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I am submitting herewith a thesis written by Thomas J. Abraham, Jr. entitled "The Degradation of Phenanthrene by a Beijerinckia Species." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemical Engineering.

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4

THE DEGRADATION OF PHENANTHRENE BY A *BEIJERINCKIA* SPECIES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Thomas J. Abraham, Jr.

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ABSTRACT

The purpose of this study was to determine the effects of temperature and pH on the degradation rate of phenanthrene by a Beijerinckia sp.

Batch experiments were employed for the experimentation with a growth medium consisting of basic mineral salts, yeast extract (0.5 grams/liter), phosphate buffer (0.1 molar) and approximately 1.29 ppm phenanthrene.

The effects of temperature at a pH of 7.0 on the specific growth rate of the Beijerinckia was Arrhenius in form exhibiting an "activation energy" of growth of approximately 16 kilocalories/gram-mole. The effect of temperature on phenanthrene degradation rate was non-Arrhenius, with phenanthrene degradation commencing at a temperature of approximately 19 °C and increasing rapidly to a temperature of approximately 25 °C. Between the temperatures of 25 °C and 29 °C the degradation rate remained essentially constant, and once the temperature was greater than 29 °C, the degradation rate declined rapidly. The maximum cell specific growth rate of 0.730 $(\text{hours})^{-1}$ occurred at 30 °C ± 2 °C and a pH of 7.0 ± 0.20. The maximum phenanthrene degradation rate of 3.59×10^{-11} milligrams of phenanthrene/cell hour occurred at 26 °C ± 2 °C and a pH of 7.0 ± 0.20.

The pH behavior of the experimental system at 25 °C exhibited a sharp maximum in terms of both specific growth rate and

phenanthrene degradation rate. The optimum pH for growth and phenanthrene degradation was 7.0 ± 0.2.

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LIST OF SYMBOLS

B	Bacterial concentration defined in equation 2.10.
C_f	Molar density of solvent, moles/cubic centimeter.
D	Bacterial diameter.
D_{AB}	Diffusivity of solute (A) in solvent (B), square centimeters/hour.
E	Activation Energy of reaction used in Arrhenius relationship.
J_A	Diffusive molar flux, moles/square centimeter-hour.
K	Reaction constant calculated from Arrhenius relationship or defined in equation 2.10. In equation 2.10, K is based on specific population growth parameters.
k	Zero-order rate constant (milligrams of phenanthrene/cell-hour) or mass transfer coefficient in equation 5.1.
KI	Inhibition constant used in equation 2.9.
K_m	Monod constant (equation 2.8). Defined as the substrate concentration at which the reaction velocity is 1/2 the maximum reaction velocity.
K_s	Constant defined in equation 2.10, based on specific growth parameters.
M_B	Molecular weight of solvent B in kilograms/kolomole.
N_A	Transport rate of Component A in moles/square centimeter-hour.
PAH	Polyaromatic hydrocarbon.
Phe	Phenanthrene.
r^2	Regression coefficient.
S	Substrate concentration used in equation 2.10.
T	Temperature (either °C or K).
t	Time
u	Solution viscosity in kilograms/meter-second.

LIST OF SYMBOLS (Continued)

V Volume of liquid in liquid film surrounding bacteria.

V_A Solute molal volume at boiling point in cubic meters/kilomole.

Z Reaction rate in equations 2.8 and 2.9 in moles/unit time.

Z_{MAX} Maximum reaction rate in equations 2.8 and 2.9.

Greek Symbols

δ Film thickness.

ε Association factor in equation 5.5.

μ Specific growth rate of bacteria in (1/hour).

CHAPTER I

INTRODUCTION

Presently two basic technologies exist that can convert coal to useable liquid and gaseous fuels--coal gasification and coal liquefaction. Both of these technologies have one problem in common: they have the potential to pollute the environment and produce oily water which contains such harmful and potentially dangerous chemicals as phenols, organic nitrogen compounds, oils, tars and in particular, polyaromatic hydrocarbons (Harrison, et al., 1974).

Some sources of oily water in the indirect liquefaction processes are the raw gas quench and refinery waste water. In direct liquefaction, some sources of oily water are wash water from desalter units and process steam which is used to remove salts and chlorides that may be harmful to refinery processes (Probstein and Hicks, 1982).

Considerable attention has been given to removal of some of the more toxic components of oily water such as phenols (Kobayashi and Rittman, 1982). Less attention, however, has been given to removal of some of the more inert components such as polyaromatic hydrocarbons (Harrison, et al., 1974), of which phenanthrene is one example.

Bacteria and fungi (Kobayashi and Rittman, 1982) have demonstrated the ability to metabolize many different organic and non-organic molecules. In particular, bacteria have demonstrated the ability to degrade a variety of polyaromatic hydrocarbons such as naphthalene, anthracene, and phenanthrene.

The objective of this work is to quantify the rate of degradation on one polyaromatic hydrocarbon, phenanthrene, which is typically present in waste water derived from coal conversion processes, by a Beijerinckia B8/36 which is known to degrade this class of compounds (Sherrill, 1982).

CHAPTER II

BACKGROUND

Microorganisms, in either laboratory or field applications, have demonstrated the ability to perform many tasks which are of interest to applied scientists and engineers. In the following sections, a few of the many applications of microorganisms to areas of engineering interest will be briefly surveyed.

2.1 Non-organic Processes: Leaching of Pyritic Sulfur from Coal

Leaching of sulfur from iron ore and coal has been demonstrated using acidophilic (Thiobacillus ferrooxidans) and certain thermophilic organisms (Murr and Mehta, 1981).

The non-bacterial leaching of pyritic sulfur from coal involves the oxidation of FeS_2 to soluble sulfate by the following reactions:

$$FeS_2 + 3.5O_2 + H_2O = FeSO_4 + H_2SO_4 \qquad (2.1)$$

$$FeS_2 + 3O_2 + H_2O = 2H_2SO_4 + Fe^{3+} \qquad (2.2)$$

$$FeS_2 + Fe_2(SO_4)_3 = 3FeSO_4 + 2S \qquad (2.3)$$

Reactions 2.1 and 2.2 proceed very slowly, while, at sufficiently high temperatures, reaction 2.3 can be quite rapid.

In the presence of catalyzing bacteria, the rate of reaction 2.1 is substantially increased, and the ferrous sulfate formed in this reaction is converted to ferri sulfate by the reaction

$$2FeSO_4 + 0.5O_s \overset{\text{bacteria}}{=} Fe_2(SO_4)_3 + H_2O) \qquad (2.4)$$

This reaction also provides $Fe^{(3+)}$ as an oxidant for reaction 2.3. Further, when bacteria are present in the leaching environment, elemental sulfur is converted to sulfuric acid by the reaction

$$2S + 3O_2 + 2H_2O \overset{\text{bacteria}}{=\!=} 2H_2SO_4 \qquad (2.5)$$

Bacterial leaching will have no effect on reaction 2.3.

Extensive work has been done to demonstrate that Thiobacillus ferrooxidans has the potential to remove significant amounts of pyritic sulfur in coal (Olsen, 1980) at 35 °C. Typically, to get significant removal of pyrite from coal, the bacterial leaching must be allowed to proceed for three to four days. The use of thermophilic bacteria in addition to Thiobacillus ferrooxidans and elevation of the leaching temperature to 55 °C will significantly increase both the rate as well as the total amount of pyrite removed. Further, bacterial leaching could possibly lower the cost of sulfur removal by as much as 30% over more convenient means of sulfur removal from coal (Detz and Barvinchak, 1979). Chakrabarti, 1980 has also presented similar results involving bacterial leaching of pyrite from iron ore.

2.2 Microbiological Degradation of Organic Compounds

Naturally occurring and genetically engineered microorganisms have been observed to degrade a multitude of organic compounds (Kobayashi and Rittmann, 1982). Table 2.1, taken from Kobayashi and Rittman, is an extensive, but by no means complete list of some

TABLE 2.1

Examples of Chemicals Which are Metabolized by Various Microorganisms

Compound	Organism(s)
Aliphatics (nonhalogenated)	
Acrylonitrile	Mixed culture of yeast mold, protozoa bacteria; activated sludge
Aliphatics (halogenated)	
Trichloroethane	Marine bacteria
Trichloromethane	Sewage sludge
Trichloroethane, trichloromethane, methyl chloride, chloroethane, dichloroethane, vinylidiene chloride, trichloroethylene, tetrachloroethylene, methylene chloride, dibromochloromethane, bromochloromethane	Soil bacteria
Trichloromethanes, trichloroethylene, tetrachloroethylene	Methanogenic (7) culture
Trichloroethane, trichloromethane, tetrachloromethane, dichloroethane, dibromochloromethane, 1,1,2,2-tetrachloroethane, bis-(2-chloroisopropyl) ether, bromoform, bromodichloromethane, trichlorofluoromethane, 1,1-dichloroethylene, 1,2-dichloroethylene, 1,3-dichloropropylene, 1,3-dichloropropylene, 1,2-transdichloroethylene	Sewage sludge
Aromatic compounds (nonhalogenated)	
Benzene	*Pseudomonas putida* Sewage sludge Stabilization pond microbes
Toluene	*Bacillus* sp. *P. putida*

TABLE 2.1 (Continued)

Compound	Organism(s)
Naphthalene	Agnenellum, Oscillatoria Anabaena
	Cunninghamella elegans
	Microcoleus sp., Nostoc sp., Coccochloris sp., Aphanocapsa sp., Chlorella sp., Dunaliella sp., Chlamydamonas sp., Cylindriotheca sp., Amphora sp.
	Pseudomonas, Flavobacterium, Alcaligenes, Corynebacterium, Aeromonas, Flavobacterium Nocardia
	Stream bacteria
Pyrene	Stabilization pond organisms
Fluoranthene	Sewage sludge
Anthracene	Stream bacteria
Phenanthrene	Beijerinckia
Benzo(a)anthracene (BA)	Cunninghamella elegans
Dibenzanthracene	Activated sludge
Biphenyl	Beijerinckia
	Oscillatoria sp.
	Pseudomonas putida
Polycyclic aromatic hydrocarbons (halogenated)	
PCBs (mono- and dichlorobiphenyls)	Pseudomonas, Vibrio, Spirillum, Flavobacterium
	Achromobacter
	Chromobacter
	Bacillus, Nocardia
4-Chlorobiphenyl; 4,4-dichlorobiphenyl; 3,3´-dichlorobiphenyl	Fungi

TABLE 2.1 (Continued)

Compound	Organism(s)
Pesticides	
Toxaphene	Corynebacterium pyrogenes
Heptachlorobornane	Micromonospora chalcea Bovine rumen fluid
Lindane	Chlorella vulgaris Chlamydamonas reinhardtii
	Chlosteridium sp., Pseudomonas Soil bacteria
	Sewage sludge
Dieldrin	Anacystis nidulans Agmeneloum quardiplicatum Pseudomonas Rumen fluid Actinomycetes
DDT (1,1´-bis(p-chlorophenyl)-2, 2,2-trichloroethane)	Klebsiella pneumoniae, E. coli, Aerobacter aerogenes, Pseudomonas, Clostridium, Proteus vulgaris
	Sewage Soil bacteria
	Rumen bacteria Yeast Trichoderma viridae Fusarium oxysporum
	Mucor alterans Cylindrotheca, Closterium, Dunaliella, Anaerobic sludge Nocardia, Streptomyces Hydrogenomonas
Parathion	Bacillus subtilus, Rhizobium, Chlorella pyrenoidosa, soil bacteria

TABLE 2.1 (Continued)

Compound	Organism(s)
Phorate sulfoxide	Soil bacteria
Pentachloronitrobenzene (PCNB)	Aspergillus niger, Fusarium solani, Glomerella congulata, Helminthosporium victoriae, Myrothecium, Penicillium, Trichoderma viridae
Methoxychlor	Nocardia sp., Streptomyces sp. Aerobacter aerogenes
Acrolein	Site water (microbes)
Aldrin	Site water (microbes) Sewage sludge
Endosulfan	Fungi, bacteria, soil actinomycetes
Endrin	Pseudomonas sp., Micrococcus sp., yeast
	Sewage sludge
Chlordimeform	Chlorella, Oscillatoria
Kepone	Treatment laggon sludge
Diuron	Mixed culture of fungi and bacteria; mineralization; single isolates ineffective
Nitrosamines	
Dimethylnitrosamine	Rumen organisms Rhodopseudomonas capsulata
Phthalate esters	Micrococcus Sediment-water

Source: Kobayashi, Hester and Rittmann, Bruce E. (1982). Microbial Removal of Hazardous Organic Compounds. Environ. Sci. Technol., 16, p. 170.

organic compounds and microorganisms that can metabolize them. The compounds listed in Table 2.1 range from simple hydrocarbons and halogenated aliphatics to both halogenated and non-halogenated aromatic and polyaromatic compounds. Even pesticides are listed as substrates for some bacteria. Though not listed in Table 2.1, bacteria and fungi have been observed to degrade synthetic oil (Sherrill, 1982), bituminous hydrocarbons found in oil and sand deposits (Wyndham and Costerton, 1981), oil sludge (Dibble and Bartha, 1979), petroleum (Walker et al, 1975), and even lignite coal (Cohen and Gabriele, 1982).

Considering the broad classes of compounds that can be degraded by microorganisms, it seems probable that a bacterium or fungus may be discovered or be engineered that possesses the ability to attack the molecular structure of coal.

2.3 Biodegradation of Polyaromatic Hydrocarbons

Polyaromatic hydrocarbons (PAH) occur both naturally and are introduced into the ecosphere by coal slurry waste waters and high temperature pyrolysis of hydrocarbons (Harrison et al, 1974).

Since PAHs are potentially dangerous to the health of humans (Zedeck, 1980), there has been a substantial amount of work done to find both naturally occurring bacteria (Sherrill, 1982) and genetically engineered bacteria (Gibson et al, 1975) that will degrade PAHs in natural systems. Although several PAHs are known to be degraded by bacteria, only the metabolism of naphthalene and phenanthrene will be discussed in the following sections.

2.3.1 The Microbial Metabolism of Naphthalene

Figure 2.1 schematically depicts the metabolism of naphthalene (Cerniglia, 1981) in biological systems. The initial steps of naphthalene metabolism are carried out by enzymes known as oxygenases which are unique to hydrocarbon-utilizing organisms (Stainer and Orston, 1977).

