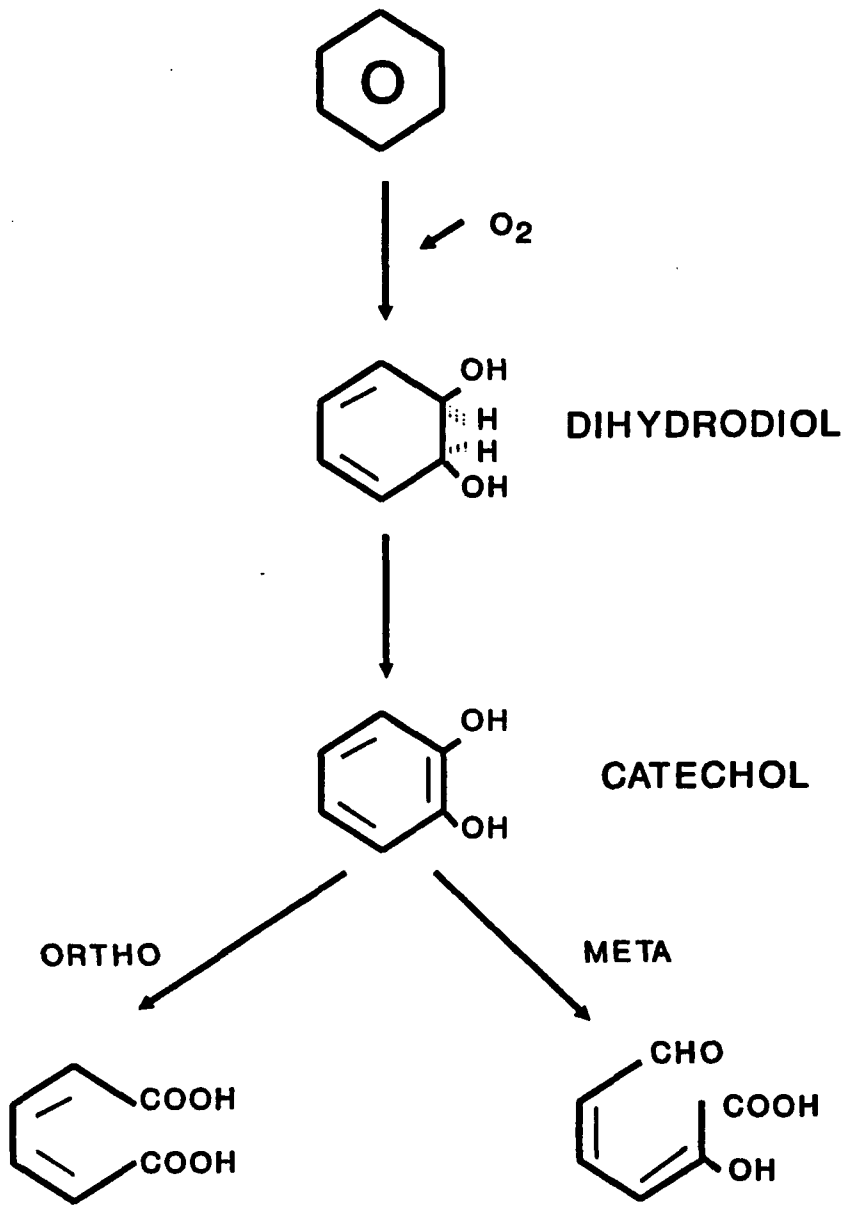

Figure 2.1: Initial reactions in the metabolic pathway for unsubstituted aromatic hydrocarbons.

2.2 Specific Considerations for Aromatic Hydrocarbon Biodegradation

2.2.1 Benzene

The bacterial degradation of benzene was first reported in 1913 by Sohngen. In 1914 two organisms, *Bacterium benzoli* a and b, were isolated that were capable of growing on benzene, toluene, and xylene (Gibson and Subramanian, 1984). For the next 40 years very little was published on the bacterial oxidation of benzene.

In 1961, two organisms were isolated from soil samples that had the capability to utilize benzene as their sole carbon source. The organisms were identified as *Mycobacterium rhodochrous* and *Pseudomonas aeruginosa.* Manometric studies showed that both organisms could oxidize benzene to carbon dioxide and water, however *P. aeruginosa* yielded a somewhat higher oxidation rate than the *M. rhodochrous* (Marr and Stone, 1961). *Pseudomonas putida* was isolated in 1968 that could oxidize benzene and ethyl benzene when grown on toluene as it's sole source of carbon and energy (Gibson et al., 1968). A mutant strain of this organism, *P. putida* 39/D was later isolated for the ability to utilize benzene as it's sole carbon and energy source (Gibson et al., 1970).

In addition to studies using pure isolates, benzene biodegradation has also been observed using wastewater

treatment plant microflora and mixed cultures obtained from soil and groundwater samples. The biodegradability of 114 priority pollutants were studied using settled domestic wastewater as the inoculum. Significant biodegradation of benzene with rapid acclimation was observed at concentrations of 5 mg/L and 10 mg/L after two weekly transfers of the culture to fresh media (Tabak, 1981).

The biodegradation of aromatic compounds was studied at an asphalt production plant at which the soil and groundwater were heavily contaminated with benzene, toluene, ethylbenzene, xylene, (BTEX); polycyclic aromatic hydrocarbons (PAHs); and phenolic compounds. A laboratory scale upflow aerated packed column and a rotating disk biological contactor (RBC) were inoculated with microorganisms obtained from the contaminated soil by percolating water through a container of the contaminated soil. After 92 days of operation, the packed column bioreactor achieved a 100% removal efficiency of benzene that was being desorbed from the contaminated soil. The RBC achieved a 94% benzene removal efficiency after 146 days of operation. The average influent benzene concentration to both reactors was 2.5 mg/L (van der Hoek et al., 1989).

During the preliminary investigations at a hydrocarbon contamination site, microorganisms capable of degrading benzene were found at a concentration of 5×10^3

cells/g-soil. Laboratory microcosm experiments using groundwater from the site showed that the miocroorganisms present in the groundwater displayed a preference for degrading the aromatic hydrocarbon contaminants, including benzene, over the aliphatic hydrocarbon contaminants. The same behavior was later seen in a field application of in situ enhanced bioremediation at this site (Geldner, 1987).

Metabolic Pathway. For many years it was believed that benzene was either oxidized to phenol or to trans-benzene dihydrodiol prior to the formation of catechol as shown in Fig 2.2. However, subsequent adaptation studies did not show the presence of phenol during the oxidation of benzene to catechol (Gibson et al., 1968; Marr and Stone, 1961).

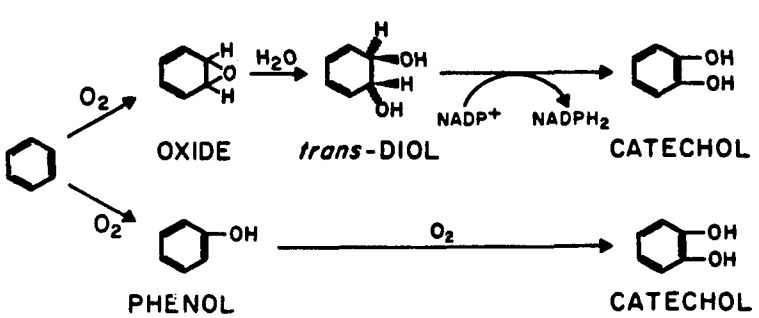

Figure 2.2: Pathways proposed for the bacterial oxidation of benzene to catechol (Gibson and Subramanian, 1984).

The suggestion that trans-benzene dihydrodiol was an intermediate in the bacterial oxidation of benzene seemed possible because it is a known intermediate in the mammalian metabolism of benzene. Studies done using *Aerobacter aerogenes* showed that crude cell extracts of the microorganism could oxidize trans-benzene dihydrodiol to catechol (Gibson and Subramanian, 1984).

In 1968, a strain of *Pseudomonas putida* was isolated from soil samples that was capable of growing on ethylbenzene or toluene as its sole source of carbon and energy. Toluene-grown cells rapidly metabolized benzene, catechol, and cis-benzene glycol whereas phenol and trans-benzene glycol were metabolized at a much slower rate compared to that of toluene. When incubated with *P.putida* cell extracts, cis-benzene glycol was stoichiometrically converted to catechol with the concomitant formation of equal amounts of NADH. Similar experiments using trans-benzene glycol did not produce catechol. This evidence suggests that *P.putida* converts benzene to cis-benzene glycol followed by catechol formation as opposed to the conversion to trans-benzene glycol (Gibson et al., 1968).

A mutant strain of this *P.putida* organism (39/D) was later isolated that did not contain cis-benzene dihydrodiol dehydrogenase which is required for the conversion of

cis-benzene glycol to catechol. When *P.putida* 39/D was grown on glucose and benzene, cis-benzene gylcol accumulated in the culture media. Experiments with isotopic oxygen showed that two atoms of atmospheric oxygen are incorporated into the cis-benzene glycol molecule. These experimental results provide conclusive evidence for the transformation of benzene to cis-benzene glycol to catechol (Gibson et al., 1970).

The enzymes responsible for the breakdown of benzene are inducible in this *P.putida* (Gibson et al., 1970). This agrees with earlier results showing that the enzymes responsible for benzene biodegradation in *P.aeruginosa* and *M.rhodochrous* are also inducible. Removal of these organisms from benzene media resulted in a loss of ability to oxidize benzene (Marr and Stone, 1960).

The pathway for the initial breakdown of benzene is shown in Figure 2.3. Bacteria initiate the oxidation of benzene through a dioxygenase enzyme system which incorporates two atoms of oxygen from an oxygen molecule simultaneously into the benzene ring. The proposed organization of the benzene dioxygenase system is shown in Fig 2.4. A *P.putida* capable of using benzene as it's sole source of carbon and energy was isolated and cell extracts were shown to contain a flavoprotein and two iron-sulfur proteins which were required for enzyme activity.

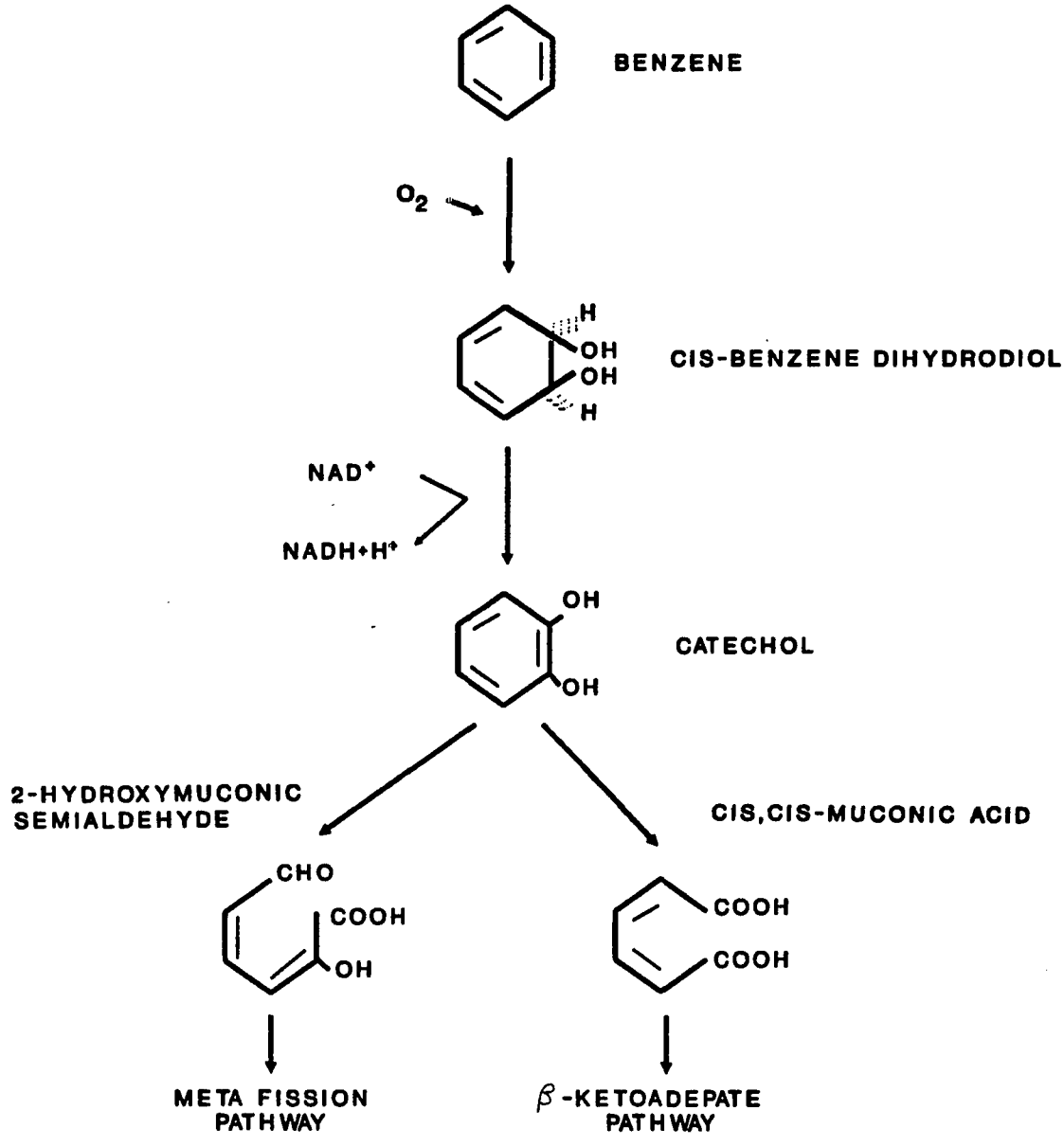

Figure 2.3: Initial reactions utilized by bacteria to oxidize benzene (Gibson and Subramanian, 1984).

