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BIODEGRADATION OF BENZENE AND A BTX MIXTURE USING IMMOBILIZED ACTIVATED SLUDGE

by
Mayur Lodaya

Dissertation submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Engineering Science in Chemical Engineering
1989

APPROVAL SHEET

Title of Thesis: Biodegradation of Benzene and a BTX Mixture Using Immobilized Activated Sludge

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"Biodegradation of 2-Chlorophenol Using Immobilized Activated Sludge". Submitted to JWPCF, April 1989.

ABSTRACT

Title of Thesis: Biodegradation of Benzene and a BTX Mixture Using Immobilized Activated Sludge

Mayur Lodaya: Doctor of Engineering Science, Chemical Engineering, 1988

Thesis directed by: Sam S. Sofer, Professor
Sponsored Chair in Biotechnology

Aerobic biodegradation of benzene was studied using activated sludge immobilized in calcium alginate (immobilization by entrapment), and also attached to a silica based catalyst support (immobilization by attachment). Hydrogen peroxide was used as a source of dissolved oxygen to eliminate physical removal of benzene due to aeration. Abiotic losses of benzene were accounted for.

A recirculation reactor, run in both batch and continuous feed mode, was used to determine the kinetic parameters. The system response was examined by following changes in benzene concentration, flow rate, and biomass loading. The system was modeled mathematically and the kinetic parameters were determined.

Biological removal of a mixture of benzene, toluene and o, m and p-xylene (BTX) was also studied.

In a typical batch experiment starting with 100 ppm benzene, the substrate utilization rate (k_M), when expressed per unit weight of dry catalyst, had a value of 0.4453 ppm/h/g dry beads for the alginate system, and 0.067 ppm/h/g dry beads for the celite catalyst carrier.

Activated sludge was characterized for biodegradation of benzene. Isolations were done for unacclimated, acclimated and end run samples. About 67% of the isolates could be assigned to a genus. These were Bacillus, Microbacterium, Plesiomonas, Kurthia, Klebsiella, Lactobacillus, and Pseudomonas.

From among the fifteen isolates found in the end run group, an isolate identified as Pseudomonas was established as a primary degrader of benzene.

ACKNOWLEDGEMENTS

I wish to take this opportunity to express my heartfelt gratitude to Professor Sam Sofer for his keen and creative guidance throughout the course of this work. I will remember his teachings including those concerning the course work. My sincere thanks are also to Professor Gordon Lewandowski and Professor Peter Strom for their continued help during the course of this work.

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Among my colleagues, I have special regards for Fayaz Lakhwala, Manjari Singh, Emilia Rus, Timothy Roche and Ajit Dastane for their sincere concern towards me. The joyful company provided by other members of biotechnology laboratory including past members, I will cherish for a long time.

It will be proper to put on record, my thanks to the Model maker Bill Guzy and Stock room personnel Yogesh Gandhi for their help.

Rajesh Singh and Bhadri Lodaya have stood by my side during the past two years for all my needs, and their indirect

contributions to the work have meant a lot to me.

Finally, I do not have words to express my gratitude to my parents and family members who have helped me in all respects through a very long academic career.

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CHAPTER I

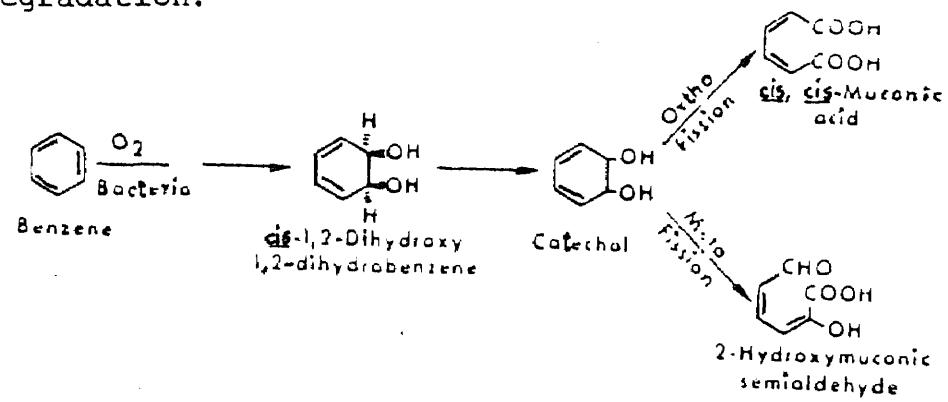
INTRODUCTION

Continuous release of xenobiotics in general, and that of organic compounds listed as priority pollutants in particular, poses a severe threat to the well-being of the human race. For the past two decades, researchers have engaged themselves in finding ways and means of containing the release on one hand, and removing the already released toxic compounds on the other. Removal methods have been either physical, chemical or biological.