Initially, naphthalene is hydrated to form cis-1,2-dihydroxyl-2-dihydronaphthalene. Then, the cis-1,2-dihydroxy-1,2-dihydronaphthalene is transformed to 1,2-dihydronaphthalene by a dehydrogenase enzyme. The 1,2-diydronaphthalene is transformed to cis-2'-hydroxybenzal-pyruvate which is subsequently cleaved to form pyruvate and salicylal-dehyde. In the following step, the salicylaldehyde is oxidized to salicylate and then further oxidized to catechol. The catechol can then be metabolized through the beta-ketoadipate pathway (Stainer, 1973), the major bacterial pathway of aromatic compound utilization. The beta-ketoadipate pathway, depicted in Figure 2.2, initially transforms catechol, through several steps, to beta-ketoadipate which, through several subsequent steps, is ultimately transformed to Acetyl-CoA and enters the citric acid cycle and ultimately is transformed into either cell mass or carbon dioxide (Herbes, 1978).

2.3.2 The Microbial Metabolism of Phenanthrene

Phenanthrene metabolism (Sherrill, 1982) depicted in Figure 2.3, is somewhat more complex than that of naphthalene (Evans et al, 1965). Initially, phenanthrene is transformed to cis-1,2-dihydroxy-1,2-dihydrophenanthrene and then immediately to cis-3,4-dihydroxy-3,

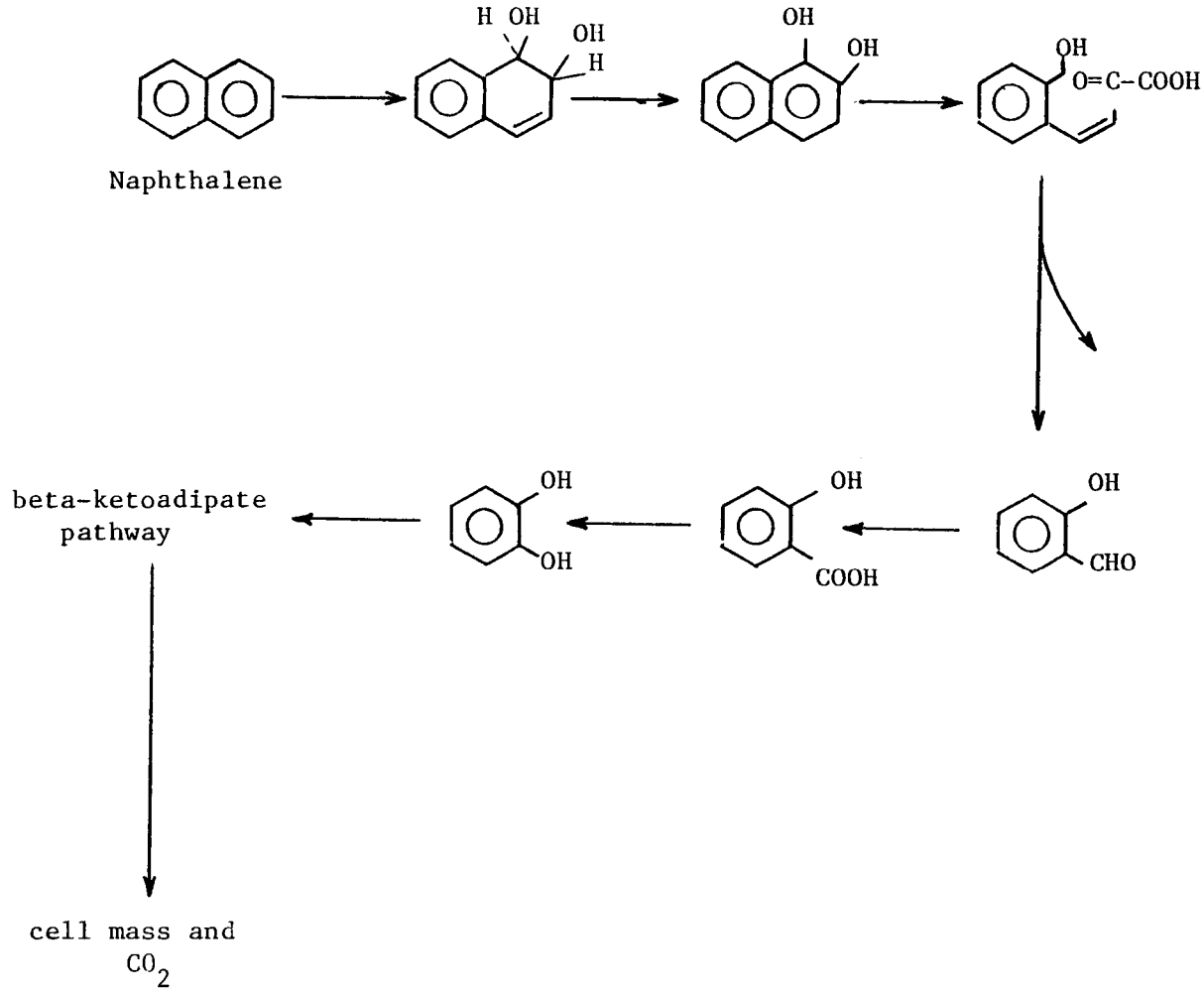

Figure 2.1. Bacterial Metabolism of Naphthalene.

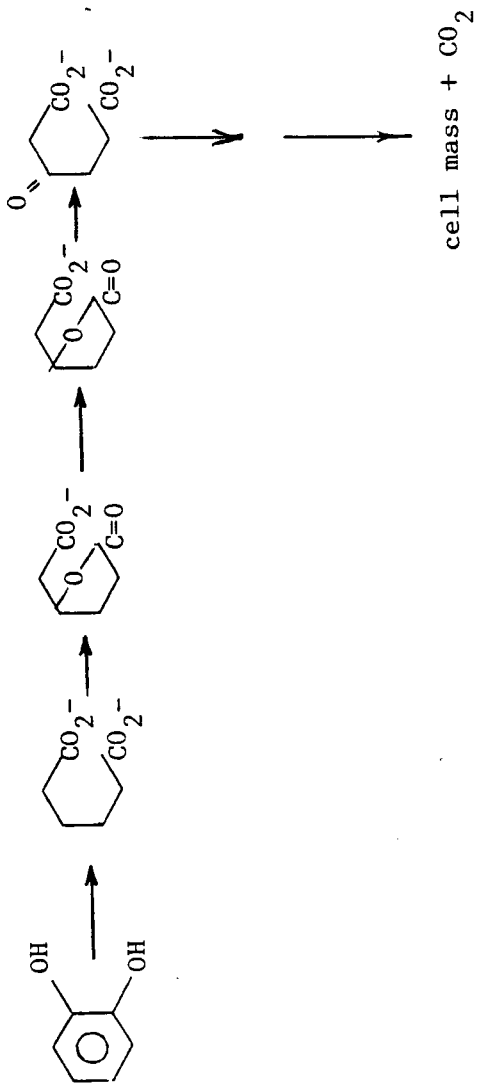

Figure 2.2. The Central Reactions of the Beta-ketoadiapate Pathway in Bacteria.

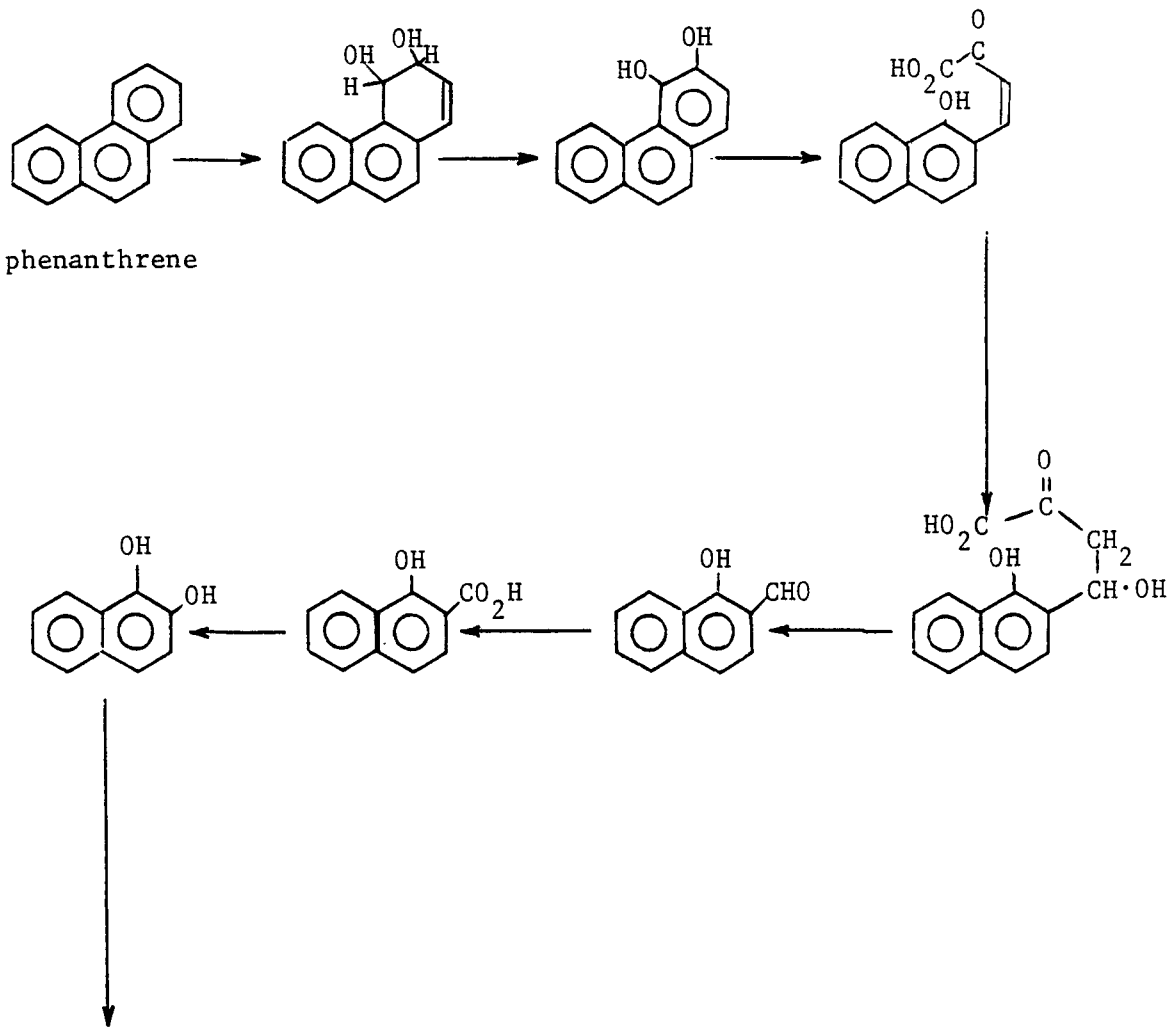

Figure 2.3. Bacterial Metabolism of Phenanthrene.

4-dihydro-phenanthrene which subsequently is cleaved to form cis-4-(1-hydroxy-naphth-2yl)-2-oxobut-3-enoic acid. The cis-4-(1-hydroxy-naphthyl-2yl)-2oxobut-3-enoic acid is further metabolized to pyruvate and 1-hydroxy-2-naphthaldehyde. The aldehyde is then oxidized to 1-hydroxy-2-napthoic acid and decarboxylated to yield 1,2-dihydroxy-naphthalene to which is metabolized through the naphthalene pathway.

The preceding has been intended only as an introduction to the major pathways of naphthalene and phenanthrene metabolism. For a more detailed treatment of the subject, the reader is referred to the cited references.

2.4 A Review of Pertinent Microbial Kinetics

2.4.1 Effects of Temperature on Growth and Metabolism

When the growth of cells is recorded under balance growth (the exponential phase of growth) then, the following equation describes growth (Pirt, 1978):

$$\ln x = \mu t + c \qquad (2.6)$$

where x is the cell concentration, μ is the specific growth rate, t is time, and c is a constant.

When the growth rate is expressed in terms of the specific growth rate μ, then both the growth characterisitcs and substrate utilization of bacteria are generally thought to be Arrhenius in form:

$$K = Ae^{-E/RT} \quad , \qquad (2.7)$$

where K is the reaction rate constant (either specific growth rate or some measure of substrate utilization rate), A is a constant particular to a given application, E is the activation energy of growth or reaction, R is the universal gas constant, and T is the absolute temperature. Therefore, if one plots lnk vs. 1/T, the resulting plot curve should display a straight line with a slope of -E/R. Figure 2.4, adapted from Pirt, demonstrates the application of the Arrhenius equation to the specific growth rate of a bacterium. Note that changes in the slopes of the lines demonstrate a change in rate controlling reactions or in metabolic regulation as temperature is varied.

As temperature increases, there is a point where the cell's metabolism and subsequent growth rate begins to decrease and eventually, the cells die. This effect is generally thought to occur from thermal degradation of one or more of the enzymes which are important to the metabolic regulation of the microorganisms (Freeman, 1979). Thus, in a given application, one might expect to observe an optimum temperature for both cell growth and substrate metabolism. These optimum temperatures may or may not coincide depending upon the particular substrate and microorganisms involved. It must be noted here that secondary or cometabolism may respond differently to temperature than growth or primary metabolism.

One of the objectives of this study is to develop temperature response curves for the degradation of phenanthrene by a Beijerinckia species (sp.) with yeast extract as the primary nutrient source.