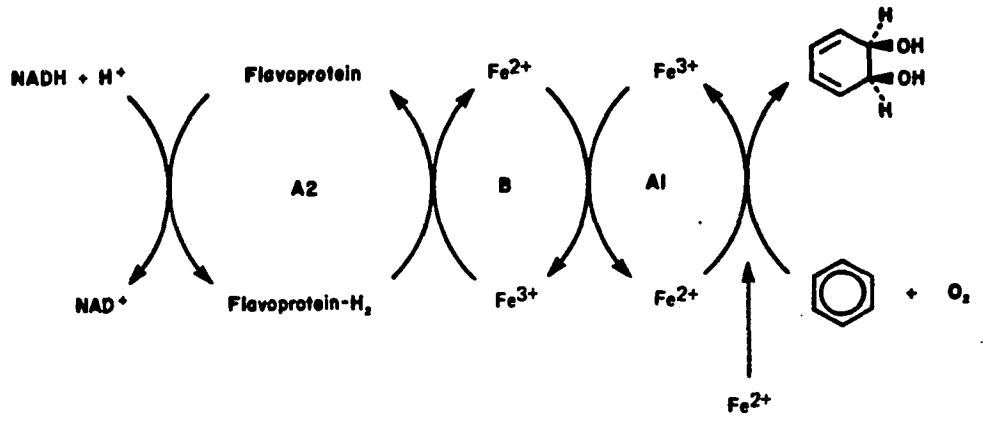

Figure 2.4: Organization of the benzene dioxygenase multienzyme complex (Gibson and Subramanian, 1984).

The cis-dihydrodiol that is produced is then transformed into catechol via a pyridine nucleotide linked dehydrogenase enzyme system. Catechols are substrates for dioxygenase enzymes that can catalyze the fission of the aromatic ring via two different pathways; meta fission or the *B*-ketoadipate pathway (Gibson and Subramanian, 1984).

2.2.2 Toluene

The biodegradation of toluene has been observed in studies using mixed cultures obtained from wastewater samples and also contaminated soil samples. Batch studies to determine the biodegradability of EPA priority pollutants showed that toluene was readily degraded using inoculum from

settled domestic wastewater (Tabak et al., 1981). The biodegradation of toluene in the gas phase was investigated during a feasibility study for using a biofilter to treat a mixture of ethylacetate, butylacetate and toluene in the process air from a lacquer factory. Degradation of toluene was achieved after inoculating the biofilter with bacteria isolated from soil samples from a nearby gas station. It was speculated that the area from which the samples were obtained was contaminated with toluene or an analog and therefore the soil bacteria were acclimated to such compounds (Vischer and Brinkman, 1989). Toluene degradation has also been achieved using small bench scale bioreactors inoculated with microorganisms isolated from contaminated soil. Using an upflow aerated packed column, 100% removal of toluene was achieved at an influent concentration of 2.9 mg/L. Treating the same waste stream, a rotating biological contactor yielded a 95% toluene removal efficiency (van der Hoek et al., 1989).

Pure cultures have also been observed to biodegrade toluene. A strain of *Pseudomonas putida* capable of using toluene as it's sole source of carbon and energy was isolated from contaminated soil. A study was done to determine if this culture could enhance the biodegradation of toluene in laboratory microcosms containing 6 grams uncontaminated soil and approximately 150 ug toluene.

Within two days, both the aqueous and the adsorbed fractions of toluene were reduced to below measureable levels (Farmer et al., 1989).

Another study investigated the biotransformation of toluene to the intermediate cis-glycol by the *P. putida* mutant, UV4. A maximum reaction rate of 1 g/hr/g-cells was observed at toluene concentrations above 2.5% aqueous saturation (aqueous solubility) (Brazier et al., 1990).

Metabolic Pathway. The presence of the methyl group on the benzene ring presents microorganisms with two alternative biodegradative pathways as shown in Fig 2.5. Bacteria can first oxidize the methyl group resulting in the formation of benzoic acid which is further oxidized to catechol, a ring fission substrate. The metabolic pathway can also be initiated by the oxidation of the aromatic nucleus to form 3-methylcatechol, also a ring fission substrate.

The biodegradation of toluene via the initial oxidation of the methyl group was first reported in 1956. *Pseudomonas aeruginosa* was shown to oxidize toluene via benzyl alcohol, benzaldehyde and benzoic acid (Kitagawa, 1956). Subsequent studies using the same organism have shown that NADH and FAD were required for the hydroxylation of toluene to benzyl alcohol. It still remains to be determined if the

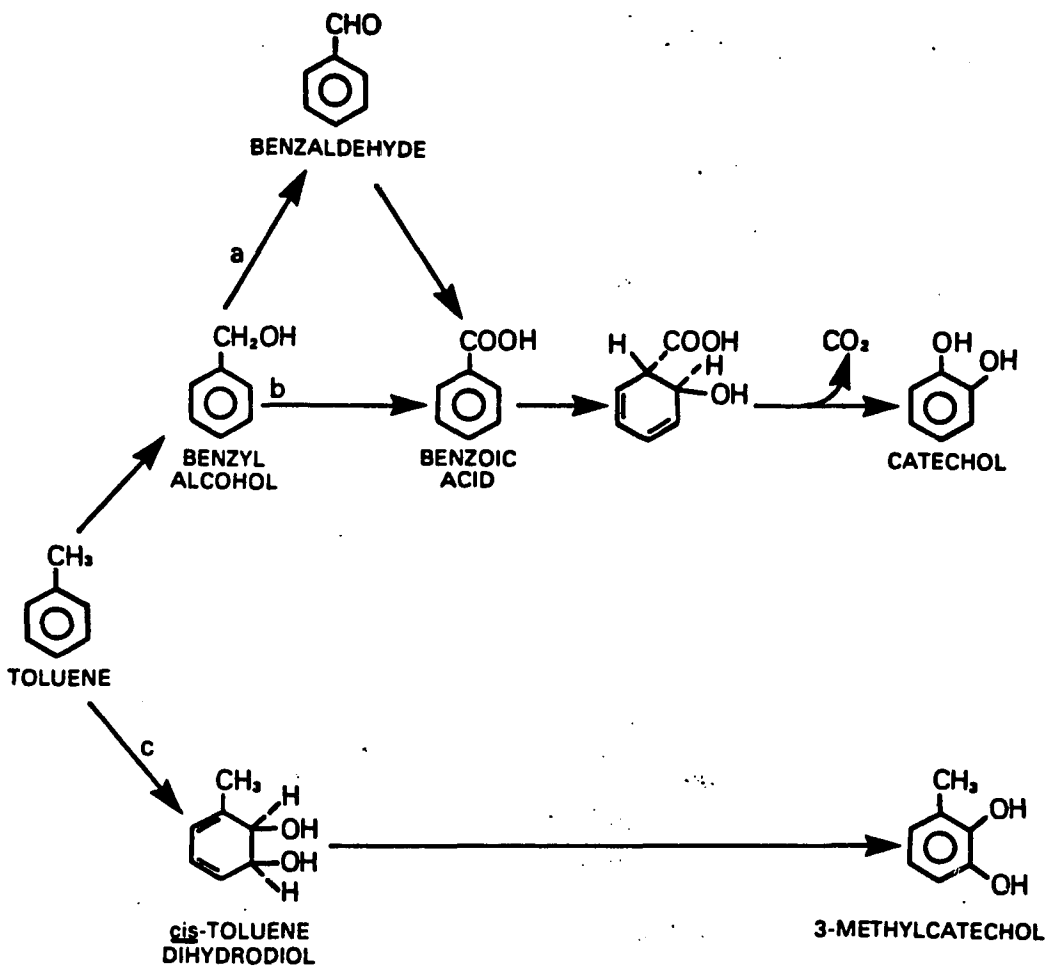

Figure 2.5: Pathways for the bacterial metabolism of toluene. Pathway (a) *P.putida* mt-2; (b) *P.aeruginosa;* (c) *P.putida; Pseudomonas* sp.; *Achromobacter* sp. (Rochkind and Blackburn, 1986).

monooxygenase enzyme system responsible for catalyzing the oxidation of the methyl group is similar to the enzyme system responsible for the hydroxylation of the terminal methyl group on linear alkanes (Gibson and Subramanan, 1984).

The oxidation of toluene to catechol via cis-toluene dihydrodiol has been observed in *Pseudomonas mildenbergei, Pseudomonas putida*, and *Achromobacter* sp. The initial oxidation of toluene to cis-toluene dihydrodiol involves the incorporation of one molecule of oxygen into the aromatic nucleus via the toluene dioxygenase enzyme system. The reaction is catalyzed by a flavoprotein and two iron-sulfur proteins. A dehydrogenase enzyme system then transforms the cis-toluene dihydrodiol to 3-methyl catechol, a ring fission substrate (Gibson and Subramanan, 1984).

Studies using two bacterial strains, a *Pseudomonas* sp. and a *Achromobacter* sp. illustrated the breakdown of toluene to 3-methyl catechol. Experiments showed that toluene metabolism by these two bacterial strains was an induced phenomenon (Claus and Walker, 1964).

2.2.3 Chlorinated Benzenes

The biodegradation of chlorinated benzenes has been reported in studies using sewage sludge, seeded granular activated carbon columns, groundwater and in soil

microcosms. Using settled domestic wastewater inoculum, chlorinated benzenes showed significant biodegradation after a gradual acclimation period. Subculturing the original culture yielded a reduction in biodegradative activity (Tabak et al., 1981). Studies were conducted to compare the removal of chlorinated benzenes in a seeded granular activated carbon column with a control column packed with glass beads and then inoculated. The columns were inoculated with primary sewage. The GAC column removed all the chlorinated benzenes tested with a 95-98% efficiency which was maintained over a two year period. The control column yielded removal efficiencies ranging from 28-98% for the different chlorobenzene compounds after varying acclimation periods (Bouwer and McCarty, 1982). In a study to determine the behavior of organic micropollutants during the infilitration of river water to groundwater, the transformation of 1,4-dichlorobenzene (1,4-DCB) was observed under aerobic conditions (Schwarzenbach et al., 1983). Subsequent laboratory column studies were performed to verify field observations. Columns filled with aquifer material from the field site were seeded with xylene enrichment cultures isolated from activated sludge. Column results compared favorably with the results from the field study. A 1,4-DCB concentration of 0.2 uM supported or enhanced the activity of 1,4-DCB degrading microbes in the

column. Batch studies proved the 1,4-DCB can be used as a sole carbon source (Kuhn et al., 1985). Soil microcosms using uncontaminated soil, showed the mineralization of both 1,2,3- and 1,2,4-trichlorobenzene. At an initial concentration of 50 ug substrate/g-soil, 1,2,4-TCB was mineralized at 1 nmol/d/20 grams soil, while 1,2,3-TCB was mineralized at approximately 1/2 - 1/3 of that rate (Marinucci and Bartha, 1979).

Using selective enrichment techniques, researchers have been able to isolate pure strains of bacteria capable of using chlorinated benzenes as their sole source of carbon and energy. A chlorobenzene degrading bacterium, strain WR1306, was isolated from soil and sewage samples after nine months of continuous enrichment on benzene followed by chlorobenzene as the growth substrate (Reineke and Knackmuss, 1984). Selective enrichment techniques also yielded an *Alcaligenes* sp. (Schraa, et al, 1986) and a *Pseudomonas* sp. (Spain and Nishino, 1987) capable of degrading 1,4-DCB; a *Pseudomonas* sp. capable of degrading 1,2-DCB (Haigler, et al, 1988); and an *Alcaligenes* sp. capable of biodegrading 1,3-DCB (de Bont et al., 1986).