Benzene and the related aromatic hydrocarbons are used extensively in various day-to-day applications. This opens more avenues for their release into the environment and subsequently accounts for their ubiquitousness. Benzene, with its toxicological profile, has been labelled as a priority pollutant. The existing standards for benzene as well as toluene and the xylenes in the drinking water supply, and for wastewater treatment are presented in Table 1.1. Given its hydrophobic nature, coupled with the high value of Henry's constant and activity coefficient, physical methods such as air stripping and carbon adsorption have been used widely for removal of benzene. These methods have an inherent drawback in that the result is a change in

the matrix of contamination, rather than the removal of contaminant. Biological removal, although a slower process, guarantees the conversion of complex molecules into simple molecules like CO_2 and H_2O, the release of which into the atmosphere is less of a threat.

It has been noted that benzene resists biodegradation in an anaerobic environment. The degradative pathway under aerobic conditions has been described by Gibson (1977), Cerniglia (1984) and Dagley (1985). The benzene molecule is first converted into a dihydrodiol, followed by catechol formation. This is then followed by ortho fission or meta fission to yield cis, cis-muconic acid or 2-hydroxymuconic semialdehyde, respectively. Both these compounds can enter the Krebs cycle to complete the biodegradation.



The use of immobilized microorganisms has many advantages over the conventional free cell system (Mattiasson, 1983; Chien and Sofer, 1985). Besides preventing washout of biomass in continuous flow reactors,

immobilization facilitates easy separation and imparts greater operational flexibility. Immobilized cells can also be much more resistant to high concentrations of toxic chemicals (Westmeier and Rehm, 1985 and Dwyer et al., 1987). In addition, the cell density of immobilized cells can be much higher than that of the free cells, resulting in higher rates of biodegradation per unit volume of the reactor.

Numerous methods have been developed for immobilized biocatalyst preparation (Dwyer et al., 1987). Microbial cells may be flocculated or aggregated; they may be attached to a suitable carrier by adsorption or ionic bonding, or they may be entrapped in a polymer matrix. Among the most used adsorption carriers are activated charcoal, porous ceramics and porous silica supports. Suitable polymers for entrapment of cells include alginate, K-carageenan, polyacrylamide and polyvinyl alcohol.

One of the many critical parameters which affects the kinetics of immobilized microbes in the case of entrapment is the diffusional or mass transfer effect. Cell entrapment in alginate is a rather simple and non-toxic method for immobilization, but the gel may create a diffusional barrier for both substrate and oxygen. The diffusion of substrates into and out of the alginate gel has been very well addressed by Tanaka et al. (1984). They found that the diffusion coefficients of substrates such as glucose

(in general for substrates with molecular weight less than 20,000) into and from calcium alginate gel beads were the same as the diffusion coefficients of the substrates in the water systems. These results suggest that these substrates can diffuse into and out of calcium alginate gel beads. In work done prior to this in our laboratory (Lakhwala et al., 1989), the physical stability and activity of alginate beads have been optimized with respect to parameters such as biomass concentration in the bead, concentration of sodium alginate, concentration of calcium chloride used for curing the beads and temperature of operation. These parameters are vital to the use of immobilization.

Attachment on the surface of the solid support offers an advantage because the support is stronger than the gel matrix. Here, the microorganisms are believed to form a film on the carrier surface. Hence, it is the film diffusion of the substrate which is important. Microorganisms are also exposed to the bulk liquid, therefore the resistance to shock loads of toxic compounds will not be as good as that with entrapped microorganisms.

Volatilization has been noted in nearly all the studies which were primarily aimed at studying biological removal of benzene. Smith and Bomberger (1979), Kincannon et al. (1983), Pai et al. (1988) and Petrasek et al. (1983) have discussed parameters pertaining

to volatilization rates of a few chemicals in water and wastewater. Smith and Bomberger (1979) and Kincannon et al. (1983) noted that, removal of benzene by volatilization is appreciable and is important while studying biodegradation.

In the present work, biodegradation of benzene has been studied in a recirculation reactor operated in batch and continuous feed mode. Various process parameters were studied, such as recycle rate, biomass loading, and concentration of benzene. A non-linear regression model was developed to evaluate the kinetic parameters for the batch mode. Unsteady state analysis of the continuous feed mode was also performed. Both calcium alginate beads (immobilization by entrapment) and celite catalyst carrier (immobilization by attachment) were used in the study. Biological removal of a mixture of benzene, toluene and o, m and p-xylene (BTX) was also studied. Finally, a preliminary economic analysis was performed for the calcium alginate system to provide a protocol for detailed economic analysis, and to assess the material and energy requirements of the process.