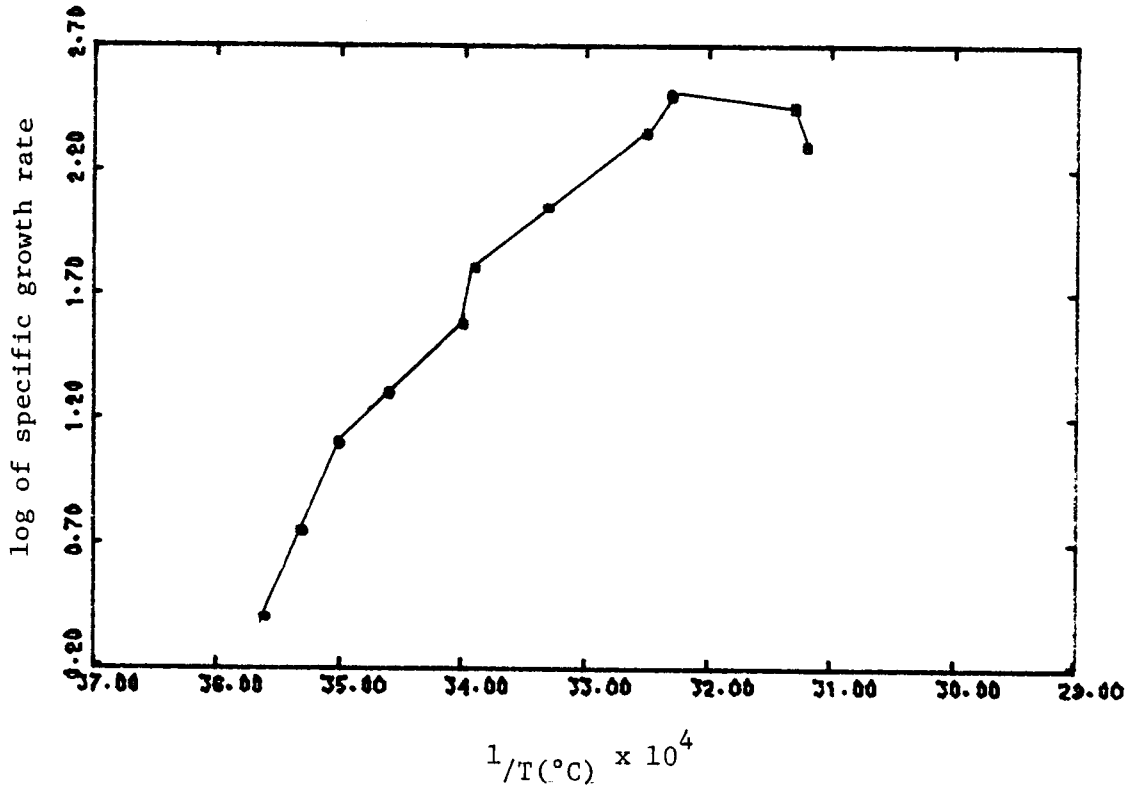

Figure 2.4. Application of Arrhenius Equation to the Specific Growth Rate of Microbes.

Source: Pirt, J.W. (1975). Principles of Microbe and Cell Cultivation. Halsted Press, New York, p. 139.

Because of the complexities of the phenanthrene degradation pathway, there is no reason to presuppose that the temperature-response will be of Arrhenius form. Data obtained from the utilization of phenol in wastewater (Vela and Ralston, 1978), demonstrated that phenol degradation rates did not vary with temperature as would be predicted by an Arrhenius equation, but temperature increased the phenol degradation between 2 and 10 °C, but between 10 and 24 °C, no effect of temperature on phenol degradation rate was observed (Figure 2.5). One of Vela and Ralston's conclusions was that, due to the complex interaction of metabolic pathways involved in phenol degradation, that quite probably, an Arrhenius equation cannot adequately describe such a system.

2.4.2 Effects of pH on Cell Growth and Metabolism

Another topic of this study is to quantify the effect of pH on the degradation rate of phenanthrene by a Beijerinckia sp. The data displayed in Figure 2.6, adapted from Pirt, are typical of the effect of pH on cell growth. Generally, there is a rather narrow range of pH which will support cell growth even though the cell membrane, to some extent, makes the cell interior somewhat insensitive to extracellular pH changes (Pirt, 1977). Also changes in extracellular pH can often cause dramatic changes in cell metabolism, and these changes might be different in terms of growth rate and primary or secondary substrate utilization rates.

It is quite apparent that cell properties change over narrow pH variations, but the molecular basis of the effects are not well understood.

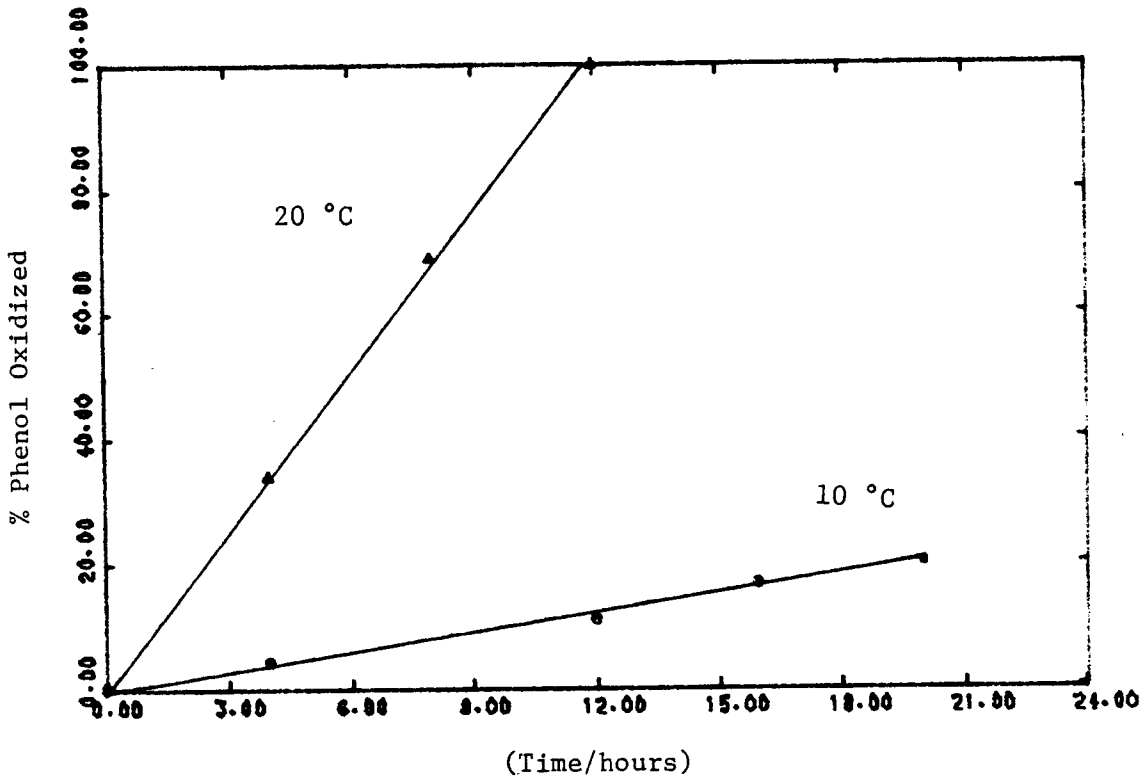

Figure 2.5. The Effect of Temperature on Phenol Oxidation by a Mixed Culture.

Source: Vela, G.R. and Ralston, J.R. (1978) - The Effect of Temperature on Phenol Degradation in Wastewater. Can. J. Microbio, 24, p. 1365.

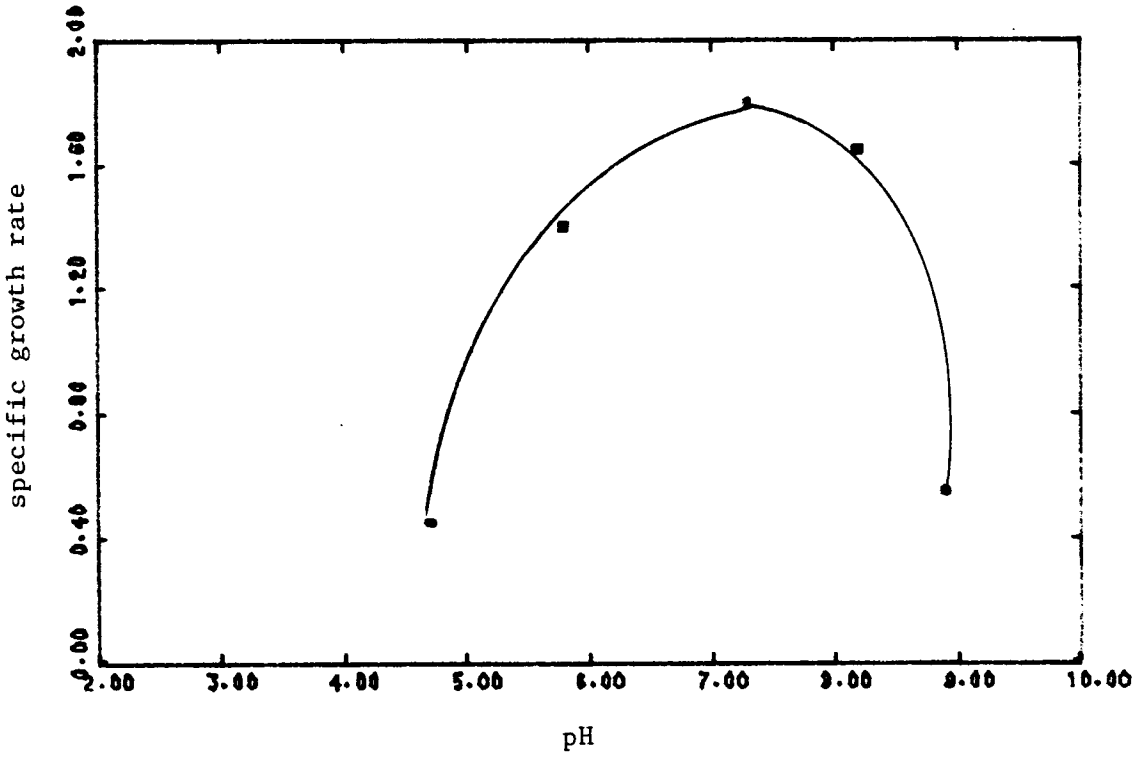

Figure 2.6. Effects of Medium pH on Maximum Bacterial Growth Rates.

Source: Pirt, J.S. (1975). Principles of Microbe and Cell Cultivation. Halsted Press, New York, p. 145.

2.4.3 Effects of Substrate Concentration on Microbial Kinetics

Substrate concentration, in primary cellular metabolism is generally thought to be of primary importance in determining the rate of substrate metabolism. There are two regions of growth supporting regimes. One is the so-called substrate-limited regime, and the other is the so-called substrate-saturated regime. In substrate-limited conditions, increases in the amount of substrate will yield an observable increase in the reaction rate of the substrate. The functional relationship proposed to explain this behavior is the well known Monod equation (Bailey and Ollis, 1977)

$$Z = Zmax\left(\frac{S}{Km + S}\right) \tag{2.8}$$

where Zmax is the maximum reaction rate corresponding to infinite substrate concentration expressed in mass or moles/time, Z is the cell reaction rate expressed in mass or moles/time, Km is an empirically determined constant, and S is the substrate concentration.

Sometimes, as substrate concentration increases, there is a point where further increase in substrate concentration actually will decrease cell reaction rates. When this behavior is observed, the cell is said to be in the substrate-saturated regime. The specifics of substrate inhibition are not well understood. A functional form explaining saturating conditions is the well known Haldene Equation (Bailey and Ollis, 1977)

$$Z = \frac{Zmax\ S}{Km + S + S^2/KI} \tag{2.9}$$

where Z is the cell reaction rate expressed in moles or mass per unit time, KI is an empirically determined inhibition constant, the other symbols are the same as the equation 2.8.

Although the concepts explained in the above discussion are generally held to be applicable in biochemical systems, their application is not necessarily universal. Boethling and Alexander (1981), in studying microbial degradation of organic compounds at trace levels demonstrated that Monod kinetics may not be applicable to such systems (Figure 2.7). In their studies, Boethling and Alexander demonstrated that, at low to trace concentrations, reaction rates for some organic compounds were far below that what the Monod kinetics predicts and further, upon varying concentration the observed degradation kinetics may change.

A modified Monod equation, applicable to cultures using organic pollutants as a sole carbon source, has also been proposed (Paris et al, 1981). The functional form of this modified Monod equation is

$$\frac{-dS}{dt} = \frac{KSB}{K_s+S} \qquad (2.10)$$

where S is the substrate concentration, B is the concentration of bacteria, and K and K_s are constants determined by specific population growth parameters.

If the substrate concentration is much less than K_s, then equation 2.10 reduces to

$$\frac{-dS}{dt} = \frac{K}{K_s} SB \qquad (2.11)$$

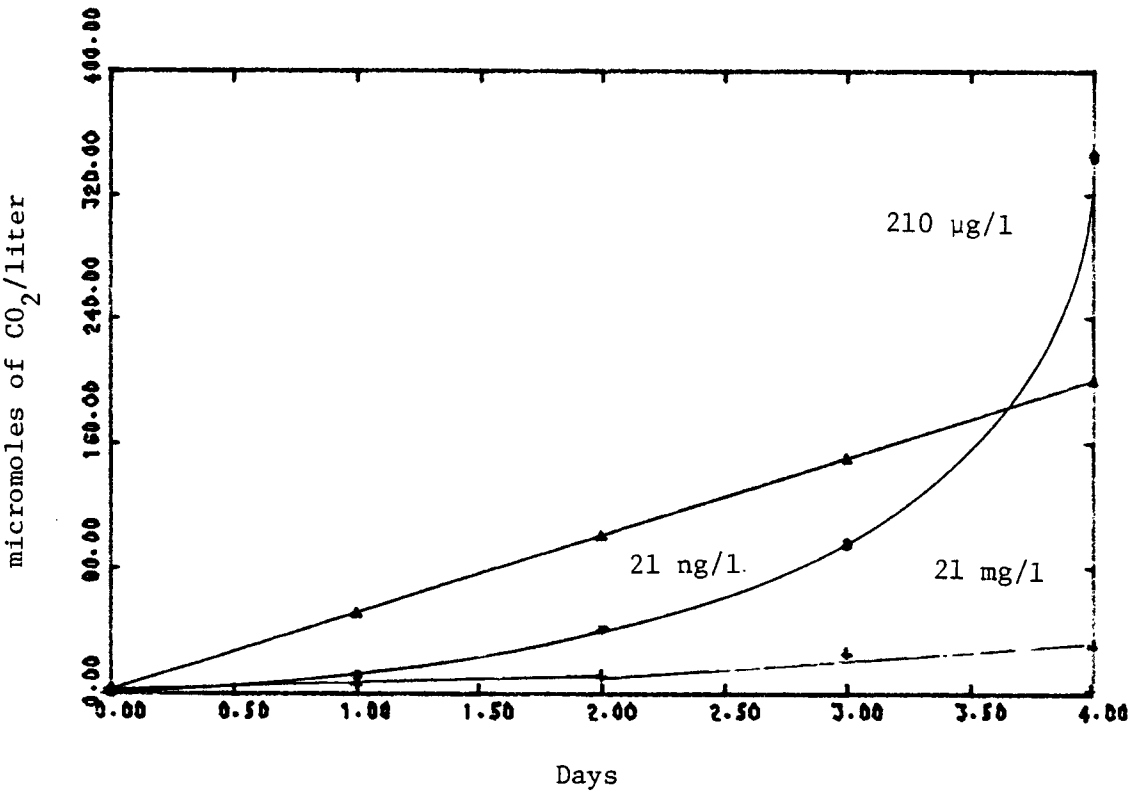

Figure 2.7. Formation of CO_2 from Diethanolamine Added to Stream Water at Three Initial Concentrations.