Metabolic Pathway. The metabolic pathway for the breakdown of all the chlorobenzene isomers is very similar. The pathway for the metabolism of chlorobenzene by strain

WR1306 is shown in Fig 2.6. The initial step involves the transformation of chlorobenzene to chlorobenzene cis-dihydrodiol via a dioxygenase enzyme system which catalyzes the insertion of molecular oxygen into the benzene ring. This step is highly specific and results in the dioxygenation at the 2,3- position only. The chlorobenzene dihydrodiol is then oxidized by a dihydrodiol dehydrogenase enzyme, resulting in the formation of 3-chlorocatechol, a ring fission substrate. These enzymes were induced in cells grown on chlorobenzene but not in cells grown on acetate. 3-chlorocatechol is oxidized via a modified ortho cleavage pathway catalyzed by a 1,2-dioxygenase. Subsequent lactonization of the 2-chloromuconic acid leads to the loss of HCl and formation of a butenolide which is hydrolyzed to form maleylacetic acid. Maleylacetic reductase then converts the maleylacetic acid to β-ketoadipate in a reaction requiring NADH (Reineke and Knackmuss, 1984).

The biodegradation of 1,2-DCB follows a similar metabolic pathway. *Pseudomonas* sp. JS100 initiates the transformation of 1,2-DCB to 1,2-DCB-dihydrodiol by a dioxygenation at the 3,4- position. This is followed by the same breakdown steps as for chlorobenzene; dehydrogenation, ring fission, lactonization and hydrolysis. The initial dioxygenase and dehydrogenase enzyme systems in JS100 are constitutive. However, the enzymes required for the ortho

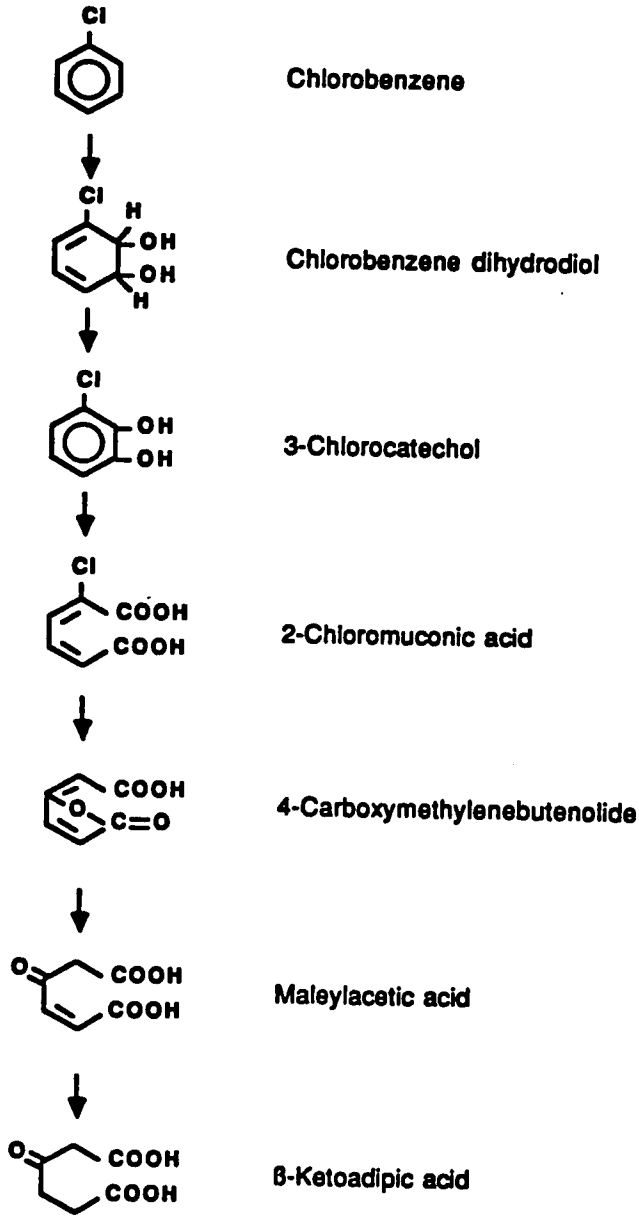

Figure 2.6: Pathway for the metabolism of chlorobenzene by strain WR1306 (Spain, 1990).

ring fission pathway are inducible. Therefore, if uninduced cells are exposed to high concentration of 1,2-DCB, toxic levels of 3,4-dichlorocatechol accumulate in the media (Haigler et al., 1988).

The biodegradation of 1,3-DCB by *Alcaligenes* sp. A175 occurs by a pathway similar to that of chlorobenzene biodegradation by WR1306. 1,3-DCB is converted to 1,3-DCB-dihydrodiol which is oxidized to 3,5-dichlorocatechol. The enzymes catalyzing these initial reactions are constitutive in A175 (de Bont et al., 1986).

The biodegradation of 1,4-DCB by *Pseudomonas* sp. strain JS6 is initiated by a dioxygenase at the 2,3- position to form 1,4-DCB dihydrodiol. This enzyme is very nonspecific and can be induced by toluene, chlorobenzene, and 1,4-DCB. The dihydrodiol is then converted to 3,6-dichlorocatechol by a NAD+ dependent dehydrogenase. A 1,2-oxygenase cleaves the catechol ring to form 2,5-dichloro-cis,cis-muconic acid. JS6 cells grown on 1,4-DCB also contained a 2,3-dioxygenase system which is somehow inhibited by the presence of chlorocatechols (Spain and Nishino, 1987).

2.3 Toxicity of Aromatic Compounds to Microorganisms

Most laboratory biodegradation studies have been conducted by applying the volatile substrate within the gas phase of a sealed batch culture. Direct injection into the

liquid phase has often been found to inhibit cell growth.

During enzyme induction studies of a *Pseudomonas* sp. on 1,4-DCB it was fortuitously discovered that liquid phase 1,4-DCB concentrations above 36.8 mg/L inhibited respiration (Spain and Nishino, 1987). Studies using a chlorobenzene-degrading bacterium showed inhibition of growth at 100-450 mg/L in the liquid phase. Chlorobenzene allowed growth when supplied at 660 mg/L in the gas phase, but even when supplied in the gas phase, the microorganism was sensitive towards the higher chlorobenzene concentrations. As chlorobenzene concentration increased, a prolonged lag phase was observed (Reineke and Knackmuss, 1984).

Volskay and Grady (1988) examined the toxicity of selected RCRA compounds to activated sludge microorganisms. Oxygen depletion rate was monitored to determine the effect of the presence of a test compound on a microbial culture degrading a high concentration of a readily degradable substrate. A 50% reduction in oxygen uptake was observed when chlorobenzene was present in the liquid phase at a concentration of 140 mg/L. The presence of 1,4-DCB or toluene at their solubility limit exhibited less than a 50% inhibition in oxygen consumption.

2.4 Effect of Additional Substrates on Biodegradation of Aromatic Hydrocarbons

The presence of additional substrates is an important

environmental factor that can influence the occurrence and rate of aromatic hydrocarbon biodegradation. Multiple substrates can have three possible efects on the biodegradation of aromatic compounds of interest; enhancement, diauxic inhibition, or no effect.

The addition of multiple substrates has been shown in some cases to enhance the biodegradation of low concentrations of toxic organic chemicals. The enhanced degradation rate is believed to be caused by increased biomass stimulated by the presence of the additional carbon sources. Examples of this include the biodegradation of methylene chloride by a *Pseudomonas* sp. Methylene chloride was more rapidly biodegraded at concentrations of 10 mg/L or less when acetate was supplied as an additional substrate. The biodegradation of very low levels of p-nitrophenol by a *Pseudomonas* sp. also has been shown to be stimulated by the presence of other substrates (Lindstrom and Brown, 1989).

Multiple substrates can also stimulate the biodegradation of high levels of toxic substrates. Microbial growth on a single toxic substrate usually requires low substrate concentrations to avoid killing the microbial population. However, the low concentration of substrate yields very little biomass and results in low degradation rates. The addition of a supplemental carbon source can increase the biomass produced and decrease the

amount of toxic organic substrate that each cell is exposed to. The enhanced culture can then biodegrade the organic substrate of interest at concentrations normally toxic to the microorganisms.

An example of this effect involves the biodegradation of pentachlorophenol (PCP) by a *Flavobacterium* sp. When PCP was provided as the sole carbon source, microbial growth was inhibited at approximately 130 mg/L. The addition of cellobiose as an additional carbon source stabilized the microbial population even at PCP concentrations exceeding 600 mg/L (Lindstrom and Brown, 1989).

The phenomenon known as diauxie was discovered by Monod and is a well known example of sequential utilization of two carbon substrates by bacteria. In diauxie, the prefered substrate must be nearly exhausted before the second substrate is utilized. Growth on the preferred substrate usually occurs at a higher rate than does growth on the second substrate. The lag phase observed between the two growth phases decreases as the ratio of the preferred substrate to the second substrate is decreased.

Two substrates which result in diauxic growth may be utilized simutaneously when they are both present at low concentration. An example of this is *P.acidovorans* exposed to acetate and phenol. Diauxie is observed when the organism is exposed to 70 ug/L acetate and 2 ug/L phenol.

However, if the acetate concentration is decreased to 13 ug/L simultaneous utilization of the substrates is observed (Schonborn, 1986).

The phenomenon of diauxie was illustrated in a study where phenol, benzene and naphthalene were exposed to a mixed microbial culture obtained from a refinery settling pond. The three compounds were readily degraded in single compound experiments. However, when phenol and benzene were exposed together, the phenol degradation rate decreased and benzene did not biodegrade during the course of the experiment (240 hrs). When phenol and naphthalene were exposed together, the phenol degradation rate again decreased and naphthalene did not degrade until the phenol concentration dropped below approximately 10 mg/L. Diauxie is proposed to account for the observed differences in the degradation rate between single compound and two compound exposures. In the benzene/phenol mixture there may have been sufficient phenol remaining at the termination of the experiment to still repress the synthesis of benzene metabolizing enzymes (Meyer et al., 1984).

2.5 Biodegradation of Mixtures

Mixtures of toxic organic chemicals pose a special biodegradative challenge to microorganisms. The various enzyme systems required for the breakdown of structurally

different chemical contaminants are often incompatible due to misrouting of intermediates. It was long believed that the simutaneous biodegradation of aromatic compounds containing chloro and methyl groups was impossible because the pathways were incompatable. Chloroaromatics are generally degraded via a modified ortho pathway catalyzed by catechol 1,2-dioxygenase. Methyl aromatic compounds are typically degraded via a meta pathway catalyzed by catechol 2,3-dioxygenase. In some bacteria both pathways are present but only one is activated at a time in response to the substrate available. When faced with both chloro and methyl aromatics, both pathways are induced. Misrouting of intermediates usually occurs and bacterial growth is disrupted. For instance the presence of chlorocatechol inactivates the catechol 2,3 dioxygenase system.

A genitically engineered strain has been developed that can biodegrade both methyl and chloro substituted aromatic compounds. *Pseudomonas* sp. B13 contains an ortho cleavage pathway but lacked a detectable meta cleavage pathway. By combining critical enzymes from 5 different catabolic pathways of three distinct soil bacteria, an ortho cleavage route was created for the degradation of methylphenols and methylbenzoates. The new engineered *Pseudomonas* sp. is able to degrade and grow on mixtures of chloro and methyl

aromatics without misrouting of the intermediates (Rojo et al., 1987).

Pseudomonas sp. JS6 has a very broad substrate range as shown in Table 2.1. This is due to the fact that JS6 can synthesize the enzymes for three different biodegradative pathways catalyzed by nonspecific enzyme systems.

JS6 contains the modified ortho pathway containing catechol 1,2-dioxygenase which transforms halogenated catechols. It also contains the meta pathway for the conversion of alkyl substituted catechols via a catechol 2,3-dioxygenase. Lastly it contains the β-ketoadipate pathways for the conversion of unsubstituted catechol (Spain, 1990).

Because of the wide substrate specificity and number of available pathways in JS6, this organism has the potential for application in the degradation of mixtures of toxic aromatic compounds. Work is still needed, however, to determine how this organism regulates its pathways and how it avoids misrouting of the intermediates.

Growth Substrate	Co-Metabolite
Chlorobenzene	o-DCB
Bromobenzene	m-DCB
Iodobenzene	1,2,4-Trichlorobenzene
p-DCB	Trichloroethylene
4-chlorotoluene	4-Chlorophenol
2-Chlorobenzoate	2-Chlorophenol
3-Chlorobenzoate	3-Chlorophenol
4-Chlorobenzoate	2,5-Dichlorophenol
Toluene	
Benzene	
Ethylbenzene	
Phenol	
Benzoate	
p-Hydroxybenzoate	
Naphthalene	
Salicylate	

Table 2.1: Partial list of substituted aromatic compounds degraded by *Pseudomonas* sp. Strain JS6 or its derivatives (Spain, 1990).