TABLE1.1 PROPERTIES AND STANDARDS FOR BENZENE, TOLUENE AND o, m, p- XYLENES

COMPOUND	SOLUBILITY[a] IN WATER (W/W %, @25°C)	DRINKING WATER STANDARD	WASTEWATER STANDARD
BENZENE	0.18	1 PPB MCL(NJDEP)	10 PPM TVOC (NJDEP)
		5 PPB MCL(USEPA)	NONE
TOLUENE	0.052	2 PPM GOAL(USEPA)	NONE
o-XYLENE	0.018		
m-XYLENE	0.02	0.44 PPM (NJDEP/USEPA)	NONE
p-XYLENE	0.19		

MCL= MAXIMUM CONTAMINANT LIMIT

TVOC= TOXIC VOLATILE ORGANIC CHEMICAL

a= From Kirk and Othmer (1978)

CHAPTER II

LITERATURE SURVEY

Activated sludge has been recognized as one of the most versatile methods known for wastewater treatment. Sawyer (1965) has given an excellent account of the developments in the activated sludge process in the first fifty years of its use. With its widespread use for the treatment of domestic and industrial waste streams, it allows for a very easily accessible source of biomass.

Apart from the work related to bacteriology of activated sludge, which is discussed in a later section, researchers have also studied process modelling, viability, oxygen requirements and fate of certain priority pollutants with respect to activated sludge. Sherrard et al. (1983) have given a comprehensive review of the work done on different aspects of activated sludge.

On the process modelling front, Gaudy and Kincannon (1977a and 1977b) have critically reviewed the models which attempt to describe the functional behaviour of the process. They also presented a step by step procedure for employing the Gaudy model proposed by them and have discussed its limitations.

Shamat and Maier (1980), Tabak et al. (1981), Stover and Kincannon (1982) and Kirsch et al.(1983) have discussed the fate of organic priority pollutants in activated sludge systems.

Shamat and Maier (1980) considered biological removal of chlorinated organics using enriched cultures obtained from a municipal wastewater treatment plant. They also correlated rates in terms of conventional activated sludge kinetics based on bacterial growth.

Tabak et al. (1981) studied the degradability and acclimation rates of 96 compounds. The compounds studied included: monocyclic and polycyclic aromatic hydrocarbons and their chlorine substituted counterparts, phenolics, and a host of pesticides and insecticides.

Stover and Kincannon (1982) studied the fate of specific organic compounds during conventional biological treatment. They also included the effects of stripping and adsorption while estimating the removals.

Kirsch et al. (1983) attempted the development of a protocol for evaluation of the fate of a priority pollutant.

The technique of using immobilized microorganisms for treatment of hazardous and toxic wastes has been recognized as a promising method (Westmeier and Rehm, 1985; Bettman and Rehm, 1985; Westmeier and Rehm, 1987; Andrews et al., 1988 and Lakhwala et al., 1989).

Rehm's group studied degradation of 4-chlorophenol by free and immobilized Alcaligenes sp. A7-2. They used different methods of immobilization; i.e., immobilization in polyacrylamide-hydrazide (PAAH), adsorptive immobilization, and immobilization in calcium alginate gel. They showed that higher concentrations of 4-chlorophenol were better tolerated and more quickly degraded by immobilized cells than by free cells. Their study included batch as well as continuous operations. However, they did not consider the kinetics of biodegradation.

Andrews et al. (1988) used a consortium of microorganisms developed by municipal sewage enrichments with a mixture of benzene, toluene and xylenes (BTX). The consortium so developed was immobilized on celite carrier (R-635). They reported a very high percentage of influent being removed biologically, but their influent concentrations were moderately low (40 ppm). They concluded that biological degradation of gasoline components contained in contaminated groundwaters using immobilized microorganisms is a viable remediation process. The use of naturally adopted organisms immobilized on diatomaceous earth supports (R-635 in this case) can provide an efficient, stable biomass population for the continuous treatment of dilute volatile organic waste streams.