Source: Boething, R.S. and Alexander, M. (1979). Microbial Degradation of Organic Components at Trace Levels. Enviro Sci. Tech., 13, p. 989.

which demonstrates that in this case, the observed reaction kinetics would be first order with respect to substrate concentration and first order with respect to cell concentration. When the substrate concentration is much greater than K_s equation 2.10 is reduced to

$$\frac{-dS}{dt} = KB \qquad (2.12)$$

which represents a zero-order degradation rate with respect to substrate concentration and a first order rate with respect to cell concentration.

The kinetics of some PAH systems have been explored (Herbes and Schwall, 1978). Using the PAH's anthracene, naphthalene, and benz(a)pyrene, Herbes and Schwall observed that the degradation of naphthalene and anthracene followed first order kinetics while that of benz(a)pyrene followed zero order kinetics where each PAH was used as a sole carbon source in accordance with equation 2.12. To the knowledge of the author, no data is available on the kinetics of phenanthrene degradation. With the above data in mind, it is logical to assume that phenanthrene degradation probably will not follow traditional Monod behavior, but will, more than likely, follow some zero or first order degradation rate.

In this study, phenanthrene will represent only about 0.1% of the total substrate available to the cells, with the rest being yeast extract (Difco).

CHAPTER III

EXPERIMENTAL METHODS AND PROCEDURES

3.1 Objectives of Work and Experimental Plan

The objectives of this experimental work were to quantify the degradation rate of phenanthrene by a mutant Beijerinckia sp. with respect to temperature and pH. Specifically, the focus of the experimentation was on quantifying the phenanthrene concentration as a function of time and concurrently determining the absolute cell concentration as a function of time. Preliminary experimentation indicated appreciable reaction and cell growth occurred over periods of about five hours, a temperature range of 15 to 40 °C, and a pH range of 5.5 to 8.5. Also, preliminary experimentation indicated that the reaction was zero order with respect to phenanthrene concentration, with the maximum reaction rate at 25 °C and a pH of 7.0. Thus, to characterize the phenanthrene degradation rate with respect to temperature, a pH of 7.0 was chosen with a nominal temperature range of experimentation from 15 to 40 °C using steps of about 5 °C. The characterization of the reaction with respect to pH was done at 25 °C in steps of 0.5 pH units from pH of 5.5 to a pH of 8.5.

Typically, the experimentation was quite straight forward with very few constraints and problems. The temperature measurements were accurate to approximately ± 1.0 °C, the pH meter was accurate

to ± 0.10 pH units, and all controlled temperature incubators were observed to control experimental temperature to approximately ± 1.0 °C.

The primary problem involved in experimentation occurred when attempting to dissolve the phenanthrene in water. Phenanthrene has a solubility limit in water of only 1.29 milligrams/liter (Mackay and Shiv, 1977) thus, this limit suggests that dissolving phenanthrene in water would be a very slow process. To alleviate the problem of dissolving phenanthrene in water, one gram of phenanthrene was dissolved in 200 milliliters methanol, water added to the mixture, the methanol distilled off, and the remaining water-phenanthrene mixture filtered through glass fiber.

3.2 Preparation of the Beijerinckia and Growth Medium

A mutant Beijerinckia sp. B8/36 known to degrade phenanthrene was obtained from the laboratory of Dr. Gary Saylor at The University of Tennessee Department of Ecology. Originally, the Beijerinckia was obtained from Gibson at The University of Texas. The Beijerinckia sp. was grown on yeast extract agar. Reagent grade phenanthrene was employed for the degradation study.

The preparation of the growth medium was as follows: one gram of phenanthrene was dissolved in approximately 200 milliliters of methanol. Two liters of distilled water were then added to the methanol-phenanthrene mixture. Following the water addition, the mixture was placed on a hot plate, stirred, and allowed to boil until the methanol had distilled off. After the methanol removal, the

water and phenanthrene mixture was filtered until optically clear using a glass fiber filter constructed from glass fiber compressed into a funnel. The solution was allowed to return to room temperature and filtered again. Following the second filtration, mineral salts and yeast extract were added to the solution. Table 3.1 shows the composition of the growth medium.

To control pH, the appropriate phosphate buffer (always 0.1 molar) was prepared and added to the medium. The medium was then autoclaved at 121 °C for 20 minutes.

The Beijerinckia sp. cells, after aseptic transfer from refrigerated stock cultures, were grown overnight in the previously mentioned growth medium on a rotary shaker at 100 rpm. The cells were then transferred to reaction flasks which contained 200 milliliters of the reaction solution that had been allowed to equilibrate to the reaction temperature for approximately ten hours. The reaction flasks themselves were 500 milliliter glass Erlynmeyer flasks which were sterilized in an autoclave at 121 °C for twenty minutes and stoppered with foam rubber stoppers. The incubation temperature was controlled by allowing the reaction flasks to either shake in a controlled temperature water-bath or in a controlled temperature incubator. pH control was not necessary since the pH of the medium did not change during experimentation. At this point, experimentation commenced.

The cells were quantified periodically using a Petroff-Hausser and Helber microscope counting chamber.

TABLE 3.1

Composition of Growth Medium

Compound	Amount of compound added to solution
KNO_3	2.0 gram/liter
$MgSO_4 \cdot 7H_2O$	0.1 gram/liter
Fe chelate	0.004 gram/liter
$CaCl_2$	0.1 gram/liter
Trace Elements (HBO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSo_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$)	10 milliliters/liter
Phosphate buffer	0.1 molar in phosphate
Yeast extract	0.5 grams/liter

3.3 Gas Chromatography

Gas chromatography is a common method employed to quantify the amount of polyaromatic hydrocarbons in aqueous environments (Sherill et al, 1981). To quantify the amount of phenanthrene in the reaction mixture, the aqueous solutions were extracted with hexane in the following manner: 30.0 milliliters of solution were added to a 100 milliliter Erlynmeyer flask with 3.0 milliliters of glass distilled hexane. The mixture was well shaken and allowed to settle for ten minutes. The hexane phase was then removed and transferred to a clean 10 milliliter test tube. The hexane-phenanthrene solution was analyzed in triplicate using a Varion Series 3700 gas chromatograph equipped with an SP2100 capillary column. To quantify the phenanthrene in hexane a flame ionization detector (FID) was employed. The specific parameters of gas chromatograph operation are tabulated in Table 3.2. The peak area was calculated using a Shimadzu Chromatopac-E1A electronic integrator.

Initially, to establish the linearity of the gas-chromatograph response with phenanthrene concentration, several hexane-phenanthrene standards were prepared. First, 2.1000 grams of phenanthrene (measured on an anlytical balance) were dissolved in 210 milliliters of hexane. One milliliter of this solution was removed and diluted with 99.0 milliliters of hexane. Subsequently, one milliliter of the diluted solution was diluted with 99.0 milliliters of hexane. This procedure was followed until a solution of 10 parts per million (ppm) was obtained. Then further serial dilutions were made to

TABLE 3.2

Parameters Employed in Gas Chromatography Analysis

Gas Chromatography	
Inert carrier gas	Nitrogen
Carrier gas flow rate	3.0 milliliters/min.
Temperature program	90 to 250 °C at 10 °C/min.
Inlet temperature	300 °C
FID temperature	350 °C
Sample size	3.0 μL
Column	
SP2100	Vendor--Supelco
Length	10 meters
Coating	100% Methyl silicone
Film thickness	0.3 μm

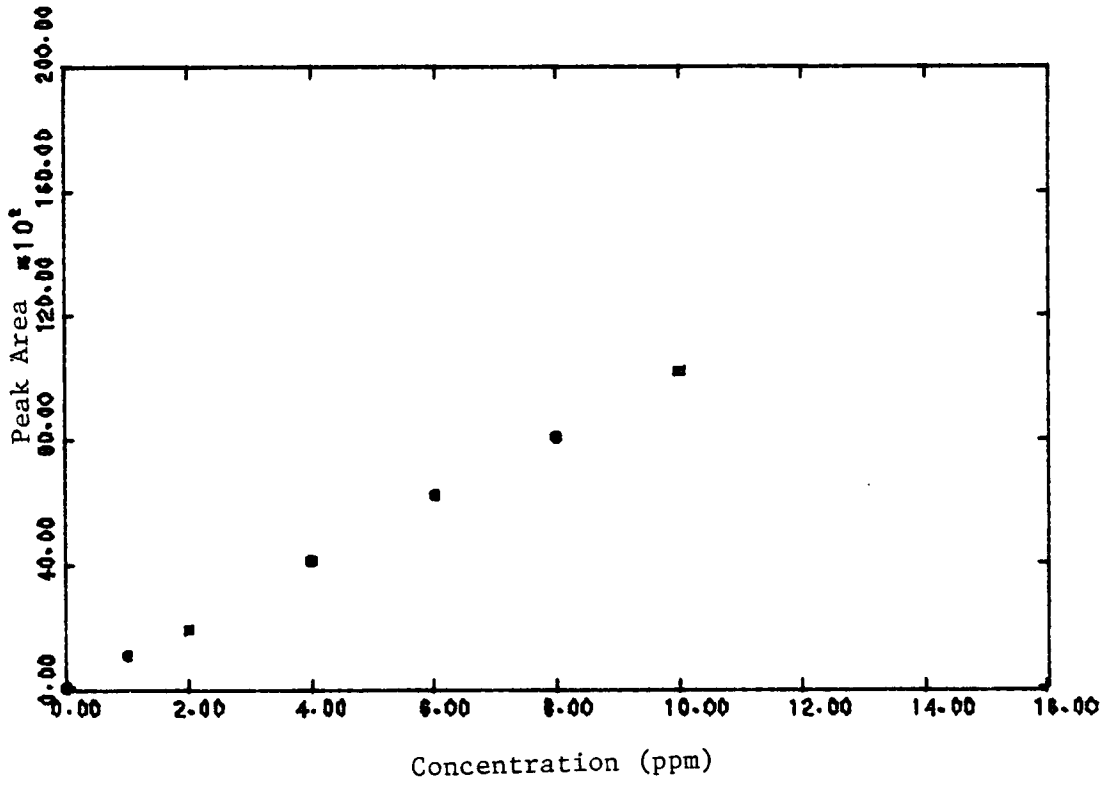

Figure 3.1. Gas Chromatograph Calibration Curve, Phenanthrene in Hexane.

obtain solutions of 8.0, 6.0, 4.0, 2.0 and 1.0 ppm. These solutions were then analyzed chromatographically and their peak areas calculated by the integrator. The results are plotted in Figure 3.1. The electronic integrator was equipped with the ability to use a preset internal standard to calculate the concentration of the phenanthrene-hexane solution.

3.4 Experimental Procedure: Temperature Dependence

All experiments to determine the temperature dependence of phenanthrene degradation were executed at pH 7.0, the pH of both maximum growth rate and maximum phenanthrene degradation rate. The following discussion will present the experimental procedure employed during the determination of the temperature dependence of phenanthrene degradation.

The growth medium (details in Table 3.1) was prepared and then autoclaved at 121 °C for 20 minutes. After allowing the growth medium to cool to the experimental temperature for approximately ten hours, four 200 milliliter portions of the growth medium were transferred to sterile 500 milliliter glass Erlynmeyer flasks. (All sterile experimental equipment was sterilized by autoclaving at 121 °C for 20 minutes.) Some of the remaining solution was transferred to sterile test tubes in 10 milliliter proportions. Approximately 24 hours prior to experimentation, several of the test tubes were innoculated with the Beijerinckia sp. and allowed to grow overnight. Three of the four flasks were innoculated with four milliliters of the 24 hour culture. The fourth flask was retained

as a sterile control. The flasks were incubated in either a water-bath incubator or a controlled air incubator at 100 rpm. pH control was not necessary since the pH did not change during experimentation.

Data were obtained by periodically sampling each flask using sterile pipets. Cell counts were made in triplicate, then each of the three solutions were combined and extracted with hexane and analyzed using the previously described gas chromatographic technique (Section 3.3). The temperature range of experimentation was from 15 to 40 °C in varied increments.

3.5 Experimental Procedure: pH Dependence

Basically, the experimental procedure to determine pH dependence of phenanthrene degradation was the same as that for the determination of temperature dependence. All of the experimental runs were performed at 25 °C, the temperature for the maximum phenanthrene degradation rate. As described earlier, the phosphate molar concentration was always 0.1 molar, but the ratio of mono to di-phosphate was varied to achieve the desired pH. The pH range for experimentation was 5.5 to 8.5 with increments of 0.5 pH units.

CHAPTER IV

EXPERIMENTAL RESULTS

4.1 A Typical Experimental Run

Plotted in Figures 4.1, 4.2, 4.3, and 4.4 are the results of a typical experimental run. In this particular run, the temperature was 25 ^{o}C and the pH was 7.0. The data are tabulated in Table 4.1. The time period of experimentation was 4.75 hours.

As was mentioned in Chapter 3, the conditions of this run correspond to the conditions at which the phenanthrene degradation rate is the greatest. However, the observed scatter and measurement precision is representative of all experimental runs.