Chapter 3

Materials and Methods

3.1 General

Biodegradation of several aromatic compounds was examined in the presence of alternate substrates using a *Pseudomonas* sp. strain, JS6. The bacteria were acclimated to either aromatic compounds, glucose or yeast extract. The biodegradability of the aromatic compounds of interest was tested using these cultures both in the presence and absence of the compound used for acclimation. A matrix of the batch experiments performed is given in Figure 3.1.

Additional batch microcosm experiments were performed to determine the concentration at which benzene and chlorobenzene is toxic to JS6 and if the enzymes responsible for the initial step in the breakdown pathway are constitutive or inducible.

Abiotic controls were included in all experiments to estimate chemical losses associated with volatilization and sorption onto the glass test tube, caps and fittings.

3.2 Maintenance and Cultivation of Bacterium

A pure culture of a *Pseudomonas* sp., JS6, was used in all batch experiments. JS6 was obtained from Dr. Jim Spain, HQ AFESC/RDVC, Tyndall AFB, FL. The inoculum was revived by

Acclimated To:	Exposed To:				
	Benz	CB	Tol	Yeast Extract	Glucose
Benzene			X	X	X
Chlorobenzene	X		X	X	X
Toluene	X	X		X	X
Yeast Extract	X	X	X		
Glucose	X		X		

Note: Substrates added in the following concentrations: Benzene, Chlorobenzene, Toluene -- approximately 10^{-4}M in the liquid phase concentration. Yeast extract -- 0.1 g/L. Glucose -- 1.0 g/L.

Figure 3.1: Description of batch microcosm biodegradation experiments.

resuspension in nutrient broth solution at 30°C. After dense growth was observed, this culture was used to inoculate nutrient agar slants. Slants were maintained at 4°C for the length of this research project and were used as the source of JS6 inocula for all experiments.

To generate cultures of JS6 acclimated to the various substrates, a culture was first grown in a nutrient broth solution (8 g/L nutrient broth and 5 g/L sodium chloride) until dense growth was observed. Cells were harvested, washed and resuspended in a defined mineral salts medium (MSB) (Table 3.1) to which the substrate of interest had been added.

3.3 Cell Preparation and Enumeration

Cells used in batch experiments were concentrated by centrifugation with an Eppendorf Model 5415 Centrifuge set at 4000 rpm for six minutes. Cells were washed twice and resuspended using sterile MSB.

Cells were enumerated using acridine orange epifluorescent methods (Hobbie et al,. 1977).

3.4 Batch Biodegradation Experiments

Fifty five milliliter screw-top test tubes fitted with mini-nert® valve caps were used for the microcosms. To acclimate JS6 to the various aromatic compounds, the

Table 3.1: Mineral salts medium (MSB) used for growth of JS6. (Spain and Nishino, 1987)

Compound	Concentration (mg/L)
$MgSO_2 \cdot 7H_2O$	112
$ZnSO_4 7H_2O$	5.0
$Na_2MoO_4 \cdot 2H_2O$	2.5
KH_2PO_4	340
Na_2HPO_4	354.9
$CaCl_2 \cdot 2H_2O$	18.5
$FeCl_3 \cdot 6H_2O$	0.22
NH_4Cl	500

Note: The pH of the MSB was adjusted to 7 using 1 M NaOH

substrate was added (to achieve 10^{-4} M in the liquid phase) via a microliter syringe to a test tube containing 25 ml MSB. The compound was allowed to come to equilibrium by placing the test tube on a shaker table at 30°C for one hour. Yeast extract and glucose were added to the MSB in the appropriate concentrations prior to autoclaving. The batch microcosms were inoculated with washed JS6 cells via a pipetteman to a cell count of approximately 10^7 cells/ml. When dense growth was observed, the acclimated cells were harvested for use in the biodegradation experiments.

Two types of test tube microcosms were prepared for the biodegradation experiments. The first contained both the chemical used for acclimation and the chemical whose biodegradability was being tested. The second contained the chemical whose biodegradability was being tested. Microcosms were prepared in duplicate.

Microcosms were inoculated with washed, acclimated cells via a 1 ml plastic syringe. An initial cell concentration was determined via direct cell counts using epifluorence microscopy methods. Final cell concentrations were determined at the conclusion of the experiment.

Substrate concentration was determined by gas chromatography prior to inoculation and periodically thereafter to determine the change in substrate concentration with time. Liquid phase substrate

concentrations were calculated from the gas phase concentrations determined from gas chromatography results and Henry's Law Constants (Oak Ridge National Laboratory, 1989).

3.5 Toxicity Experiments

A culture of JS6 was grown in a nutrient broth solution to a turbidity reading of approximately 50 NTU. Turbidity was measured with a Hach model 2100A Turbidimeter. Cells were harvested and washed using MSB.

Fifty-five milliliter screw-top test tubes fitted with mini-nert® valve caps were used for the microcosms. Test tubes containing 25 ml sterile MSB were spiked with varying volumes of benzene or chlorobenzene via a microliter syringe and allowed to equilibrate for one hour at 30° C on the shaker table. After equilibration, the tubes were inoculated with washed JS6 cells to achieve a turbidity reading of 1-4 NTU.

Substrate utilization was monitored over time by headspace analysis using gas chromatography and bacterial growth was monitored by turbidity measurements. Abiotic controls were run using uninoculated tubes of MSB containing the same amounts of benzene or chlorobenzene. Substrate concentration was monitored at time zero and at the

conclusion of the experiment. Table 3.2 summarizes the range of substrate concentrations tested.

3.6 Enzyme Induction Experiments

Microcosms were prepared in the same manner as for the toxicity experiments described above. Prior to substrate addition the test tubes were spiked with chloramphenicol to yield a final concentration of 161.5 mg/L. Chloramphenicol inhibits protein synthesis by interfering with the transfer of amino acids from their soluble RNA complex to the ribosomes (Pelczar et al., 1977). After chloramphenicol and substrate addition, the test tubes were inoculated with the appropriate volume of washed JS6 cells suspended in MSB to achieve a total cell count of approximately 10^9 cells/ml. A very high initial cell density was desired to provide adequate enzymes to demonstrate substrate utilization if the enzymes proved to be constitutive. Substrate utilization was measured with time by headspace analysis using gas chromatography. Bacterial cell counts were determined by direct counts. Table 3.2 summarizes the test variables for the enzyme induction experiments.

3.7 Chemicals

Benzene, chlorobenzene, 1,2-dichlorobenzene and 1,3-dichlorobenzene were obtained from Aldrich Chemical Co.,

Table 3.2: Summary of variables for toxicity and enzyme-induction experiments.

SAMPLE	INITIAL LIQ. PHASE SUBSTRATE CONC. (mg/L)	CHLORAMPHENICOL CONC (mg/L)	INITIAL CELL COUNT (cells/mL) $x10^{-8}$
B1	12.4 mg/L benzene	-------	3.2
B2	44.1 mg/L benzene	-------	2.5
B3	72.6 mg/L benzene	-------	4.0
B4	144.1 mg/L benzene	-------	2.8
B5	660.0 mg/L benzene	-------	3.4
B6	1402.4 mg/L benzene	-------	3.9
B7	8.4 mg/L benzene	161.5	16
B8	19.4 mg/L benzene	161.5	13
CB1	13.8 mg/L chlorobenzene	-------	0.44
CB2	27.1 mg/L chlorobenzene	-------	0.44
CB3	62.2 mg/L chlorobenzene	-------	0.53
CB4	88.9 mg/L chlorobenzene	-------	0.40
CB5	113.0 mg/L chlorobenzene	-------	0.53
CB6	133.9 mg/L chlorobenzene	-------	0.48
CB7	10.8 mg/L chlorobenzene	161.5	9.3
CB8	29.3 mg/L chlorobenzene	161.5	7.3

Inc., Milwaukee, Wisconsin. Toluene was obtained from Fisher Scientific Co. Fairlawn, New Jersey.

Gas standards were made for the aromatic compounds by adding varying amounts of the pure chemical via a microliter syringe to 120 ml serum vials fitted with teflon-lined® septa and aluminum crimp caps. The pure chemical was allowed to volatilize inside the serum bottle prior to withdrawing gas samples for GC analysis. Care was taken not to exceed the air saturation limit of the chemical to ensure complete volatilization. Calibration standards were prepared within 2 hours of analysis.

The retention time of oxygen was identified by injecting pure oxygen into the GC. Room air was routinely used to estimate the oxygen concentration in the headspace of the microcosm. Room air (0.4 ml) was collected in a one ml syringe and injected into the GC. The chromatographic peak area associated with oxygen was assumed to correlate with 21 percent oxygen in air. Oxygen concentrations in the microcosms were determined by assuming a linear relationship between percent oxygen in air and chromatographic peak area.

3.8 Analytical Methods

Concentrations of the aromatic compounds in the microcosms were determined using a 30-meter DB-Wax Megabore (0.53 mm) column (J&W Scientific) in a Hewlett Packard 5890

gas chromatograph equipped with both a flame ionization detector (FID) and a thermal conducting detector (TCD). High purity helium was used for the carrier gas. Analytical data was collected on a Hewlett Packard Model 3392 A integrator.

The gas chromatographic conditions were as follows:

Helium Carrier Gas	380 kPa
Injector Temp	110°C
FID Temp	200°C

To analyze the headspace for aromatic compounds, 100 ml gas samples were introduced into the GC via a gas tight microliter syringe. Oven temperatures and pressure was altered for each experiment based on the organic compound being analyzed for to achieve good peak separation but also minimize retention times.

Oxygen concentration in the headspace was measured using the following GC conditions:

Injection Temperature	110°C
TCD Temperature	200°C
Initial Oven Temperature	35°C
Initial Hold Time	13.5 min.
Rate of Increase	70°C/min.
Final Oven Temperature	200°C
Final Hold Time	2 min.

Headspace gas samples (0.4 ml) were introduced into the GC for the oxygen analysis.

Chapter 4

Results and Discussion

4.1 Batch Biodegradation Experiments

The presence of an easily degraded substrate can have three different effects on the biodegradation of a xenobiotic compound. It may cause the cell density to increase so that the xenobiotic compound can be degraded at a faster rate by the higher cell population. The secondary substrate may also inhibit biodegradation of the xenobiotic compound by being degraded preferentially over the xenobiotic compound. This is termed diauxic growth. The presence of a secondary substrate may also have no effect at all on the biodegradation of a xenobiotic compound.

To determine the effect of the presence of a non-aromatic substrate on the biodegradation of an aromatic compound, JS6 cells were exposed to solutions of the aromatic compound alone and a mixture of the aromatic compound and the non-aromatic compound. The results (Figure 4.1-4.3) indicate that the presence of yeast extract at 100 mg/L enhanced the biodegradation of benzene, chlorobenzene, and toluene. Benzene was removed to non-detectable levels within 1 day when JS6 was grown on a mixture of benzene and yeast extract, and 2-2.5 days when grown on benzene alone