In an earlier study conducted in this laboratory (Lakhwala et al., 1989), biodegradation of 2-chlorophenol was studied using immobilized activated sludge. This study also demonstrated the ability of immobilized microorganisms to withstand high concentrations of organic compounds. Parameters important to bead design were optimized to obtain a physically stable and viable microbial population. Different reactor configurations were employed and parameters influencing reactor design were studied. A non-linear regression model was developed to estimate kinetic parameters under varying conditions of flow, 2-chlorophenol concentration, and biomass loadings.

Biodegradation of benzene and related aromatic hydrocarbons has been attempted by Lee and Ryan (1976), Thomas et al. (1986),and Weber et al. (1987), among others.

Lee et al. (1976) and Thomas et al. (1986) studied the biodegradation of benzene using microorganisms of marine origin. Although these studies pointed towards biodegradability of benzene and some of its derivatives, the studies were qualitative in nature.

Weber et al. (1987) studied removal of nine organic compounds including benzene, toluene, and o-xylene in activated sludge treatment systems. Addition of powdered activated charcoal to enhance the removal was evaluated. They concluded that although the removal of biodegradable

compounds did not change much, significant removals of poorly biodegradable and non-biodegradable compounds were noticed.

CHAPTER III

OBJECTIVES

The objectives of the present study were to:

(I) Conduct biodegradation studies using activated sludge immobilized by entrapment and attachment.

1. Conduct biodegradation studies in batch feed mode using a recirculation reactor, in order to:

(a) Determine flow rate of recycle stream, biomass loading, and spiking concentration of benzene.

(b) Develop a model to verify the kinetics of biodegradation.

2. Conduct biodegradation studies in continuous feed mode using a recirculation reactor, in order to:

(a) Determine variation in effluent concentration of benzene with retention time.

(b) Develop a model to verify the kinetics of biodegradation.

(II) Conduct biodegradation studies of a BTX mixture and study the pattern and extent of biodegradation.

(III) Isolate the mixed culture microorganisms and identify the microbial biota.

(IV) Develop preliminary economic analysis of the calcium alginate system.

CHAPTER IV

MATERIALS AND EXPERIMENTAL METHODS

4.1 MICROORGANISMS

Activated sludge (a mixed microbial community) was obtained from the Parsippany-Troy Hills Water Pollution Control Plant (N.J.). A portion of the mixed liquor was centrifuged (3,000 rpm and 15°C) soon after it was brought from the plant, and stored as unacclimated pellets at 4°C for about a month. Following this, the microorganisms were acclimated to a mixture of benzene, toluene and o, m and p-xylene (10 ppm each) at room temperature. The microorganisms were spiked daily over a period of seven days, and during this period, the oxygen consumption of a fixed amount of sludge was measured daily in the microassay reactor (Figure 4.1), which is described later in the text. The oxygen consumption rate decreased initially and later remained constant, at which point acclimation was considered to be complete. The culture was then centrifuged at 3000 rpm and 15°C to obtain concentrated pellets. These were stored at 4°C as acclimated pellets for about three months.

4.2 NUTRIENT MEDIUM

Two different compositions of the nutrient medium were used in the study. Whereas nutrient medium (1) was used for the most part, nutrient medium (2) was used for the preparation of inoculum for attachment of activated sludge on celite carrier. The composition of nutrient medium (1) was developed by us and the composition of nutrient medium (2) was obtained from Manville Corporation.

Nutrient Medium (1)

The nutrient medium (1) consisted of the following:

Magnesium chloride	100 mg
Manganese sulfate	10 mg
Ferric chloride	0.5 mg
Potassium phosphate	10 mg
Ammonium sulfate	10 mg
Distilled Water	100 ml

The above solution was then diluted to 1000 ml by adding distilled water. One can note that the concentration of potassium phosphate was very low. Initially, potassium phosphate was used at a concentration of 1 g/l, but this led to a shorter bead life. The concentration of potassium phosphate was then reduced to 10 mg/l. It was observed that this change did not affect the microbial activity. Also, by making this change it was possible to increase the bead life

from about 24 hours to more than a month without any observable deterioration in the bead structure.

Nutrient Medium (2)

The nutrient medium (2) consisted of the following:

Magnesium chloride	0.02 g
Sodium dihydrogen phosphate	2.50 g
Potassium monohydrogen phosphate	1.25 g
Ammonium sulfate	0.500 mg

The above salts were dissolved in distilled water and the volume made up to 1000 ml. The pH was adjusted to 7.0 with either acid or base.

4.3 MEASUREMENT OF BIOMASS

A known weight of pellets obtained after centrifugation was dried at 120°C for 24 hours. The dry weight so obtained was expressed as dry biomass per unit weight of pellets. For the present set of experiments, biomass concentration was 55 mg dry biomass per gram of pellets. In the case of alginate beads, estimation of the number of viable cells was done by plate count method. The details are discussed in the chapter on materials and experimental methods of Part II.