Since all experiments were run under periods of balanced growth (the exponential phase of growth), the cell growth data can be shown to follow first order kinetics. The following expression describes first order kinetics:

$$\frac{1}{x}\frac{dx}{dt} = \mu \qquad (4.1)$$

where x is the cell concentration, μ is the specific growth rate, and t is time. Upon integrating equation 4.1, we have

$$\ln x = \mu t + c \qquad (4.2)$$

where c is a constant of integration and the other symbols are defined as in equation 4.1. Thus, to find μ, the specific growth rate, one may plot the logarithm of cell concentration vs. time and generate the slope of the curve. Figure 4.1 is a linear plot of

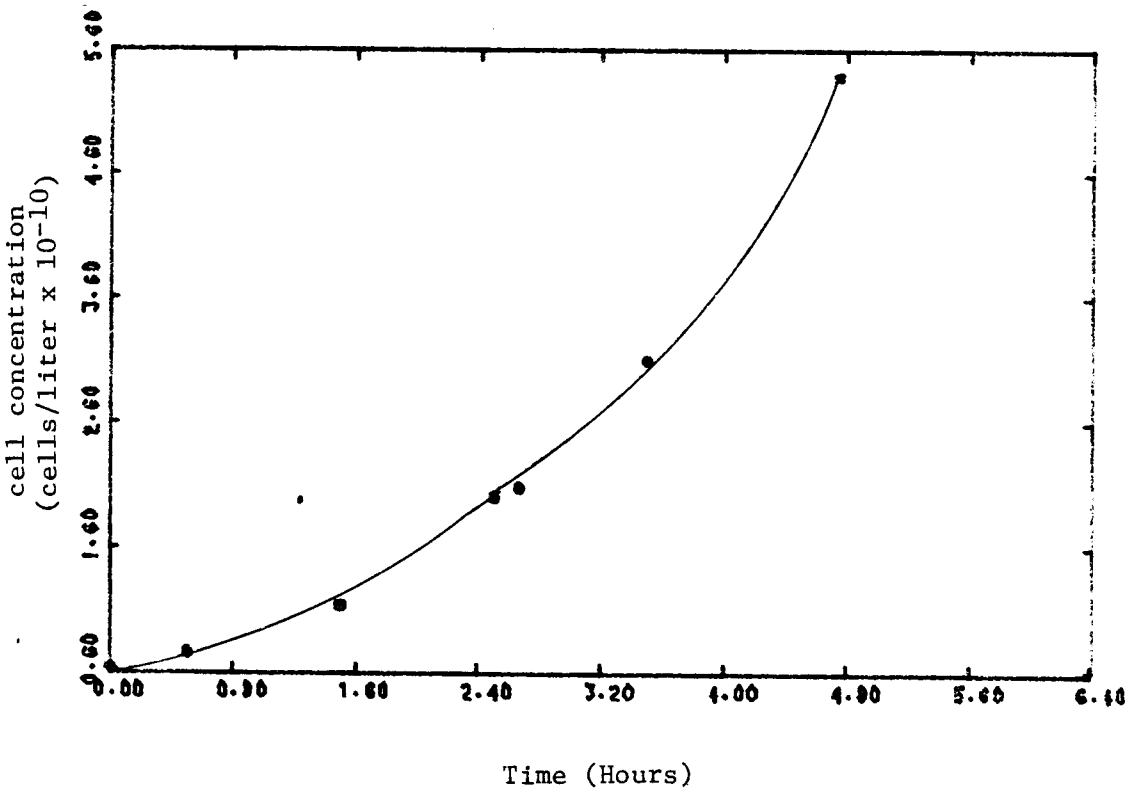

Figure 4.1. Beijerinckia Growth Curve at 25 °C and a pH of 7.0 on Yeast Extract and Phenanthrene.

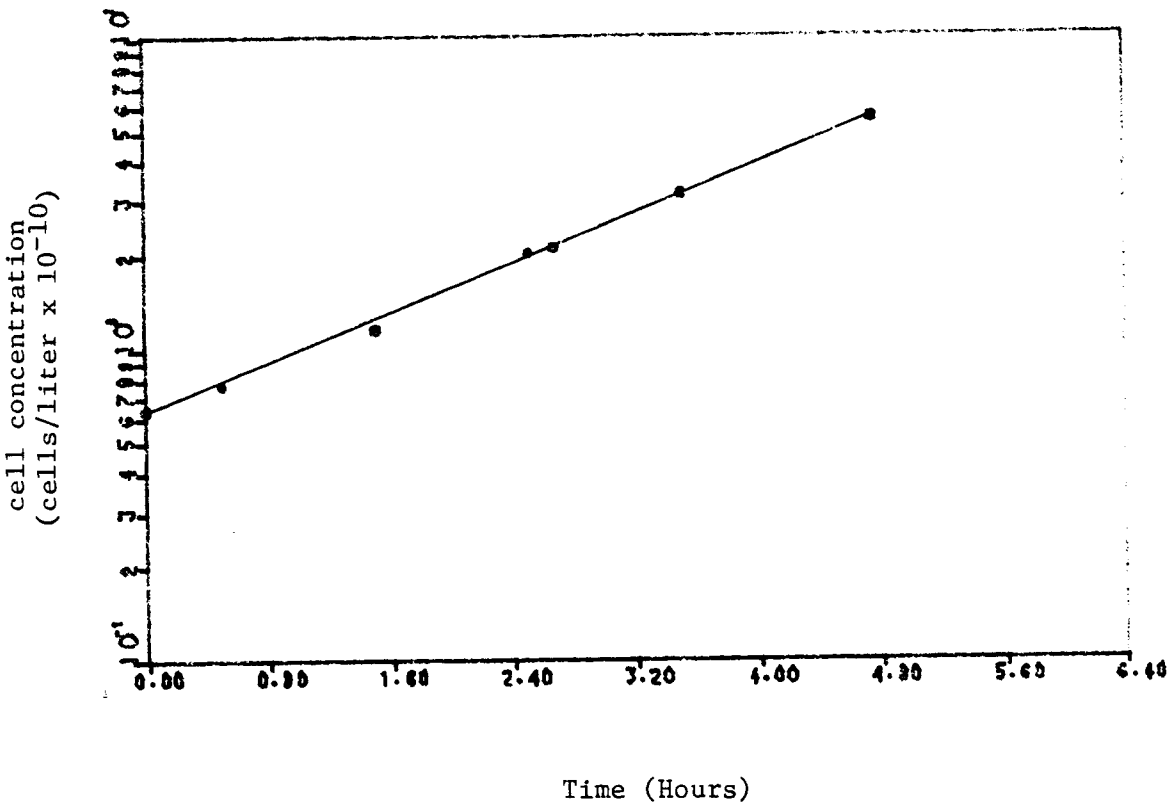

Figure 4.2. Beijerinckia Growth Rate at 25 °C and a pH of 7.0 on Yeast Extract and Phenanthrene.

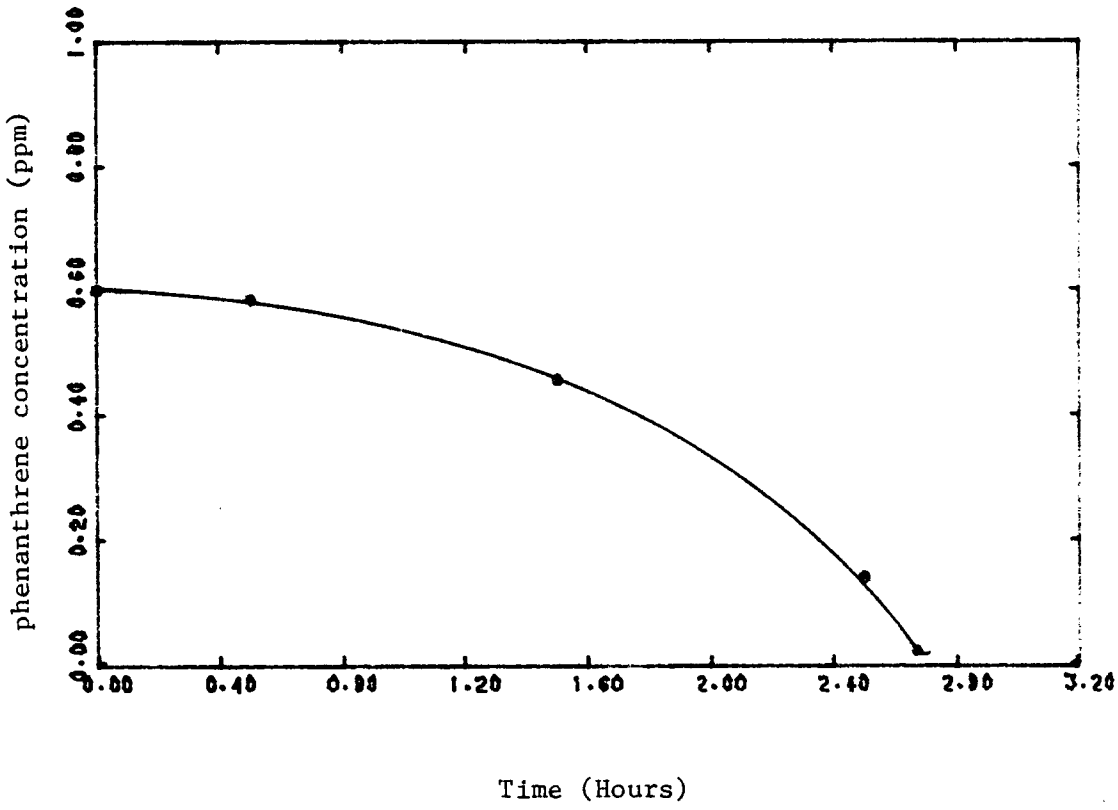

Figure 4.3. Phenanthrene Degradation by Beijerinckia at 25 °C and a pH of 7.0 in Presence of Yeast Extract.

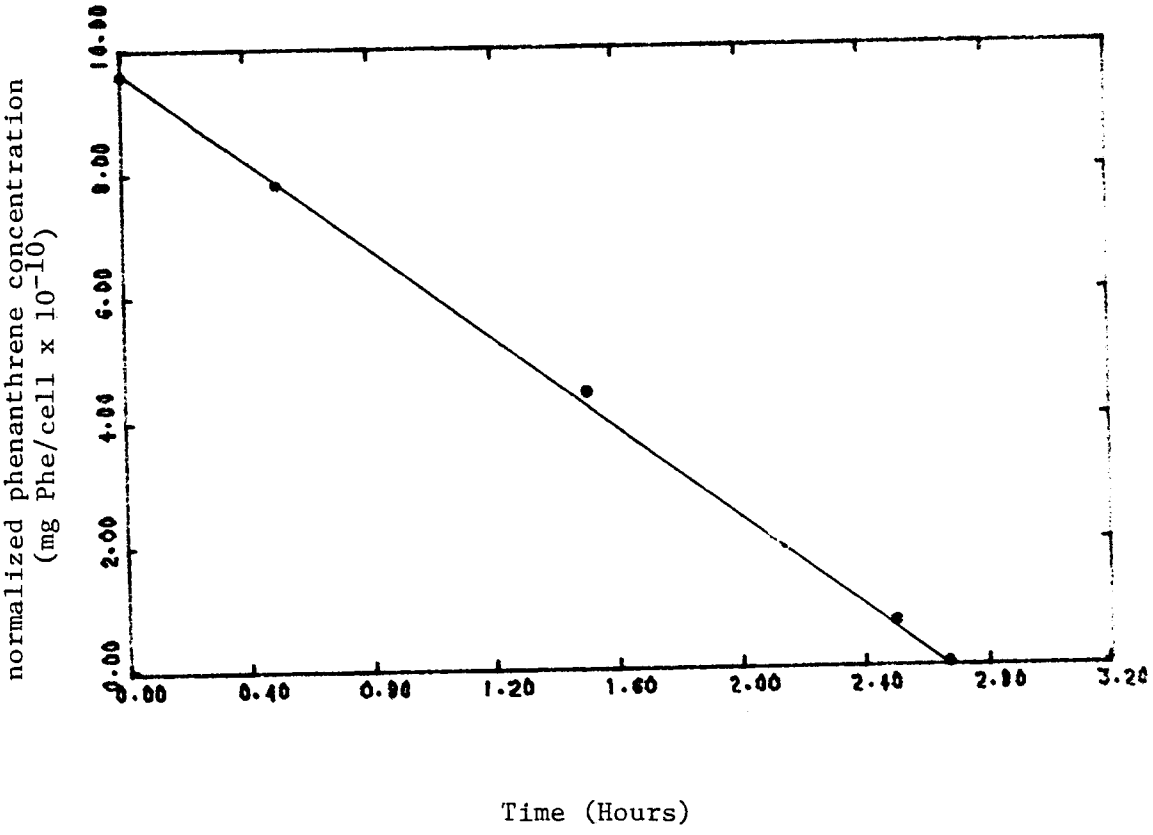

Figure 4.4. Cell Normalized Phenanthrene Degradation by Beijerinckia at 25 °C and a pH of 7.0 in Presence of Yeast Extract.

TABLE 4.1

Data From a Typical Experimental Run
(Run Number 7)

Time (hrs.)	Average Cell Concentration (cells/1) x 10^{-10}	Phenanthrene Concentration (ppm)	Cell Normalized Phenanthrene Concentration (x 10^{11})
0.0	0.63	0.60	9.60
0.50	0.75	0.59	7.81
1.50	1.13	0.46	4.44
2.50	2.00	0.14	0.69
2.67	2.08	0.02	0.01
3.50	3.13	0.00	0.00
4.75	5.39	0.00	0.00

Temperature - 25.0 °C

pH - 7.0

Shaker Speed - 100 RPM

Specific cell growth rate (1/hour) 0.472 (From Figure 4.2).

Phenanthrene degradation rate 3.59×10^{-11} milligrams/cell-hour.

cell concentration vs. time and Figure 4.2 is a plot of the same data according to the natural logarithm of cell concentration vs. time. Observe from Figure 4.2 that the data do, in fact, fall on a straight regression line with a correlation coefficient, r^2, of .998. Also, observing Table 4.1, the average standard deviation as a percent of measurement is only 1.7. Thus, the data displays little scatter. The specific cell growth rate, μ, at 25 °C and pH 7.0 is 0.472 1/hour.

Figure 4.3 is a plot of the phenanthrene concentration of Table 4.1 vs. time. As with the cell growth data, very little scatter is observed in the concentration data (Table 4.1) with the average standard deviation as a percent of the measured phenanthrene concentration being only 1.1. Figure 4.4 is a plot of cell normalized concentration vs. time with these data also provided in Table 4.1. The data in Figure 4.4 are obtained by dividing the phenanthrene concentration by the cell concentration in order to determine the degradation rate per cell.