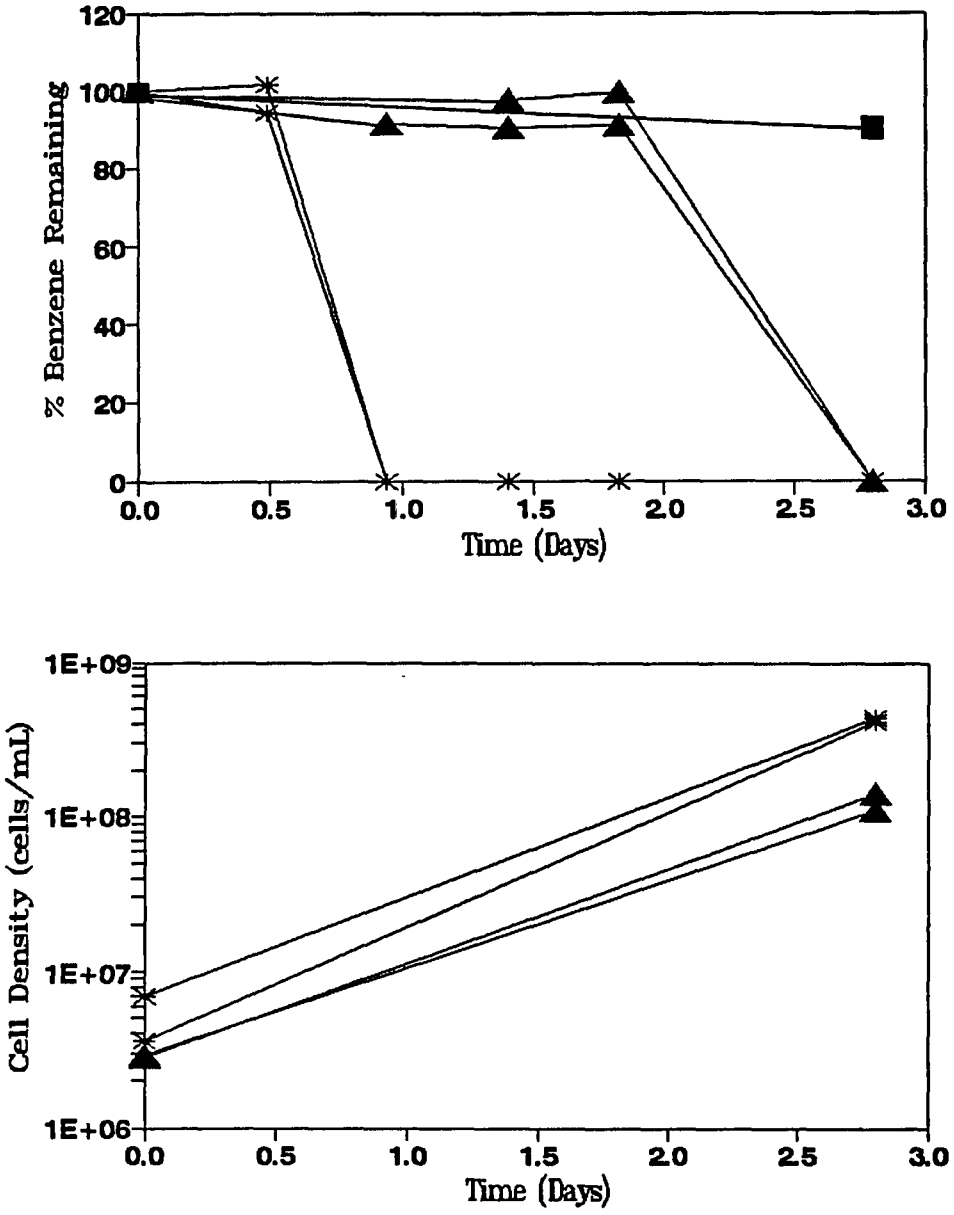

Figure 4.1: Batch experiments using JS6 cells grown on yeast extract (100 mg/L), then exposed to benzene (20 mg/L) (▲) and a mixture of benzene and yeast extract (*). Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

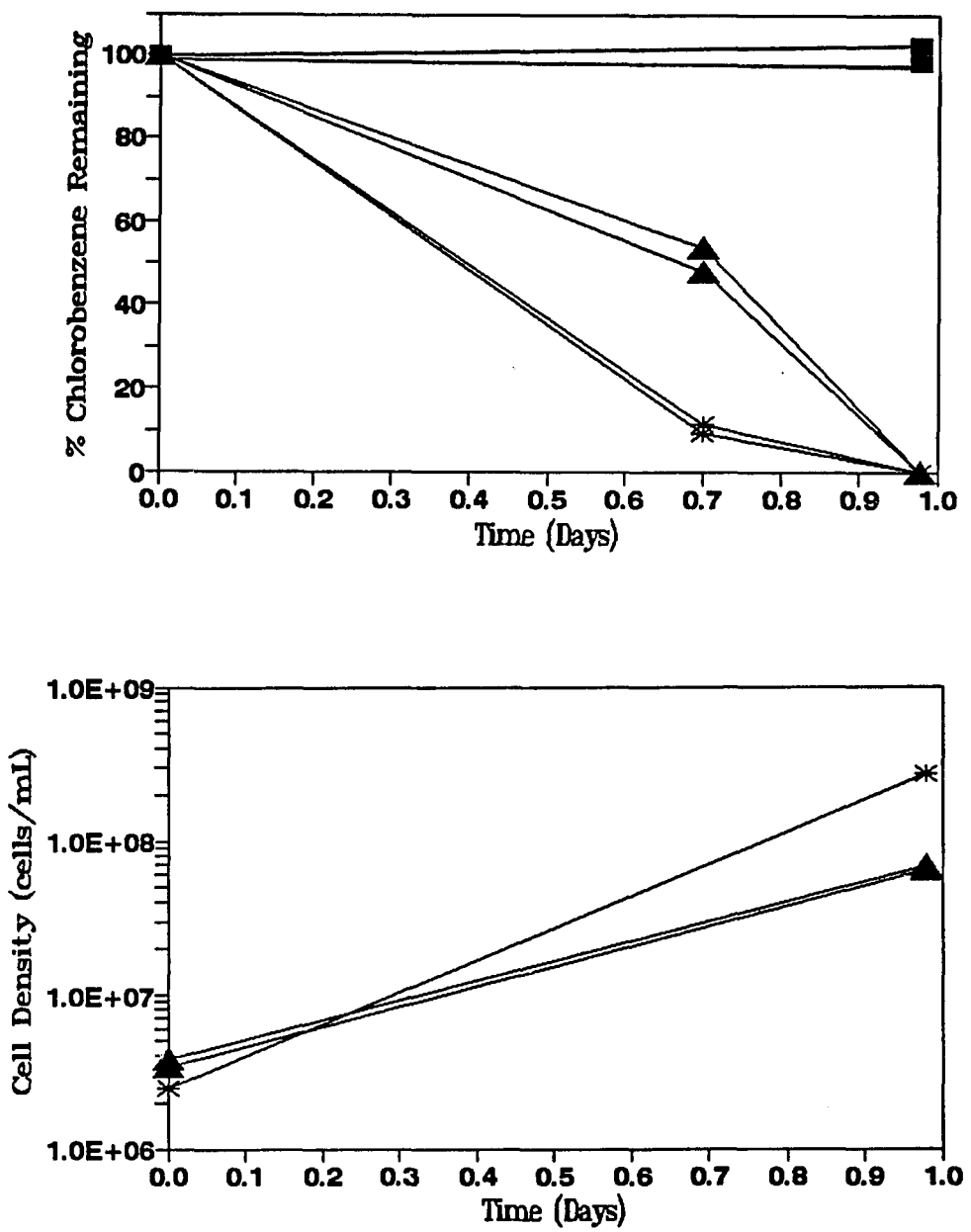

Figure 4.2: Batch experiments using JS6 cells grown on yeast extract (100 mg/L), then exposed to chlorobenzene (33 mg/L) (▲) and a mixture of chlorobenzene and yeast extract (✳). Abiotic controls were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

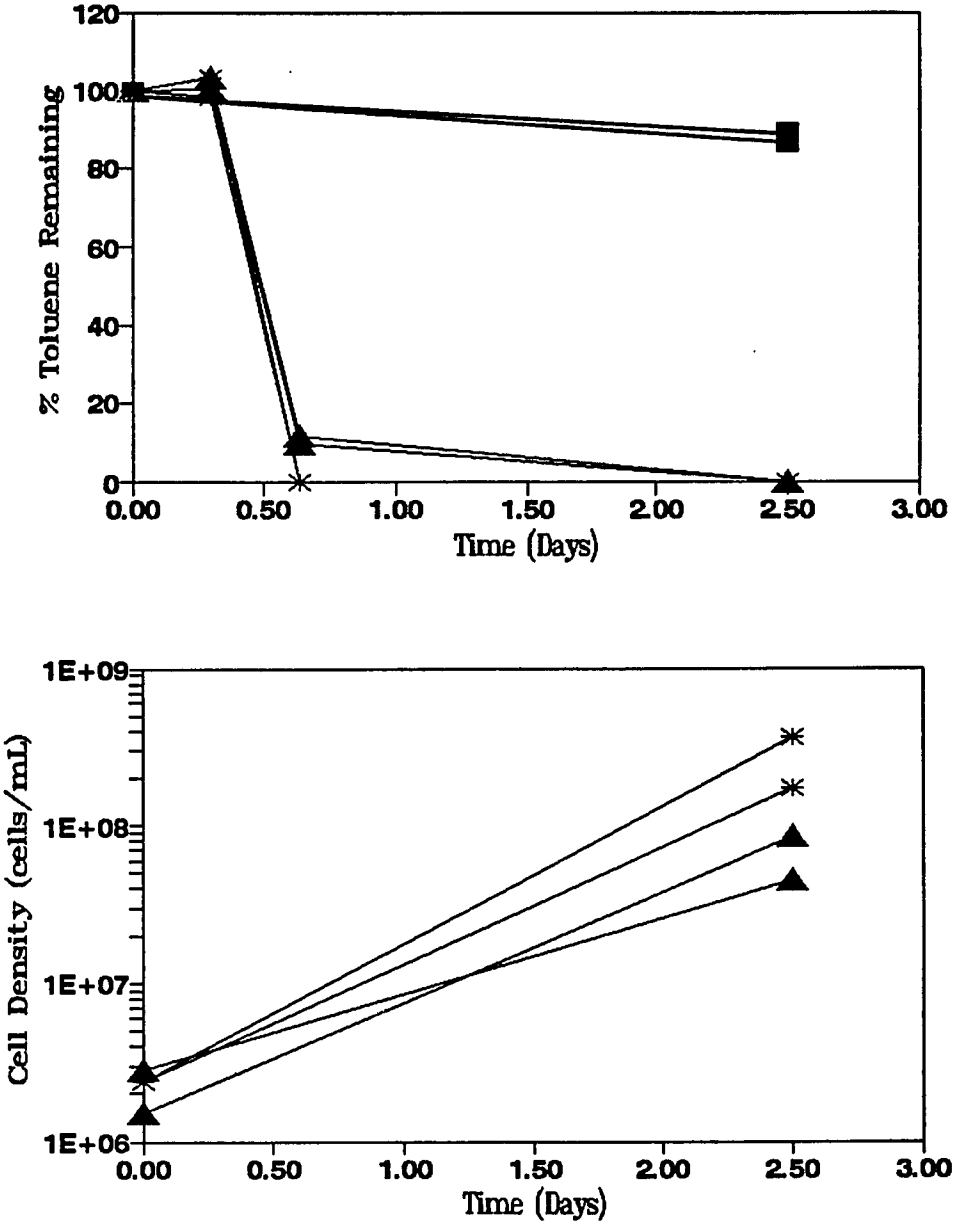

Figure 4.3: Batch experiments using JS6 cells grown on yeast extract (100 mg/L), then exposed to toluene (17 mg/L) (▲) and a mixture of toluene and yeast extract (✳). Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

(Fig 4.1). Similar, but less dramatic results, were observed for chlorobenzene and toluene.

These data suggest that the presence of yeast extract decreases the lag time prior to the initiation of degradation of the aromatic compound. Yeast extract may contain a substantial fraction of amino acids which may be an effiecient source for building proteins. Thus, when the enzymes required for degradation of the aromatic compound are needed, the proteins can be manufactured more quickly and thereby reduce the lag time associated with enzyme induction.

A similar experiment was run with 1 g/L glucose as the secondary substrate. Results (Figure 4.4) also indicate enhanced biodegradation of benzene. Cell density data were not collected for this experiment.

In the batch experiments described above, JS6 was initially grown on the non-aromatic substrates (yeast extract or glucose). To determine if the initial growth substrate had any subsequent effect on biodegradation of the aromatic compounds, a set of experiments was run with JS6 initially being grown on an aromatic compound (benzene, chlorobenzene, or toluene), then exposed to a mixture of the aromatic compound and yeast extract. These results are presented in Figures 4.5-4.7.