4.4 IMMOBILIZATION

4.4 (i) Entrapment of activated sludge in calcium alginate gel

For a given batch of pellets (50 g), a typical procedure for making beads was as follows. Distilled water and concentrated pellets (55 mg dry biomass/g of pellet) were taken in a ratio of 5:2 by weight along with sodium chloride (0.05% w/w) in a blender. Sodium alginate (1.5% w/w) was then added slowly over a period of 2 to 3 minutes to the mixture, with continuous stirring to obtain a homogeneous cell suspension. The homogeneous cell suspension was then extruded as discrete droplets using a syringe pump, into a slowly stirred 500 ml solution of 0.1 M calcium chloride. On contact with the calcium chloride solution, the droplets hardened to form beads about 3 to 3.5 mm in diameter. The beads were then cured in 0.1 M calcium chloride for 24 hours at $4^{o}C$ before use.

4.4 (ii) Attachment of activated sludge on celite^{R} carrier (R-635)

The carrier is a diatomaceous earth based proprietary material of Manville Corporation (Denver, Colorado). It is in the form of a cylindrical pellet (0.25"D X 0.5"L) with a mean pore diameter of 20 um. The physical properties of celite carrier are listed in Table 4.1. The first step in

the process was preparation of inoculum in a shaker flask. For this purpose, 200 ml of nutrient medium (2) was inoculated by the mixed culture, and benzene (20 ppm) was fed as the only carbon source once everyday for a period of 7 days during which enough growth was observed. This was then poured on to a dry celite carrier contained in a reactor (described in the section on "batch experiments using recirculation reactor"). This was followed by setting up of a recirculation system in which 2 liters of nutrient medium (2) containing 100 ppm benzene was recycled between a reservoir and a reactor for two days. This treatment ensured attachment of microorganisms on, to, and within the porous structure of the carrier. The above mentioned procedure was adopted following Manville Corporation's instructions.

4.5 MEASUREMENTS FOR PHYSICAL REMOVAL

Benzene is known to be a very volatile compound and hence aeration of any kind may result in physical removal being the primary mode of removal, rather than biological removal. As seen from Figure 4.2, simple high speed stirring (enough to renew the gas/liquid surface) is enough to remove benzene completely from a starting concentration of 100 ppm in approximately 8 hours. Thus the experiments were done at a very low stirring speed and the resulting physical removal

was characterized and accounted for, both benzene and the BTX mixture in batch mode (Figure 4.2 and 4.3).

4.6 SUPPLY OF H_2O_2 AND MONITORING OF DISSOLVED OXYGEN

On the basis of the results of the physical removal studies, it was evident that routine methods of aeration and / or supply of pure oxygen, as a means of maintaining the dissolved oxygen (DO) could not be applied in this case. Catalase, a terminal respiratory enzyme, is present practically in all aerobic living cells and it breaks down H_2O_2 into H_2O and O_2 (Gabbita and Huang, 1984). A very dilute solution of H_2O_2 (30 g/l) was injected periodically to provide the dissolved oxygen needed for aerobic biodegradation. As can be seen from Figure 4.4, periodic addition of very small volumes of H_2O_2 (1 ml/h) was enough to keep the DO concentrations above 2 mg/l, while making sure that this did not result in chemical oxidation of benzene. Concentration of dissolved oxygen was monitored using a Clark-type dissolved oxygen probe. The oxygen probe was connected through a signal conditioner and amplifier to a chart recorder assembly. Conditions under which H_2O_2 will oxidize benzene have been discussed among others by (Tamagaki et al., 1983 and Dedov et al., 1985). Given that a very small quantity of H_2O_2 is added, and the very short

half life due to large quantities of the catalase present in the activated sludge, it can be safely assumed that there is no chemical oxidation of benzene.

4.7 DISSOLVED OXYGEN MEASUREMENTS IN MICROASSAY REACTOR

The experimental set up of the microassay reactor is shown in Figure 4.1. The microassay reactor is a small jacketed vessel of 1.8 ml capacity, with provision for a Clark-type DO probe. This reactor has been a valuable tool in enzyme kinetic studies because the data produced are accurate and reproducible. It is also economical in the sense that it requires very small amounts of reagents. In the present work, the reactor proved suitable for easy yet explicit measurements of DO. Water at the required temperature was circulated through the jacket, and the reactor was mounted on a magnetic stirrer plate.