From literature sources previously discussed (Section 2.4.2) and preliminary experimentation, it was expected that a plot of cell normalized phenanthrene concentration vs. time should show a zero-order response. Thus, linear regressions were performed which would correspond to the following equation:

$$\frac{d(\frac{[Phe]}{x})}{dt} = k \qquad (4.3)$$

$$\frac{[Phe]}{x} = kt+c \qquad (4.4)$$

where [Phe] is the concentration of phenanthrene, x is the cell concentration, k is a zero order rate constant, t is time, and c is a constant of integration. As is observed from data in Table 4.1, a very good fit of the data is obtained through a linear regression of the data displayed in Figure 4.4 with a regression coefficient (r^2) of .998. The phenanthrene degradation rate in this particular case is 3.59×10^{-11} milligrams of phenanthrene/cell-hour. In all experimental runs, when the cell normalized concentration vs. time was regressed in accordance with equation 4.4, the regression coefficient (r^2) was greater than or equal to .970.

4.2 The Effects of Temperature on the Phenanthrene Degradation Rate

To some degree, all biochemical reactions are temperature dependent often, but by no means exclusively, following Arrenhius behavior (Pirt, 1977). Generally, however, as temperature increases so does the rate of cell reaction (both growth rate and substrate utilization rate) until the proteinaceous enzymes catalyzing the reactions begin to denature at some critical temperature (Freeman, 1979). Thus, the temperature response curve of such a system (either growth rate or substrate reaction rate) should have a maximum or optimum operating temperature, which, in terms of cell growth, is reflected in a largest (in absolute terms) value of the specific growth rate, μ, as defined by equation 4.1. The purpose of this group of experiments was to develop temperature-response

curves for the specific growth rate μ and phenanthrene degradation rate of the mutant Beijeninckia sp. B8/36.

The temperature response curves for specific growth rates and phenanthrene degradation rates are shown in Figures 4.5 and 4.6. Figure 4.5 is the specific growth rates plotted in an Arrenhius type plot, and Figure 4.6 is simply a plot of cell normalized reaction rate vs. temperature. The data are provided in Tables 4.2 and 4.3.

Inspecting Table 4.3, several data points are presented at 25 °C and two data points are presented at 22.0 °C. Even though each sample was analyzed in triplicate, there was interest in the reproducibility of the experiment. Four total runs were performed at 25 °C, three of which were performed during some of the late preliminary work. Thus, the accuracy of these latter points is somewhat questionable, but they can be used to provide at least an approximate meausre of the data reproducibility. The average of the four runs at 25 °C is 3.52×10^{-11} with a standard deviation of $.09 \times 10^{-11}$. The standard deviation as a percent of the mean is 3.6. At 22.0 °C, the average of the two data points is 1.65 with a range of 0.049. The range as a percent of the mean is ±3.0. Thus, the data seems to be reproducible to within or better than about ±3%, and, at worst, the 95% confidence interval (using the data at 25 °C) is ±7.2%.

The shape of the growth curve (Figure 4.5) is as anticipated, with an activation energy of growth (calculated over the

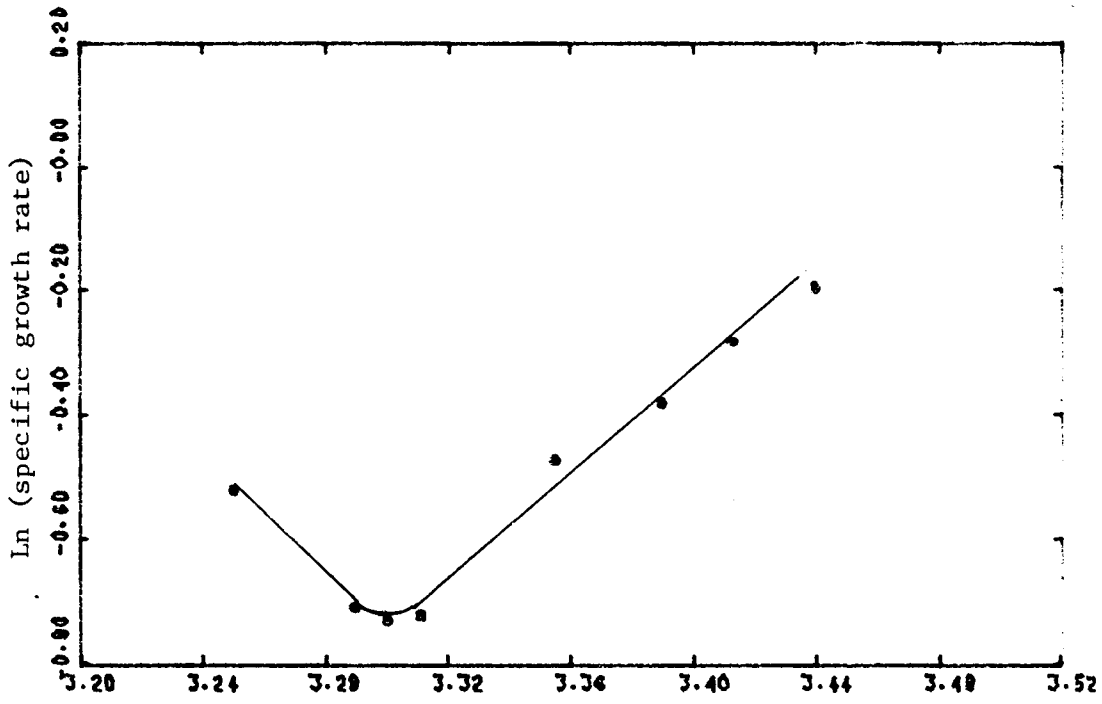

Figure 4.5. Arrhenius Plot, Beijerinckia Specific Growth Rate at pH of 7.0 in Presence of Yeast Extract and Phenanthrene.

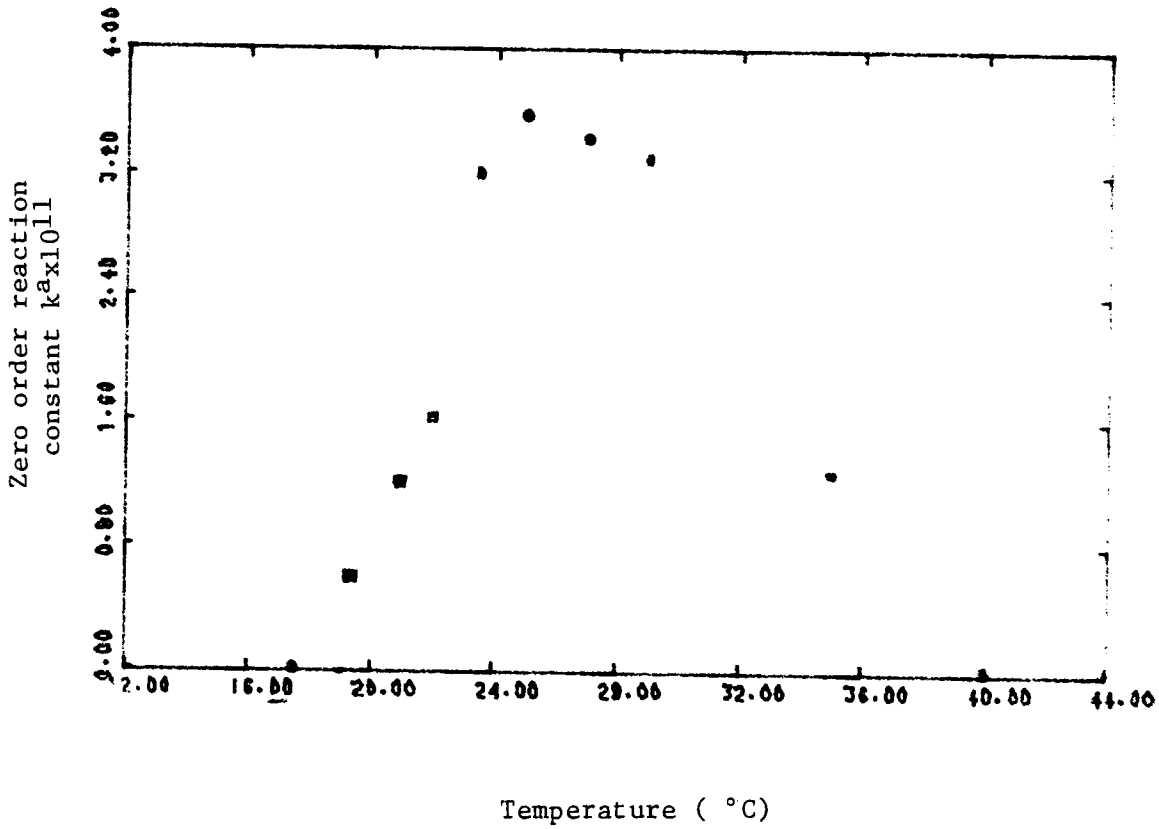

Figure 4.6. Temperature Dependence of the Zero-Order Rate Coefficient, Phenanthrene Degradation by Beijerinckia at pH of 7.0 in presence of Yeast Extract.

[a]The units of k are milligrams of Phenanthrene/cell-hour.

TABLE 4.2

Beijerinckia Species Growth Data as a Function of Temperature[a]

Run Number	Temperature °C	Specific Growth rate (1/hour)	Regression Coefficient[c] r^2
1	17.5	0.201	0.983
2	19.0	0.247	0.975
3	20.5	0.285	0.993
4	22.0	0.381	0.994
6	23.5	0.435	0.989
7	25.0	0.472	0.998
11	27.0	0.595	1.000
12	29.0	0.723	0.991
12A	30.0	0.730	0.980
12B	31.0	0.710	0.991
13	35.0	0.522	0.976
14	40.0	b	

[a]All experiments were run at a pH of 7.0 and a shaker speed of 100 RPM. This table only contains growth activity.

[b]Little or no detectable growth activity.

[c]The regression coefficient is that obtained by a least square regression of equation 4.2.

TABLE 4.3

Results of Temperature Dependence Experimentation[a]

Run Number	Temperature °C	Run Duration (hours)	Phenanthrene Degradation rate, K, in milligrams/cell-hour x 10^{11}	Regression Coefficient[d] (r^2)
1	17.5	4.0	b	
2	19.0	4.0	0.793	.994
3	20.5	4.5	1.250	.993
4	22.0	3.0	1.624	.994
5	22.0	3.25	1.672	.998
6	23.5	4.25	3.190	.997
7	25.0	4.75	3.560	.998
8	25.0[c]	4.50	3.400	.997
9	25.0[c]	4.50	3.613	.997
10	25.0[c]	4.75	3.490	.996
11	27.0	2.50	3.420	.997
12	29.0	3.75	3.290	.989
13	35.0	2.75	1.270	.996
14	40.0	4.0	b	

[a]All experiments were run at a pH of 7.0 and a shaker speed of 100 RPM. This table does not contain the growth rate data.

[b]Little or no detectable phenanthrene degradation activity.

[c]These data were taken during preliminary experimentation. Their accuracy may be less than the other points.

[d]This regression coefficient is that obtained by a least square fit of the equation 4.4.

temperature range from about 15 °C to 30 °C) of 16.0 kilocalorie /mole.

The cell normalized phenanthrene degradation rate, however, demonstrated quite different behavior (Figure 4.6). Degradation of phenanthrene does not commence until a temperature of 19 °C, rises from a rate of 0.793×10^{-11} milligrams/cell-hour at 19 °C to a rate of 3.560×10^{-11} milligrams/cell-hour at 25 °C, stays approximately constant from the range of 25 °C to 29 °C, but, by 35 °C, the reaction rate has dropped to 1.270×10^{-11} milligrams/cell-hour. At 40 °C and 17.5 °C, little or no activity was detectable over the course of experimental times of up to five hours.

The observed non-Arrehenius behavior in phenanthrene degradation rate can probably be explained by the series of very complex reactions involved in phenanthrene degradation. Many different enzyme systems are involved in the degradation of phenanthrene (Section 2.3.2), and, in the coupling of all of the involved reactions, the overall observed reaction rates may take on very complex relationships with changes in process variables. A similar result involving the degradation of phenol by a mixed culture was observed by Vela and Ralston. Since analysis of intermediates was impossible with available equipment, trying to suggest a specific reaction scheme would yield an unsubstantiated guess.

The maximum cell growth rate of 0.472 1/hour was observed at 30 °C while the maximum phenanthrene degradation rate of 3.590×10^{-11} milligrams/cell-hour was observed at 25 °C.

4.3 Effect of pH on Phenanthrene Degradation Rate

It is well known that pH influences growth rate and substrate utilization in microbiological systems although no general theory exists for the complex effects of pH on cellular metabolism (Pirt, 1977). Even without an understanding of the specific mechanisms, the effects of pH on both growth and phenanthrene degradation can be recorded.

The preparation of the growth medium for this set of experiments was the same as that for the temperature response studies, except that the phosphate buffer and medium solution was adjusted to control the pH in the range from 5.5 to 9.0. External pH control was not necessary since the pH did not change detectably throughout experimentation.

Figures 4.7 and 4.8 are plots of the specific growth rate and cell normalized phenanthrene degradation rate vs. pH respectively at 25 °C. The data are tabulated in Tables 4.4 and 4.5. As before, the data had very little scatter and the regression coefficients (r^2) were all close to 1.000. In this set of experiments, a replication was performed at pH 8.0. Inspecting Table 4.5, the two values of the reaction constant recorded for pH 8.0 were 2.50×10^{-11} milligrams/cell-hour and 2.450×10^{-11} milligrams/cell-hour. The spread is 0.05, and the spread as a percent of the mean is 2.0.