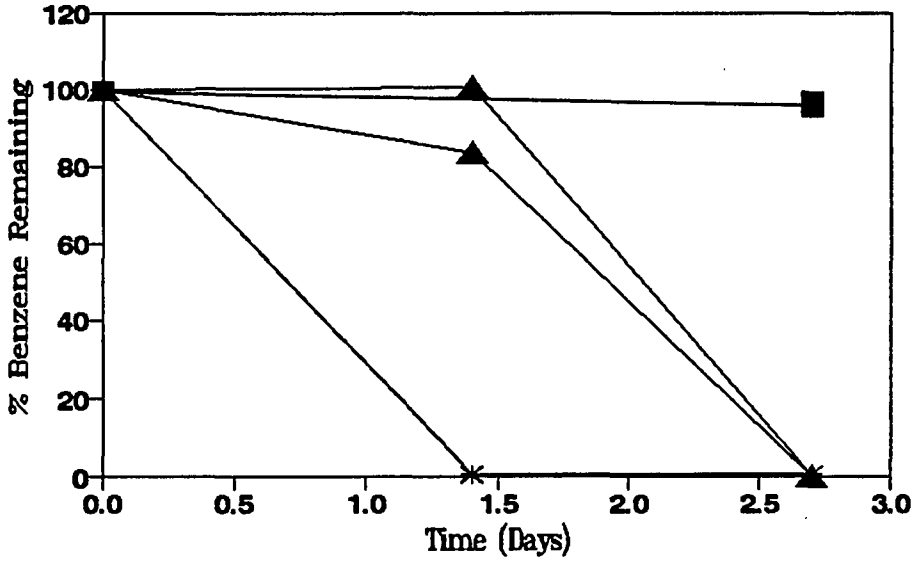

Figure 4.4: Batch experiments using JS6 cells grown on glucose (1 g/L), then exposed to benzene (20 mg/L) (▲) and a mixture of benzene and glucose (✳). Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

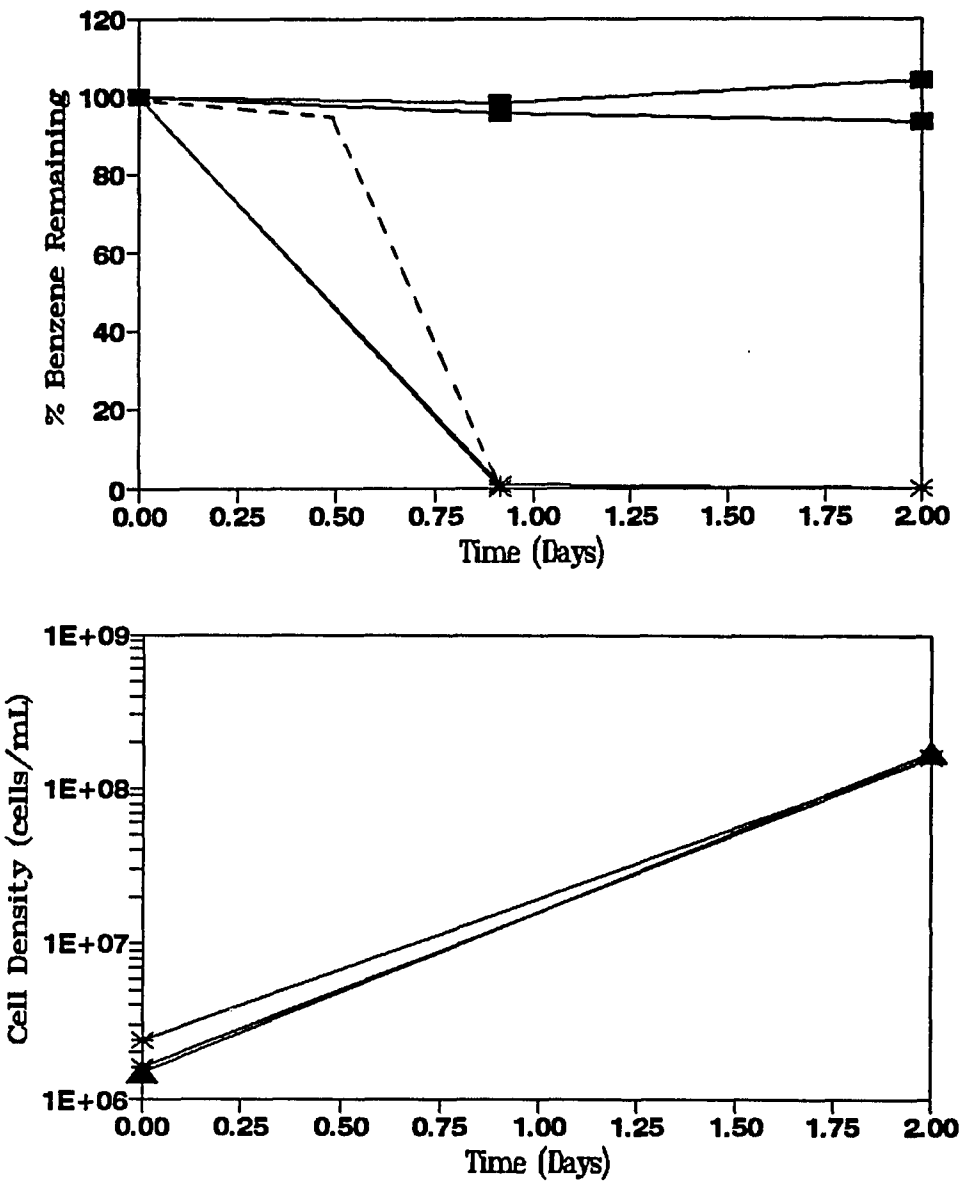

Figure 4.5: Batch experiments using JS6 cells grown on benzene (20 mg/L), then exposed to a mixture of benzene and yeast extract (100 mg/L) (✳) and yeast extract alone (▲). Dashed line represents data from Fig. 4.1 for yeast-extract-grown cells exposed to a mixture of benzene and yeast extract. Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

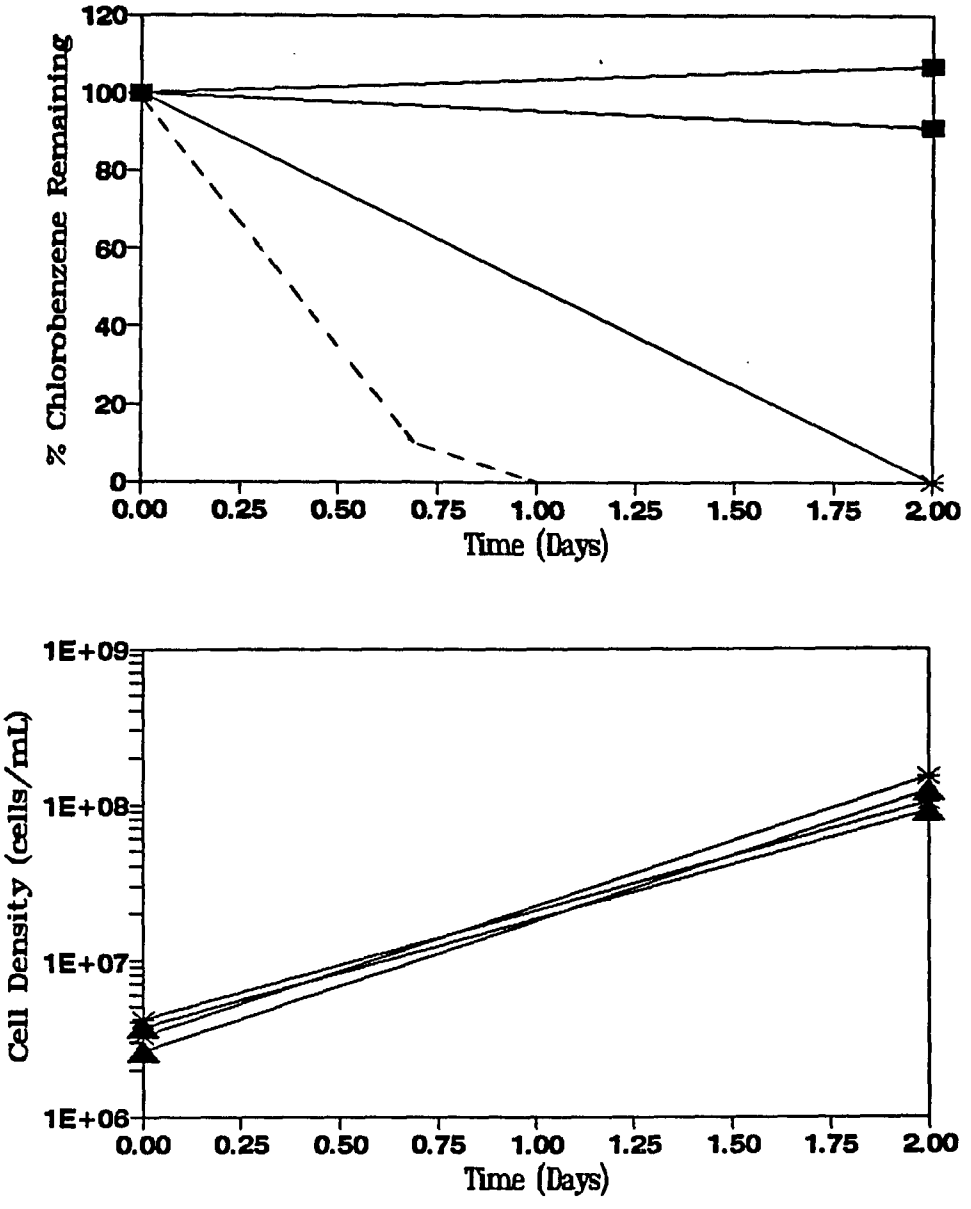

Figure 4.6: Batch experiments using JS6 cells grown on chlorobenzene (35 mg/L), then exposed to a mixture of chlorobenzene and yeast extract (100 mg/L) (✳) and yeast extract alone (▲). Dashed line represents data from Fig. 4.2 for yeast-extract-grown cells exposed to a mixture of chlorobenzene and yeast extract. Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

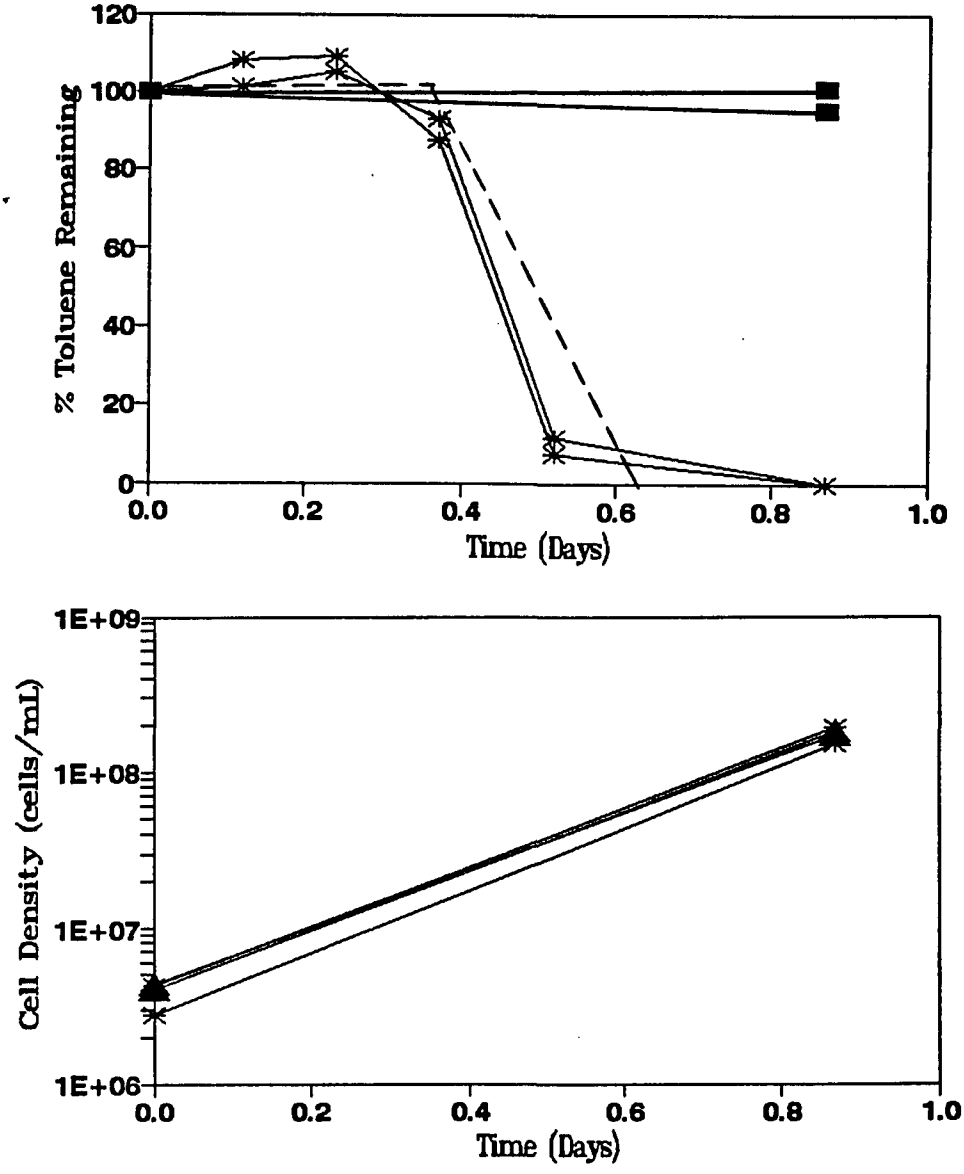

Figure 4.7: Batch experiments using JS6 cells grown on toluene (20 mg/L), then exposed to a mixture of toluene and yeast extract (100 mg/L) (✳) and yeast extract alone (▲). Dashed line represents data from Fig. 4.3 for yeast-extract-grown cells exposed to a mixture of toluene and yeast extract. Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

Due to the infrequency of sampling it could not be concluded whether the aromatic compound biodegradation rate was dependent on whether JS6 was initially grown on yeast extract or the aromatic compound of interest.