4.8 BIODEGRADATION STUDIES OF BENZENE IN BATCH MODE

The recirculation reactor used in the present study has been described by Chambers et al. (1972). The scope of its use in a relevant work has been described by Yang (1988). It is a tool designed to determine a regime of operation with respect to specific kinetic parameters. This

helps to accurately determine operating windows while optimizing with respect to reaction rate. Since oxygen is a co-substrate in aerobic biodegradation, the importance of monitoring oxygen concentration is evident. The configuration of the recirculation flow reactor enables continuous measurement of the rate of dissolved oxygen consumption. Thus, biodegradation of the compound in question can also be assayed in terms of oxygen consumption.

The experimental set up of the recirculation reactor is shown in Figure 4.5. The bioreactor is 2.5 inches in diameter by 8 inches long (640 ml). The reservoir is 4.5 inches in diameter by 10 inches long. The total reaction volume is 2 liters. The reaction medium was circulated between substrate reservoir and reactor using a centrifugal pump at a flow rate of 300 ml/min. The reactor contained a thermometer and a pH probe. Liquid samples were taken periodically from the reservoir and analyzed by gas chromatography for benzene. All experiments in the recirculation reactor were done at room temperature (25 to 27 ^{o}C).

Oxygen consumption was continuously determined by measuring the slope of dissolved oxygen concentration vs. time from the chart recorder.

Whereas the weight of celite carrier used was 300 g for all the runs (this means essentially constant biomass),

the biomass loading in the case of calcium alginate beads was varied from 2.5 g to 15 g dry bead weight. The two reactors were the same in all other respects.

4.9 BIODEGRADATION STUDIES OF BENZENE IN CONTINUOUS FEED MODE

The dimensions of the reactor used in these studies were similar to those of the reactor used in the batch mode. The reservoir used in this case is 10.7 inches in diameter by 49.5 inches long.

4.9 (i) Plug flow regime

In this case, the H_2O_2 solution required to satisfy the oxygen demand had to be supplied to the reservoir. Depending on the desired retention time and knowing the oxygen consumption rates from batch studies, the amounts of H_2O_2 needed were calculated. Unfortunately, these amounts turned out to be high enough to cause chemical oxidation of benzene in the reservoir which was noted immediately by the change in color of the reservoir solution. The solution gradually changed from colorless to dark brown. Following this, no further studies were done in plug flow regime.

4.9 (ii) Mixed flow regime

In this case, additional inlet and outlet streams were provided to mix the reactor contents by recirculating it at high flow rates using a centrifugal pump (Figure 4.6). Liquid samples were taken periodically from the reactor case, and analyzed by gas chromatography for benzene. Oxygen measurements were done as with the batch experiments.

In this set of experiments, the weight of celite carrier used was 300 g, the biomass loading in the case of calcium alginate entrapped beads was kept at 150 g of wet beads, and the total reaction volume was 600 ml.

4.10 ANALYSIS OF BENZENE AND BTX MIXTURE BY GAS CHROMATOGRAPHY

A Perkin Elmer gas chromatograph (8500 series) was used for analysis of benzene and BTX mixture. The column used for this purpose was 5% sp-1200 containing 1.75% Bentone-34 on 100/120 Supelcoport^{R}. For calibration purposes, standards were prepared in hexane. Saturated solution of benzene, was prepared by adding excess of it to nutrient medium (1), and stirring the resulting two phase for over a period of one week. In practice, a 3 l conical flask (stoppered) containing two phases was stirred all the time. These solutions were diluted depending on the desired initial concentrations. Similar procedure was followed for BTX mixture.

TABLE 4.1 PHYSICAL PROPERTIES OF CELITE^{R} CARRIER

Form (cylindrical pellet)	approx. 0.25"D x 0.5"L
Mean pore Diameter	20.0 um
Surface area B.E.T.	0.27 m^2/g
Total Pore Volume	0.61 cm^3/g
Water Absorption % by weight pellet method	60.0
Hardness-Monsanto Hardness test	8.0
Bed Density (compacted) lbs/ft^3	32.0