In the cases of both cell growth and phenanthrene degradation, there is quite a sharp maximum around pH 7, thus, the cells are quite intolerant to pH changes at this temperature. The cells

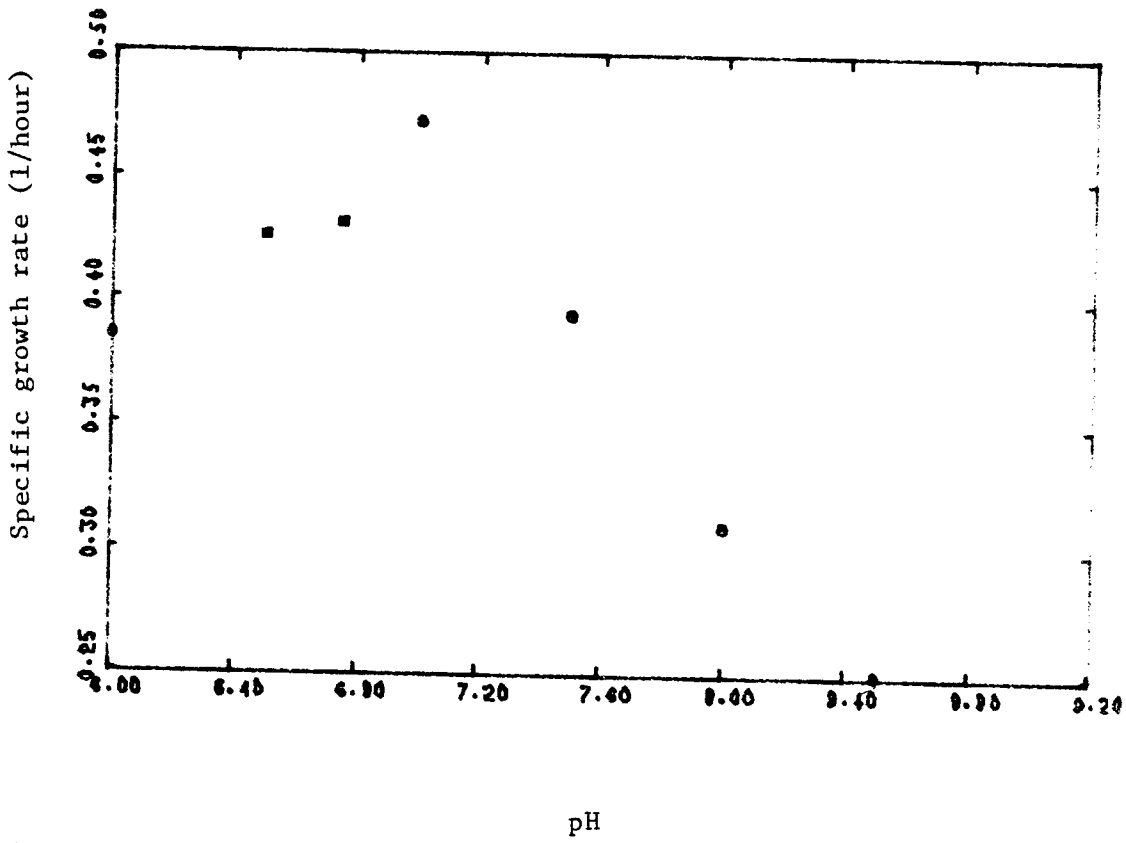

Figure 4.7. Beijerinckia Specific Cell Growth Rate at 25 °C in Presence of Yeast Extract and Phenanthrene.

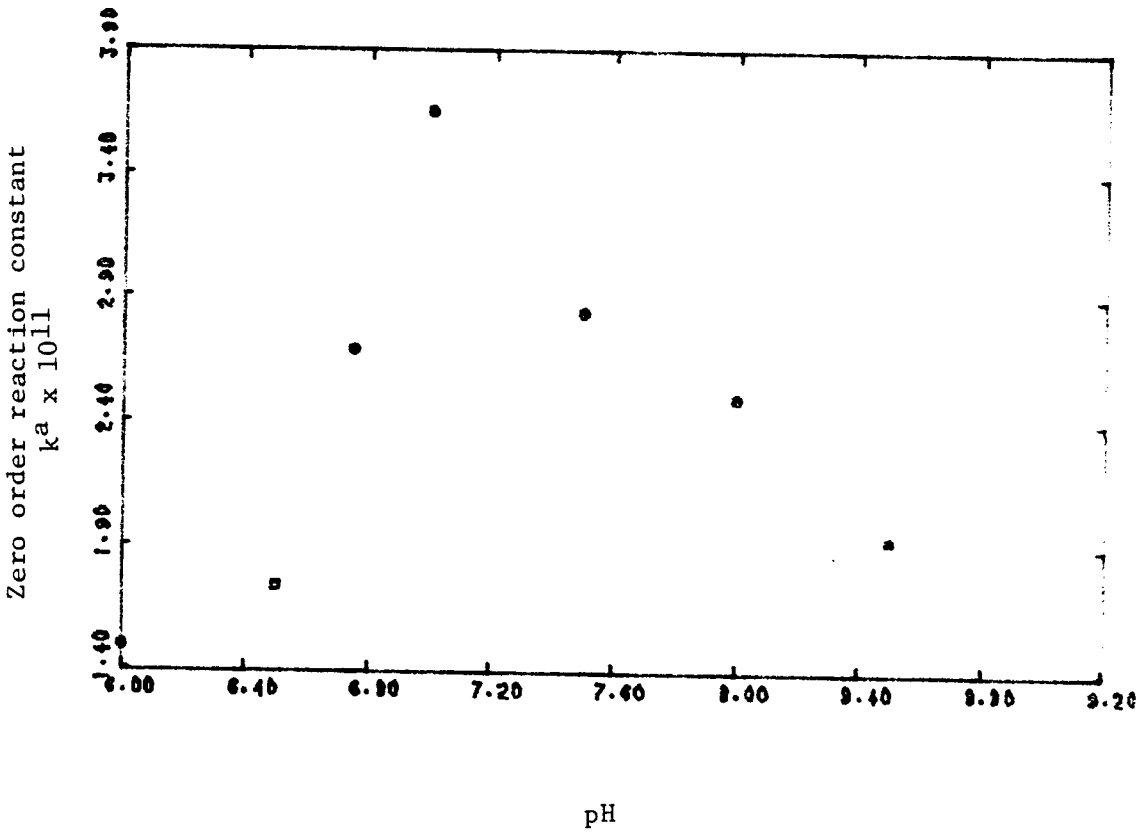

Figure 4.8. pH Dependence of the Zero-Order Rate Coefficient, Phenanthrene Degradation by Beijerinckia at 25 °C in Presence of Yeast Extract.

[a]The units of k are milligram of Phenanthrene/cell-hour.

TABLE 4.4

Results of pH Dependence Experimentation[a]

Run Number	pH	Run Duration (hours)	Phenanthrene Degradation rate (1/hour)	Regression Coefficient r^2 [c]
15	5.5	4.00	b	
16	6.0	4.00	1.490	.985
17	6.5	3.50	1.740	.993
18	6.75	4.00	2.695	.999
19	7.0	4.75	3.560	.998
20	7.5	2.50	2.840	.979
21	8.0	3.00	2.500	1.000
22	8.0	3.00	2.450	.970
23	8.5	4.00	b	

[a]All experiments were run at a temperature of 25 °C and a shaker speed of 100 RPM. This table does not contain the growth rate data.

[b]Little or no phenanthrene degradation activity.

[c]The regression coefficient is that obtained by a least square fit of equation 4.4.

TABLE 4.5

Beijerinckia Species Growth Data as a Function of pH[a]

Run Number	pH	Specific Growth rate (1/hour)	Regression Coefficient[c] (r^2)
15	5.5	b	
16	6.0	0.385	.980
17	6.5	0.426	.993
18	6.75	0.431	.999
19	7.0	0.472	.998
20	7.5	0.394	.976
21	8.0	0.310	.990
23	8.5	b	

[a]All experiments were run at a temperature of 25 °C and a shaker speed of 100 RPM. This table contains only growth rate data.

[b]Little or no growth activity.

[c]The regression coefficient is that obtained by a least squares regression of equation 4.1.

ceased to grow and metabolize phenanthrene at the pH extremes of 5.5 and 9.0. The relationship between phenanthrene degradation and pH seems to be more sensitive than the relationship between growth and pH. From pH 6 to 7, the phenanthrene degradation rate changes by a factor of 3 while specific growth rate changes by a factor of only about 1.25. In a study of adipate transport by a Psuedomonas (Ondrako and Ornston, 1980), similar sharp pH dependent behavior was observed for the uptake of adipate. Although the uptake of adipate is not the same as metabolism of phenanthrene to adipate, the beta-ketoadipate pathway is ultimately involved in the degradation of phenanthrene, and it is quite possible that the phenanthrene system could show similarities to the beta-ketoadipate system.

The maximum growth rate (0.472 1/hour) and phenanthrene degradation rate (3.56×10^{-11} milligrams of phenanthrene/cell-hour) occurred at pH 7.0.

4.4 Metabolism of Phenanthrene in the Absence of Yeast Extract

An experiment was performed to determine the phenanthrene degradation rate in the absence of yeast extract. The pH and temperature chosen for this experiment were 7.0 and 25 °C respectively, the optimum pH and temperature for phenanthrene degradation.

The Beijerinckia cells were grown overnight in the standard mineral salts, yeast extract, and phenanthrene broth (see Table 3.1, page 28). The cells were then transferred to several

erlynmeyer flasks (as done in the previous experimentation) and allowed to grow for approximately four hours. The cells were then collected, centrifuged, washed in a mineral salts solution, centrifuged again, and then resuspended in a prepared mineral salts-phenanthrene broth. This growth medium was identical to the standard medium except that yeast extract was not added to the broth.

In order to assure a high cell concentration, the harvested cells were resuspended in several different test tubes, each containing 10.0 milliliters of the mineral salts-phenanthrene broth. The contents of each test tube was then combined into one flask.

Periodically throughout the experiment, the cell concentration of a flask was measured and a ten milliliter sample was extracted with hexane. The extract was then analyzed on the gas chromatograph and the phenanthrene concentration quantified.

The results of the experiment were that the phenanthrene degradation rate in the absence of yeast extract, observed to be 3.35×10^{-11} milligrams of phenanthrene/cell-hour was within 6.0% of the phenanthrene degradation with yeast extract present.

CHAPTER V

DISCUSSION

In Chapter IV, the experimental results were presented. In summary, the major results are as follows:

1. the reaction proceeded with zero or pseudo-zero order kinetics,
2. with temperature the degradation of phenanthrene demonstrated non-Arrhenius behavior, and
3. with pH the degradation rate of phenanthrene displayed a very sharp peak at pH 7.

The aim of this chapter is to suggest a plausible explanation of the results and to predict the controlling mechanisms involved in the reaction system by answering the following questions:

1. Is the reaction limited by the diffusion of phenanthrene to the cell?
2. Is the reaction limited by transfer phenanthrene through the cell wall into the cell interior?
3. Is the reaction rate control inside the cell itself? In other words, is some specific enzymatic reaction involved in phenanthrene degradation controlling the overall degradation rate?
4. Could the reaction rate be controlled by transfer of reaction products from the cell to the surroundings or could reaction products actually hamper the inter-cellular reactions or

phenanthrene transport from the cell surroundings to the cell interior?

5.1 Diffusion of Phenanthrene to the Cell

To determine if the observed rates of phenanthrene degradation were controlled by diffusion through the aqueous media to the cells, one might model the degradation system assuming that the bacterial cells are spheres. Actually, Beijerinckia is a small, cylindrical cell with a diameter of approximately 1.0 micron and a length of approximately 3.1 microns (Berges, 1976). For a diffusion model, however, a Beijerinckia cell can be thought of as an equivalent sphere with a diameter 1.76 microns which is the diameter of a sphere that has a surface area equal to a 1.0 micron by 3.1 micron cylinder.

Mass transfer to a small sphere can be estimated by the formula

$$J_A = k(X_{AB}-X_{AS}) \tag{5.1}$$

where J_A is the molar flux of phenanthrene from the bulk aqueous phase to the cell, k is the mass transfer coefficient in moles/square centimeter-hour, X_{AB} is the bulk concentration of phenanthrene, and X_{AS} is the cell surface concentration of phenanthrene.

If the degradation of phenanthrene is diffusion controlled, then equation 5.1 also should closely approximate the observed phenanthrene degradation rate when multiplied by the total cell surface area. Further, assuming that the degradation rate of

phenanthrene inside the cell is very fast with respect to the mass transfer rate, then X_{AS} from equation 5.1 may be taken as zero, and equation 5.1 reduces to

$$N_A = k(X_{AB}) \tag{5.2}$$

where N_A is the total molar flux of phenanthrene to the cell in moles/square centimeter-hour and the other symbols are the same as in equation 5.1.

To approximate the mass transfer coefficient k, the following correlation was employed (Bird, Stewart, and Lightfoot, 1976):

$$\frac{kD}{C_f D_{AB}} = 2.0 + 0.6\ (Sc)^{1/3}\ (Re)^{1/2} \tag{5.3}$$

where k is the mass transfer coefficient in moles/square centimeter-hour, D is the diameter of the particle in centimeters, C_f is the molar density of the solvent in moles/cubic centimeter, D_{AB} is the diffusivity of the solute (phenanthrene) in the solvent (water) in square centimeters/hour, Sc is the Schmidt number, and Re is the Reynold's number.

Even though the reaction flask is shaken, one can get an estimate of the minimum phenanthrene transfer rate to the cells by assuming the cells are following the motion of the aqueous phase, or in other words, by assuming that the velocity of a cell is zero relative to the aqueous phase. This would certainly be the case if the density of a cell is essentially the same as that of the

aqueous medium. Allowing the Reynold's number to approach zero, equation 5.3 reduces to

$$\frac{kD}{C_f D_{AB}} = 2.0 \quad . \tag{5.4}$$

To estimate the diffusivity of phenanthrene in water, the Wilke and Chang correlation (Treybal, 1980) was employed:

$$D_{AB} = \frac{(117.3 \times 10^{-18})(\varepsilon M_B)^{0.5} T}{u(V_A)^{0.6}} \tag{5.5}$$

where D_{AB} is the diffusivity of compound A (phenanthrene) in very dilute solution in solvent B (water) in square meters/second, M_B is the molecular weight of solvent B in kilograms/kilomole, T is the temperature in K, as is the solution viscosity in kilograms/meter second, V_A is the solute molal volume at its normal boiling point in the cubic meters/kilomole and ε is the association factor for solvent B and has a value of 2.26 for water.

The value for V_A was estimated from atomic and molecular volumes obtained from Treybal, Chapter 2. The specific parameters employed in the diffusivity calculation are listed in Table 5.1. The value for the diffusivity of phenanthrene in water at 298K is calculated as 0.021 square centimeters/hour.

Taking the calculated value for D_{AB} and solving equation (5.4) for k, a value of 1.84×10^{-3} moles/square centimeter-second is obtained, leading to a calculated mass transfer rate of 6.96×10^{-9} milligrams of phenanthrene/cell-hour. Comparing this calculated

TABLE 5.1

Parameters Employed in Diffusivity Calculation

Parameters Included in Equation 5.5
M_B = 18.02 Kilomoles/Kilogram
T = 298 K
μ = 0.00100 Kilograms/meter-second
V_A = 0.200 cubic meters/kilomole
ε = 2.26

[B] Refers to water

[A] Refers to phenanthrene

value of the mass transfer rate to the observed maximum phenanthrene rate of 3.59×10^{-11} milligrams of phenanthrene/cell-hour (Section 4.2), it can be concluded that if the controlling mechanism of mass transfer of phenanthrene from the solution to the cell surface is simple diffusion, the degradation rates of phenanthrene observed in this experimentation is not transfer controlled.