Another observation that can be made from Figures 4.5-4.7 is that the small amount of aromatic compound that was added for the biodegradation experiments did not cause any noticable biomass growth above that which was caused by yeast extract alone. It appears that the majority of biomass generation is caused by the utilization of the non-aromatic compound. Cell density data from Figures 4.1-4.3 support this. The cultures exposed to a mixture of yeast extract and the aromatic compound show a greater increase in cell density than do the cultures exposed to only the aromatic compound.

Batch biodegradation experiments were also run to determine if two aromatic compounds could be simultaneously biodegraded. It is known that JS6 possesses three pathways for the biodegradation of aromatic compounds (Spain et al., 1990). It uses the ortho or β-ketoadipate pathway for the unsubstituted aromatic compounds such as benzene; the modified ortho pathway for the chlorinated aromatic compounds; and the meta pathway for the alkyl substituted aromatic compounds. The enzymes associated with these three pathways can be induced when expopsed to a single

substrate, such as benzene, chlorobenzene, or toluene. But it was unknown if JS6 would synthesize the enzymes for more than one pathway simultaneously.

Toluene-grown cells were exposed to benzene and chlorobenzene and a mixture of toluene and each of these aromatic compounds. The results (Figure 4.8) indicate that toluene and benzene can be simultaneously biodegraded. This fact suggests that the initial enzymes required for the ortho and meta ring fission pathways can be synthesized and utilized simultaneously. The fact that benzene was biodegraded faster by the culture exposed to the toluene and benzene mixture versus the culture exposed to benzene alone may be a result of the higher cell density in the culture exposed to the mixture.

When toluene-grown cells were exposed to chlorobenzene and a mixture of toluene and chlorobenzene, the results (Figure 4.9) also indicated that the two required pathways could be turned on simultaneously. The enzymes required for the initial steps in both the meta and modified ortho pathways were induced at the same time. In this case however, the presence of toluene seemed to inhibit the biodegradation of chlorobenzene.

Chlorobenzene-grown cells were also exposed to benzene and toluene and a mixture of chlorobenzene and these two aromatic compounds. Results (Figures 4.10 and 4.11)

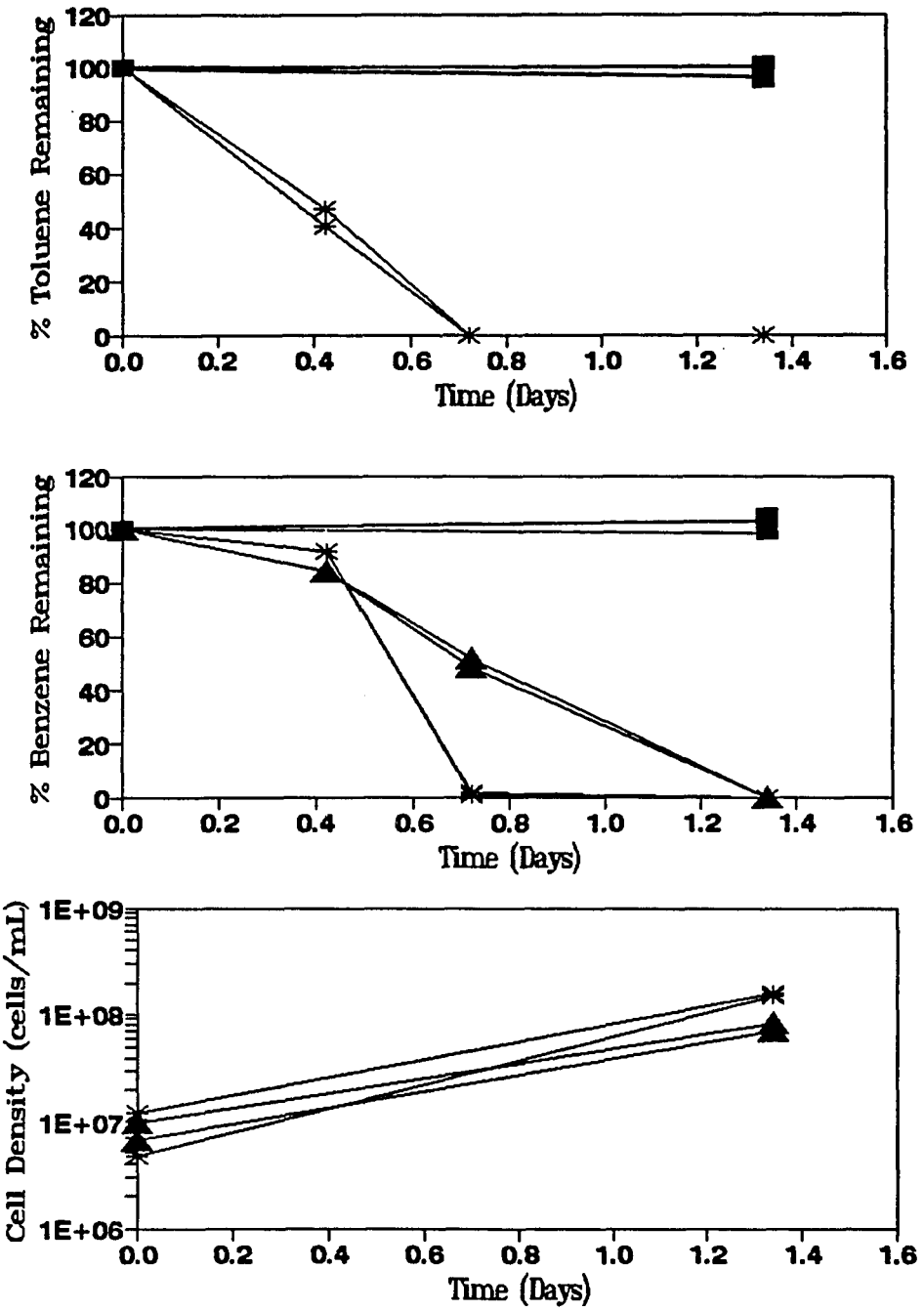

Figure 4.8: Batch experiments using JS6 cells grown on toluene (20 mg/L), then exposed to a mixture of toluene and benzene (20 mg/L) (✳) or benzene alone (▲). Abiotic controls (■) were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

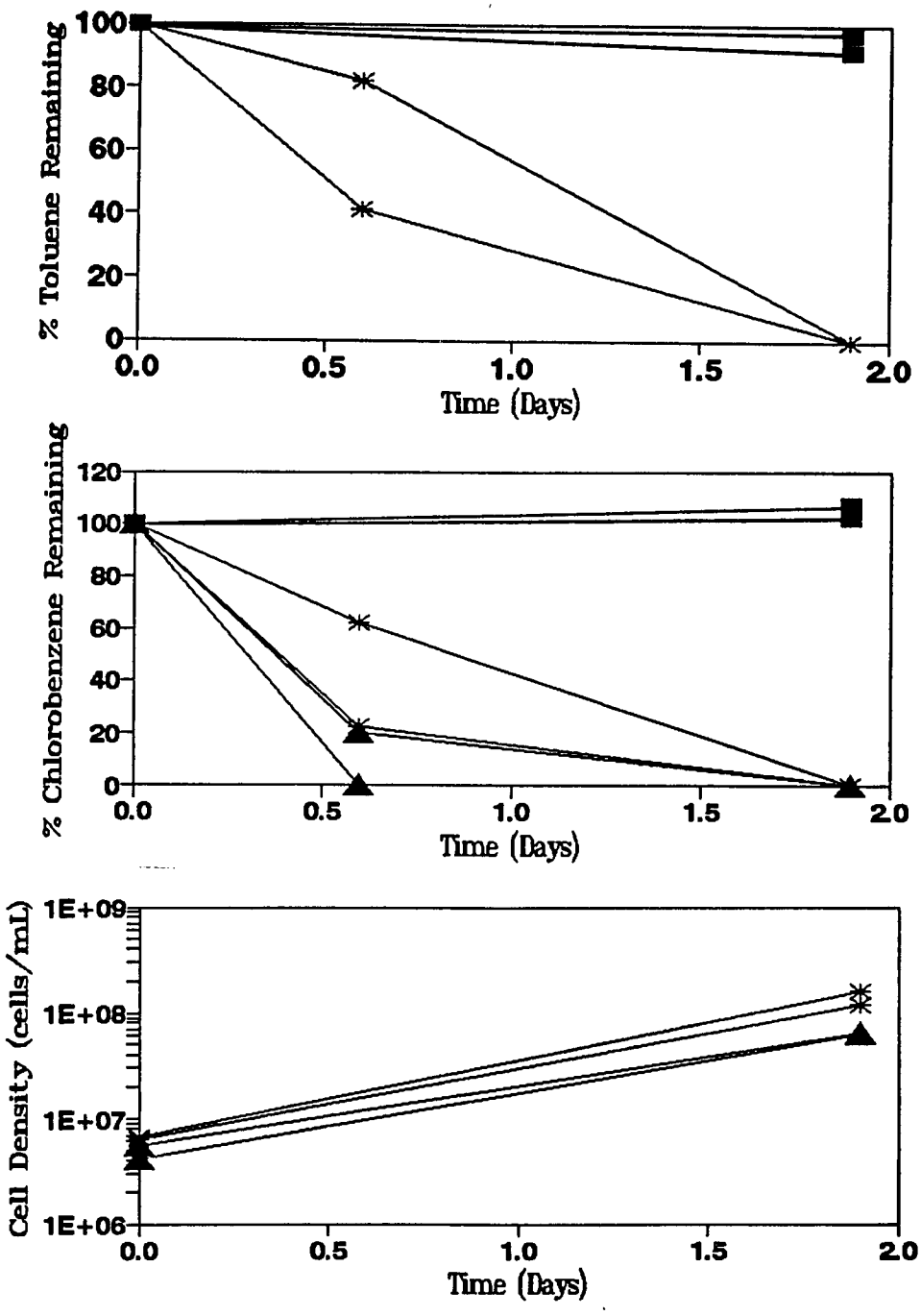

Figure 4.9: Batch experiments using JS6 cells grown on toluene (20 mg/L), then exposed to a mixture of toluene and chlorobenzene (30 mg/L) (✳) or chlorobenzene alone (▲). Abiotic controls were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

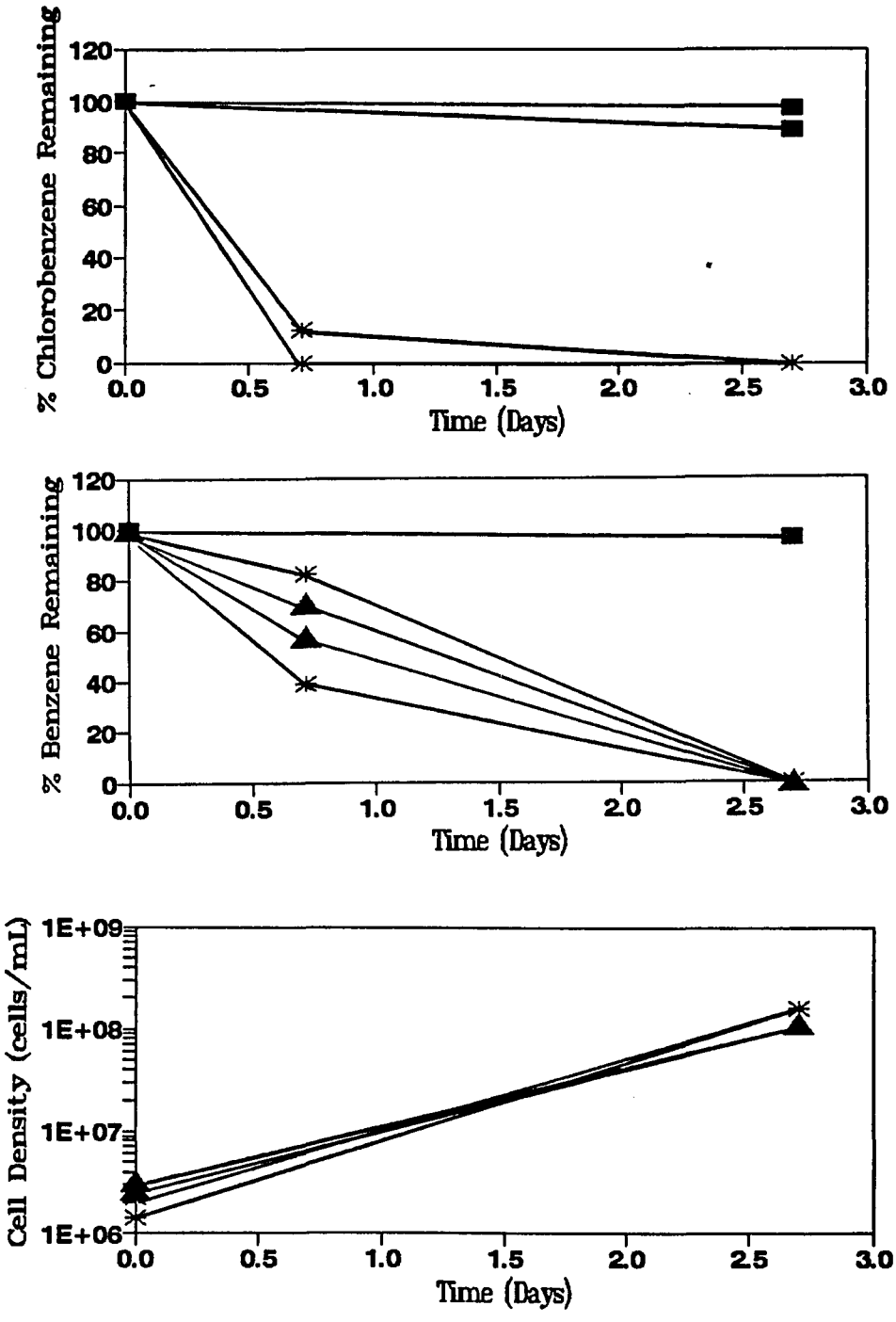

Figure 4.10: Batch experiments using JS6 cells grown on chlorobenzene (30 mg/L), then exposed to a mixture of chlorobenzene and benzene (20 mg/L) (✳) or benzene alone (▲). Abiotic controls were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