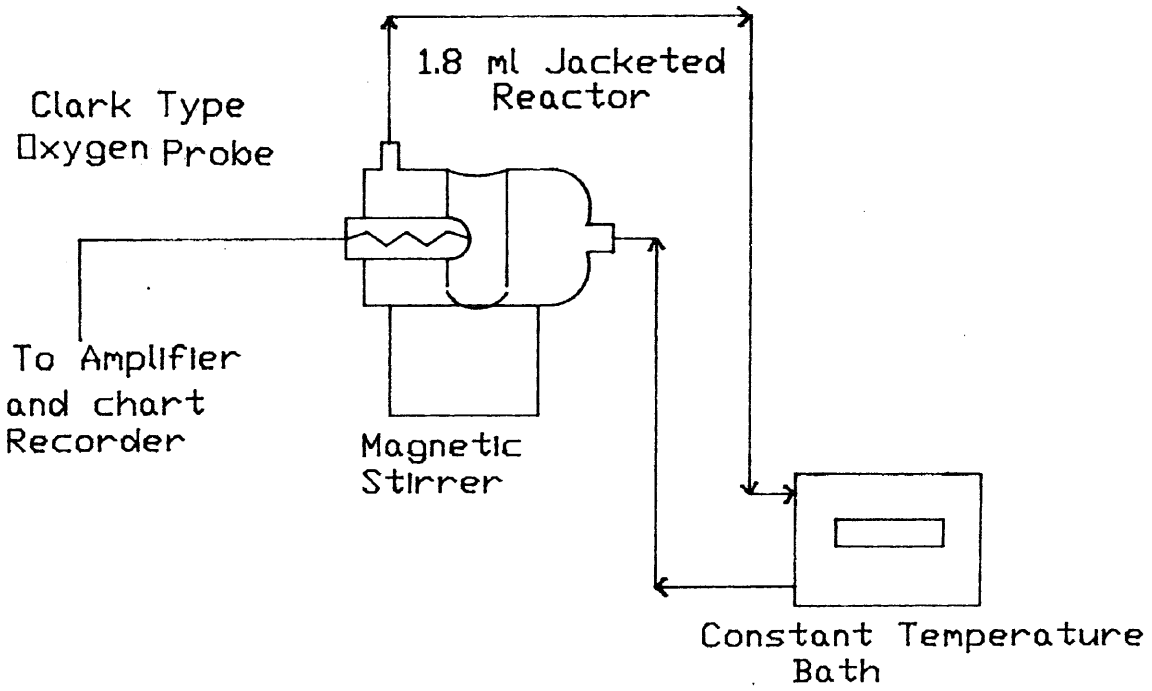


Fig. 4.1 Microassay Reactor

Fig.4.2 Physical removal of benzene in batch recirculation reactor