Further, if one assumes Fickian diffusion, then the following equation describes the diffusive flux of phenanthrene from the bulk liquid to the cell:

$$J_A = -D_{AB} \frac{dC_A}{dz} \qquad (5.6)$$

where J_A is the molar flux of phenanthrene from the bulk aqueous phase to the cell in moles/square centimeter-hour, D_{AB} is the diffusivity of phenanthrene in water in square centimeters/hour, C_A is the molar concentration of phenanthrene in moles/cubic centimeters and z is the length of the medium through which the diffusion takes place in centimeters.

Assuming a constant flux of phenanthrene through a non-diffusing film of water, then one may separate and integrate equation 5.6 and obtain the following expression assuming that the concentration of phenanthrene on the cell surface is zero and a film thickness of δ:

$$N_A = -D_{AB} \frac{C_A}{\delta} \quad . \qquad (5.7)$$

Substituting $\frac{dVC_A}{dt}$ for N_A where V is the volume of liquid in the film, one obtains

$$\frac{VdC_A}{dt} = -D_{AB} \frac{C_A}{\delta} \quad . \qquad (5.8)$$

Separating and integrating equation 5.8 the following expression is obtained:

$$\ln \frac{C_A}{C_{AO}} = - \frac{D_{AB}}{V\delta} (t-t_0) \qquad (5.9)$$

Thus, if the degradation of phenanthrene were diffusion controlled, equation 5.9 demonstrates that the reaction should demonstrate first order kinetics. The observed reaction rate, however, is zero order (Section 4.2). Thus, one can assume that the reaction is not transport controlled and some other mechanism must be rate limiting in phenanthrene degradation.

Since the degradation of phenanthrene is not transport controlled, then it must be assumed that either there is a rate limiting step in either the transport of phenanthrene through the cell membrane, in the cell interior biochemical reactions or in the transport or inhibiting effects of reaction products or intermediates.

5.2 Transfer Through the Membrane, Reactions Inside the Cell, and the Transfer of Reaction Products out of the Cell

Further experimental work designed to identify both final and intermediate reaction products is required in order to clarify the metabolic pathway of phenanthrene degredation and to identify the controlling step or steps in this reaction sequence. However, this system is of special interest because of its rather sharp temperature behavior, and perhaps some speculation as to what the possible controlling mechanisms may be of interest.

Most molecules of molecular weight less than 1000 daltons freely pass through cell membranes (Freeman, 1979). Phenanthrene has a molecular weight of approximately 178 daltons, and further, the phenanthrene molecule contains no functional groups and thus is non-polar. Therefore, one would expect phenanthrene to readily pass through the cell membrane. The final reaction products of phenanthrene degredation are probably carbon dioxide and water which are two molecules that easily pass through cell membranes. Also, intermediate reactions products such as cis-1,2-dihydroxyl-2-dihydronaphthalene and cis-1,2-dihydroxy-1,2-dihydrophenanthrene are of relatively low molecular weight and also would be expected to easily pass through cell membranes. From the facts stated previously, it can be concluded that the passage of phenanthrene or reaction products across the cell membrane is probably not the rate controlling step leading to the observed temperature behavior.

If phenanthrene can, in fact, freely pass through the cell membrane, then, the controlling step of steps in phenanthrene degredation presumably must occur in the metabolic pathway of phenanthrene degredation.

The dominate mechanism of reversible enzyme modulation is called allosteric control. Basically, allosteric control can either inhibit or activate the catalytic ability of a given enzyme. Further, the substance which is responsible for the inhibition of a particular enzyme may or may not have some resemblance to the substrate on which the enzyme is acting. Thus,

formation of some reaction product either in a particular biochemical pathway or in some parallel biochemical pathway can either promote or inhibit an enzyme which is catalyzing an earlier or parallel reaction. This type of action can be considered a form of feedback control in a cellular system. (Bailey and Ollis, 1977).

Another method by which cells may control the utilization of substrates is by enzyme induction or repression at a genetic level. (Bailey and Ollis, 1977). Generally, the theories of repression and induction are based upon the fact that either a repressor or an aporepressor are present in the cellular system, and whenever an inducer (in the case of induction) or a co-repressor are present, the production of specific enzymes is respectively induced or repressed. For a more complete treatment of this material the reader is referred to Bailey and Ollis.

As mentioned in Chapter II, biochemical reactions are generally temperature dependent obeying Arrhenius' law, but not all pathways will necessarily demonstrate such behavior. For instance, in a complex, multi-step pathway, such as the degredation of phenanthrene, many different enzymatic reactions are involved and thus, changes in temperature can cause rather complex behavior as the results of this work demonstrate.

In view of the preceding discussion, one possible means by which the temperature behavior observed in this experimentation could take place is if, after enough thermal energy is present to start phenanthrene degredation, the reaction rate increases

dramatically with temperature to about 25 ^{o}C. If some reaction further on in the reaction scheme of phenanthrene degredation is not quite as sensitive to temperature as the initial reactions, then, at some temperature (in this case 25 ^{o}C) the controlling step in phenanthrene metabolism shifts and there is a subsequent increase in concentration of intermediate degredation products. One or more of these products could act to either allosterically inhibit an enzyme catalyzing an earlier reaction or act as a co-repressor to inhibit cellular production of enzyme required in the phenanthrene degredation pathway.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The basic conclusions of this research follow.

1. Phenanthrene is degraded by the chosen Beijerinckia sp. employed in the temperature range of 19 to 35 °C and pH range of 5.5 to 8.5. Degradation was not detectable outside the ranges of experimental observation up to six hours.
2. The maximum phenanthrene degradation rate of 3.59×10^{-11} milligrams/cell-hour occurs at a temperature of 26.0 °C ± 2 °C and a pH of 7.0 ± 0.20.
3. The maximum specific growth rate of 0.730 $(\text{hours})^{-1}$ occurs at a pH of 7.0 ± 0.2 and a temperature of 30.0 °C ± 2 °C.
4. The observed degradation reaction is zero order and is not diffusion controlled.
5. The degradation reaction is very sensitive to pH changes.
6. The degradation reaction does not demonstrate Arrhenius behavior.
7. The rate of phenanthrene degradation is independent of the presence of yeast extract.

The literature, in terms of polyaromatic hydrocarbon degradation or mineralization is, at present, quite sketchy. Therefore, no data exists with which to compare that obtained in this study.

Much more work has been done to assess whether certain naturally occurring microorganisms or mutant bacteria can degrade compounds such as phenanthrene, napthlene, and even benzo-o-pyrene, but, very few, if any, controlled kinetic studies have been reported.

6.2 Recommendations

Some recommendations for further work would be to try to devise a method to attempt to study degradation rates with higher concentrations of phenanthrene and to try to identify some important intermediates which are reaction rate limiting. Further, experimentation to determine rate limiting steps and further explanation of the temperature response recorded in this work would be helpful to determine exactly which enzyme system is limiting the degradation of phenanthrene.

Work on the degradation of coal particles themselves would be the next step in trying to isolate organisms that could aid in coal conversion to useable liquid and gas fuel sources. Exploring organisms, especially in anerobic growing conditions, could possibly yield a viable organism that could degrade coal.

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APPENDICES

APPENDIX A

EXPERIMENTAL DATA, PHENANTHRENE DEGRADATION BY *BEIJERINCKIA* IN PRESENCE OF YEAST EXTRACT

TABLE A.1

Experimental Data, Phenanthrene Degradation by *Beijerinckia* in Presence of Yeast Extract

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #2	pH = 7.0	Temperature = 19.0 °C	
0.0	1.59	0.59	3.71
1.0	2.00	0.45	2.27
1.5	2.63	0.41	1.57
3.5	3.38	0.23	0.68
4.0	4.25	0.00	0.00
Run #3	pH = 7.0	Temperature 20.5°C	
0.0	0.75	0.45	6.00
0.5	0.85	0.44	5.18
1.5	1.16	0.43	3.71
2.5	1.55	0.41	2.65
3.5	2.10	0.36	1.60
4.5	2.58	0.10	0.39
4.75		0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #4	pH = 7.0	Temperature = 22 °C	
0.0	1.13	0.64	5.00
0.5	1.44	0.59	4.10
1.0	2.13	0.52	2.40
2.0	2.98	0.24	0.81
3.0	3.75	0.06	0.15
3.50		0.00	0.00
Run #6	pH = 7.0	Temperature = 22°C	
0.0	0.75	0.41	5.50
0.5	0.91	0.40	4.61
1.0	1.10	0.39	3.76
2.0	1.60	0.35	2.16
3.0	2.35	0.10	0.48
3.25		0.03	0.01

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #6	pH = 7.0	Temperature = 23.5 °C	
0.00	0.60	0.60	10.00
0.50	0.75	0.60	8.12
1.50	1.20	0.58	4.83
2.50	1.82	0.36	2.00
3.50	2.70	0.00	0.00
4.25	3.83	0.00	0.00
Run #7	pH = 7.0	Temperature = 25°C	
0.00	0.60	0.58	9.59
0.50	0.75	0.58	7.81
1.50	1.13	0.46	4.44
2.50	2.00	0.14	0.92
3.50	3.12	0.01	0.00
4.75	5.38	0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #8	pH = 7.0	Temperature = 25 °C	
0.0	0.60	0.57	9.50
1.0	0.90	0.55	6.10
1.5	1.13	0.50	4.40
2.5	2.10	0.22	1.03
3.5	3.13	0.00	0.00
4.5	5.20	0.00	0.00
Run #9	pH = 7.0	Temperature = 25°C	
0.00	0.60	0.62	10.33
0.50	0.75	0.60	8.01
1.50	1.13	0.56	4.88
2.50	2.01	0.26	1.27
3.50	3.20	0.00	0.00
4.50	5.17	0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #10	pH = 7.0	Temperature = 25 °C	
0.0	1.20	0.60	5.00
0.5	1.52	0.49	3.25
1.0	1.88	0.28	1.48
1.5	2.46	0.00	0.00
Run #11	pH = 7.0	Temperature = 25 °C	
0.00	0.92	0.61	6.52
0.50	1.25	0.60	4.83
1.00	1.68	0.50	3.00
1.50	2.24	0.30	1.35
2.50	4.09	0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #12	pH = 7.0	Temperature = 29 °C	
0.00	0.61	0.48	7.90
0.50	0.77	0.47	0.22
1.67	1.81	0.44	2.43
2.00	2.50	0.33	1.30
2.40	3.75	0.03	0.01
3.00	5.28	0.00	0.00
Run #13	pH = 7.0	Temperature = 35.0 °C	
0.00	1.30	0.68	5.23
0.50	1.62	0.54	3.35
1.50	2.75	0.21	0.76
2.25	3.50	0.12	0.33
2.75	4.36	0.01	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #16	pH = 6.0	Temperature = 25 °C	
0.0	0.69	0.44	6.38
0.5	0.79	0.44	5.63
1.5	1.00	0.43	3.85
2.5	1.75	0.36	2.06
4.0	2.75	0.17	0.62
4.33		0.00	0.00
Run #17	pH = 6.5	Temperature = 25 °C	
0.0	0.61	0.36	5.91
0.5	0.75	0.35	5.04
1.5	1.13	0.35	3.13
2.5	1.62	0.26	1.55
3.5	2.75	0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #18	pH = 6.75	Temperature = 25 °C	
0.0	0.75	0.600	8.00
0.5	0.92	0.580	6.30
1.5	1.40	0.360	2.55
2.5	2.18	0.00	0.00
3.5	3.40	0.00	0.00
4.0	4.18	0.00	0.00
Run #20	pH = 7.5	Temperature = 25 °C	
0.0	0.80	0.50	6.25
0.5	1.00	0.48	4.81
1.75	1.50	0.18	1.17
2.00	1.87	0.11	0.60
2.50	2.40	0.00	0.00

TABLE A.1 (Continued)

Time (hrs.)	cell concentration (cells/1 x 10^{10})	phenanthrene concentration (ppm)	cell normalized phenanthrene concentration milligrams/cell x 10^{11}
Run #21	pH = 8.0	Temperature = 25 °C	
0.0	0.86	0.513	6.00
0.5	1.00	0.472	4.72
1.75	1.25	0.189	1.52
2.00	1.50	0.130	0.87
2.50	1.87	0.00	0.00
Run #22	pH = 8.0	Temperature = 25 °C	
0.00	0.740	0.594	8.00
0.67	0.87	0.586	6.74
1.67	1.06	0.258	2.434
3.00	1.75	0.006	0.034
3.15	1.85	0.000	0.000

APPENDIX B

EXPERIMENTAL DATA, PHENANTHRENE DEGRADATION BY *BEIJERINCKIA* IN ABSENCE OF YEAST EXTRACT

TABLE B.1

Experimental Data, Phenanthrene Degradation by *Beijerinckia* in Absence of Yeast Extract

Time (hrs.)	cell concentration (cells/liter)	phenanthrene concentration (ppm)	normalized phenanthrene concentration
Run #1			
0.0	1.5×10^{10}	0.70	4.67×10^{-11}
0.25	1.5×10^{10}	0.57	3.72×10^{-11}
0.50	1.5×10^{10}	0.43	2.90×10^{-11}
0.75	1.5×10^{10}	0.30	2.00×10^{-11}
1.00	1.5×10^{10}	0.19	1.25×10^{-11}
1.25	1.5×10^{10}	0.05	0.39×10^{-11}
1.50	1.5×10^{10}	0.00	0.00
Run #2			
0.00	1.5×10^{10}	0.70	4.67×10^{-11}
0.25	1.5×10^{10}	0.55	3.37×10^{-11}
0.50	1.5×10^{10}	0.47	3.31×10^{-11}
0.75	1.5×10^{10}	0.32	2.13×10^{-11}
1.00	1.5×10^{10}	0.20	0.80×10^{-11}
1.25	1.5×10^{10}	0.07	0.47×10^{-11}
1.50	1.5×10^{10}	0.00	0.00

VITA

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