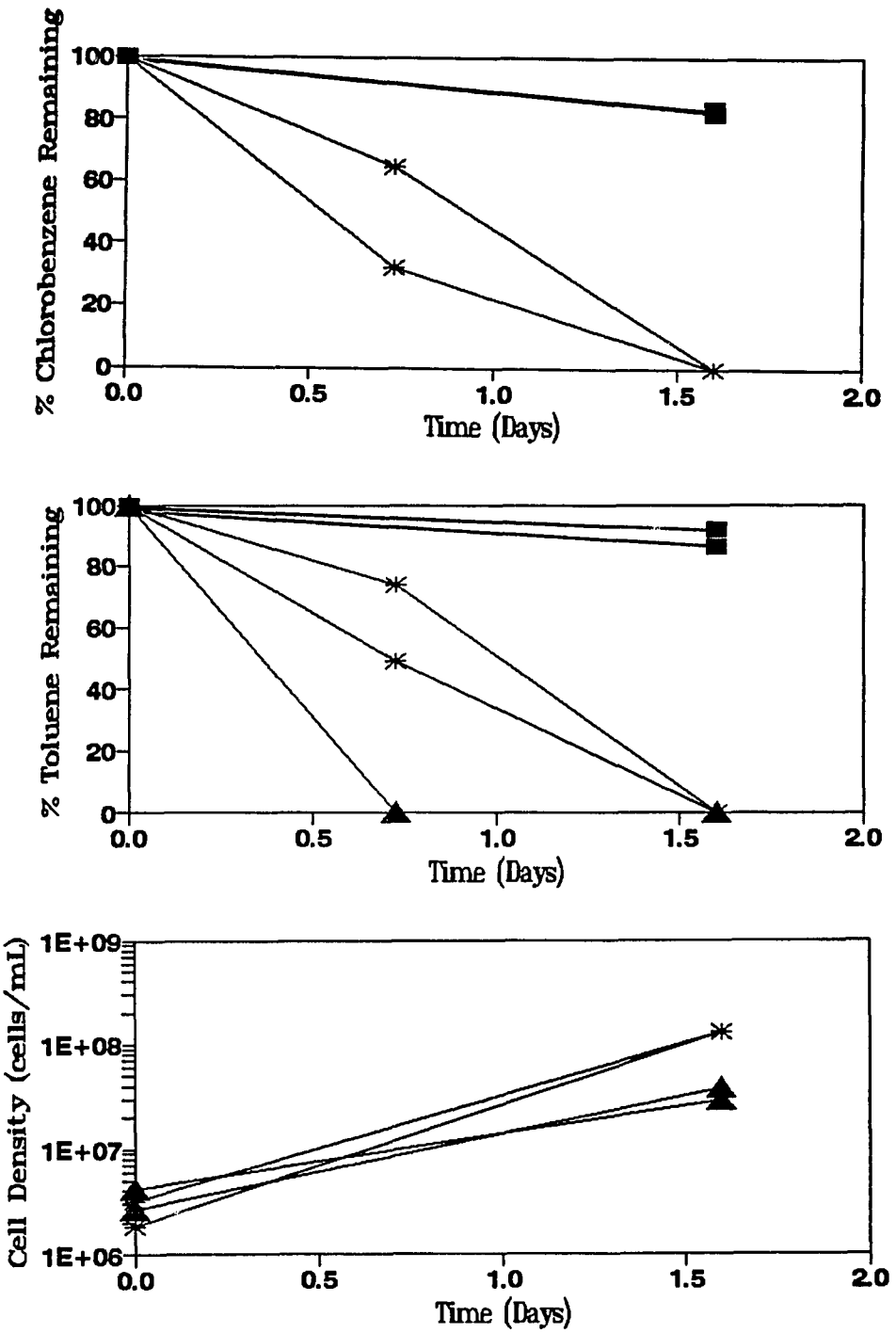

Figure 4.11: Batch experiments using JS6 cells grown on chlorobenzene (30 mg/L), then exposed to a mixture of chlorobenzene and toluene (20 mg/L) (✳) or toluene alone (▲). Abiotic controls were run in duplicate. Each data point represents the average of two GC injections from the same sample vial.

indicate that chlorobenzene could be biodegraded simultaneously with both benzene and toluene. This suggests that the modified ortho pathway can be induced simultaneously with the ortho pathway and with the meta pathway. Toluene is biodegraded more slowly when in the presence of chlorobenzene than when it is the sole substrate. When switched to toluene as a sole substrate, chlorobenzene-grown cells reduced the concentration of toluene to non-detectable levels within 0.75 days. However, when switched to a mixture of toluene and chlorobenzene, it took from 1-1.5 days for the toluene concentration to be reduced to non-detectable levels. The presence of chlorobenzene may somehow slow down the induction of the meta pathway. This could be caused by misrouting of intermediates which may result in the buildup of an intermediate that has a toxic or inhibitory effect on the growth of the culture (Spain, 1990).

4.2 Toxicity Experiments

Batch experiments were done to determine the acute toxicity of benzene and chlorobenzene to JS6. Acute toxicity was defined to be the point at which no growth was observed as measured by a change in turbidity. The results (Figure 4.12) indicate that the toxic level of benzene to JS6 lies between 144 - 660 mg/L and the toxic level for

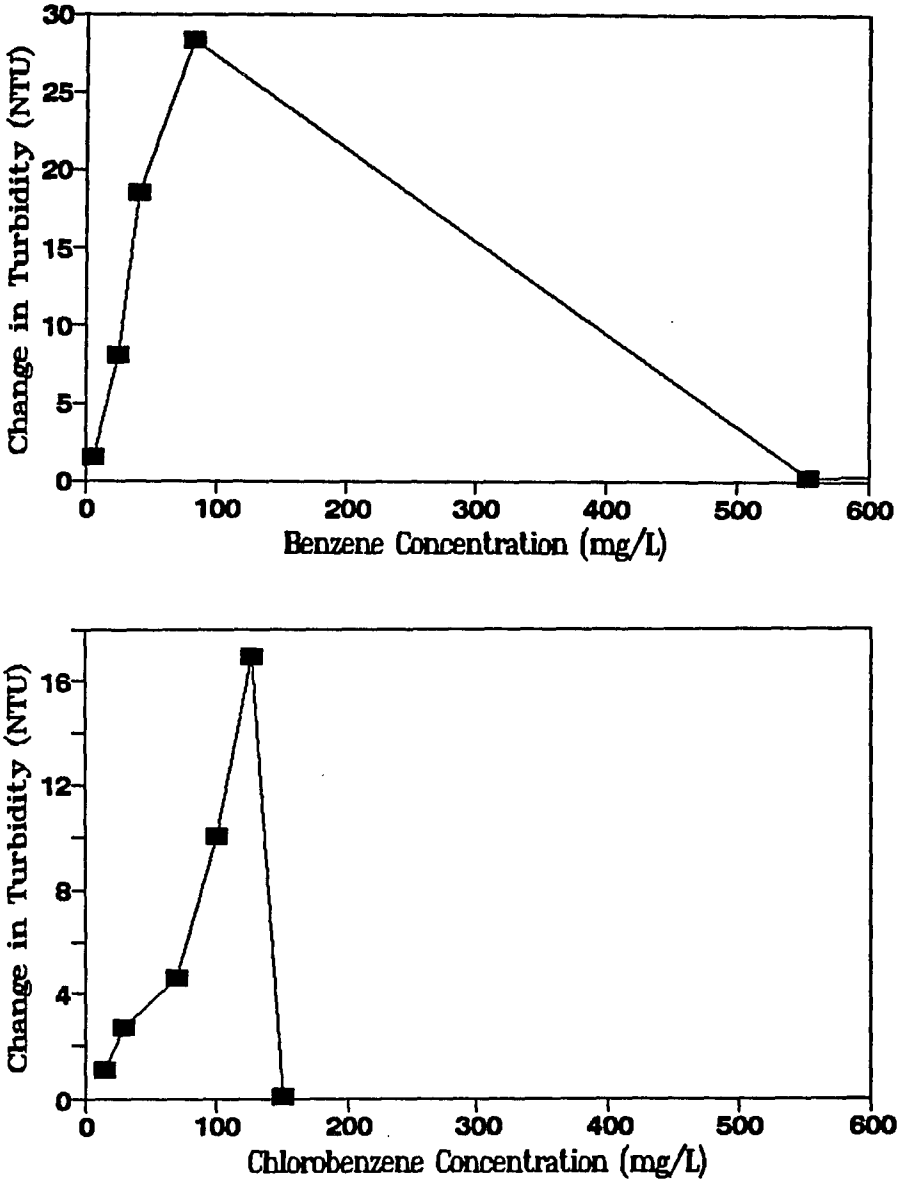

Figure 4.12: Effect of varying concentrations of benzene and chlorobenzene on the growth of JS6 in liquid medium.

chlorobenzene lies between 113 - 134 mg/L.

4.3 Enzyme Induction Experiments

Batch experiments were conducted concurrently with the toxicity experiments to determine if the enzymes required for the initial breakdown for benzene and chlorobenzene were constitutive or inducible. Chloramphenicol was added to the batch test tube at a concentration sufficient to inhibit growth (Table 3.2). Chloramphenicol inhibits protein synthesis by interfering with the transfer of amino acids from their soluble RNA complex to the ribosomes. No cell growth or substrate utilization was observed in any of the benzene or chlorobenzene test tube cultures to which chloramphenicol had been added. This indicates that the enzymes responsible for the initial breakdown of benzene are inducible. The initial cell density of these cultures was sufficiently high enough so that if the enzymes were constitutive, some substrate utilization would have been observed.

Chapter 5

Conclusions

The following conclusions are justified on the basis of the data and discussion presented here:

1. The presence of a non-aromatic substrate (yeast extract or glucose) enhanced the biodegradation of benzene, chlorobenzene, and toluene. The data suggest that the presence of a non-aromatic substrate decreases the lag time prior to the initiation of degradation of the aromatic compound. The non-aromatic compound may contain a substantial fraction of amino acids which may be an efficient source for building proteins. Thus, when the enzymes required for degradation of the aromatic compound are needed, the proteins can be manufactured more quickly and thereby reduce the lag time associated with enzyme induction.

2. Enzyme induction experiments indicated that the initial enzymes required for the breakdown of the aromatic compounds tested are inducible, not constitutive. No cell growth or substrate utilization was observed in dense liquid cultures containing chloramphenicol (protein synthesis inhibitor) and benzene or chlorobenzene.

3. JS6 has the ability to simultaneously degrade two aromatic compounds when the following mixtures were tested; toluene and benzene, toluene and chlorobenzene, and chlorobenzene and toluene. This indicates that JS6 has the ability to induce the enzymes for any two of the three pathways it possesses simultaneously.

4. The acute toxicity levels observed for benzene (144 - 660 mg/L) and chlorobenzene (113 - 134 mg/L) are magnitudes higher than the concentrations these compounds are typically found at in groundwater contamination sites. If JS6 were to be used in a groundwater treatment application, it would most likely not be harmed from the standpoint of toxicity.

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