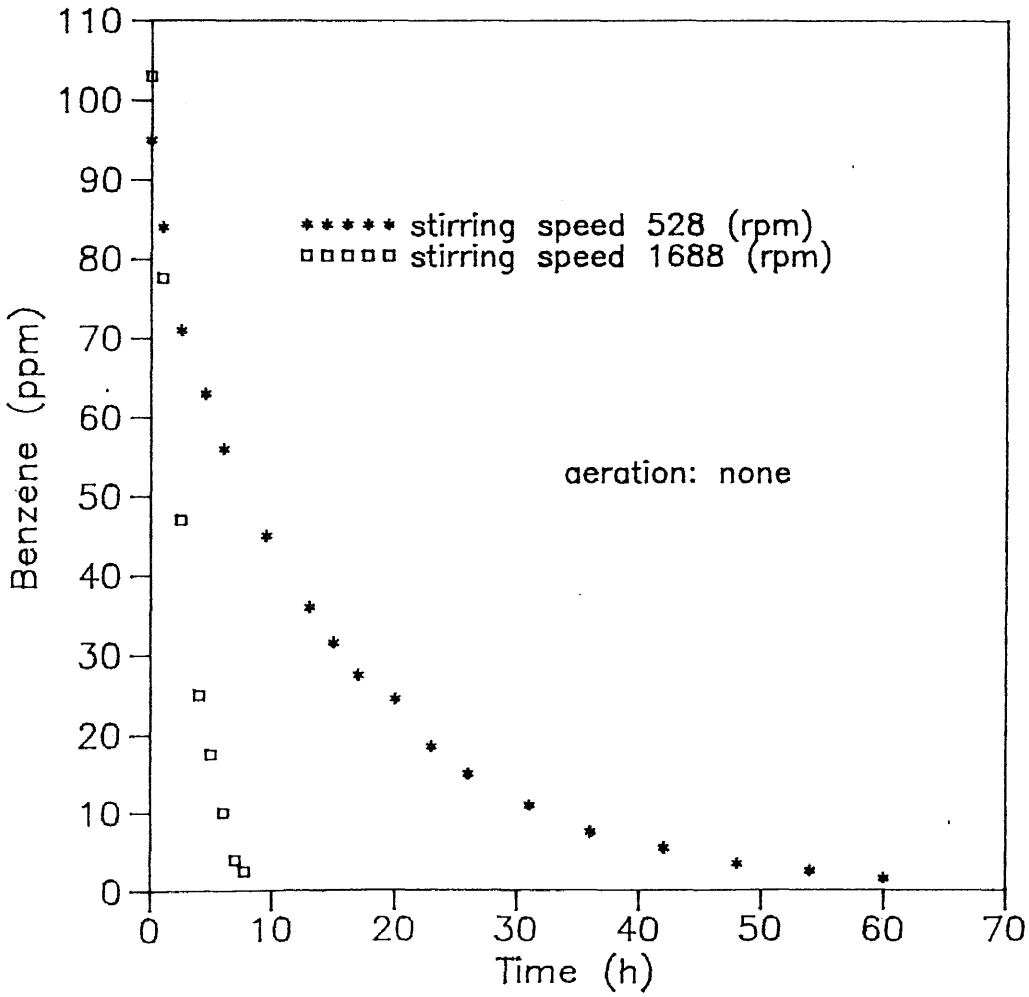


Fig.4.3 Physical removal of BTX mixture in batch recirculation reactor

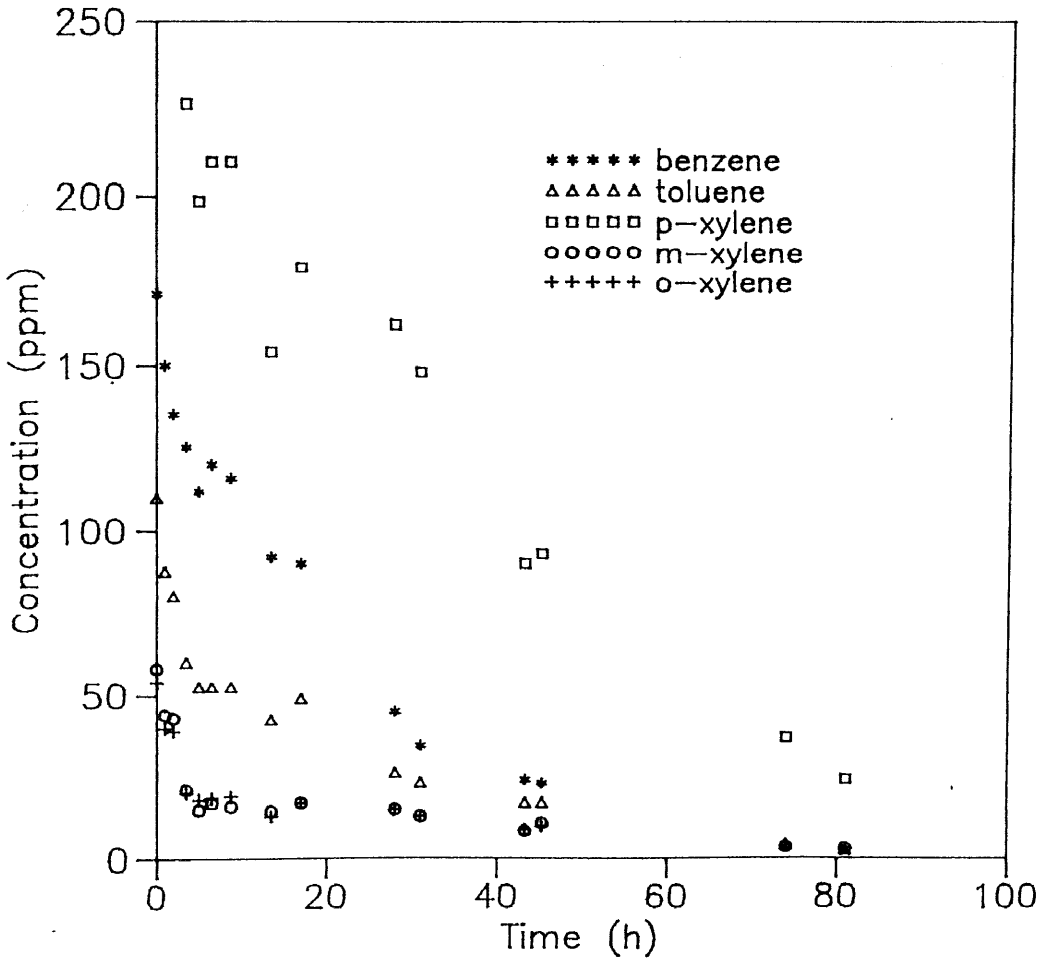
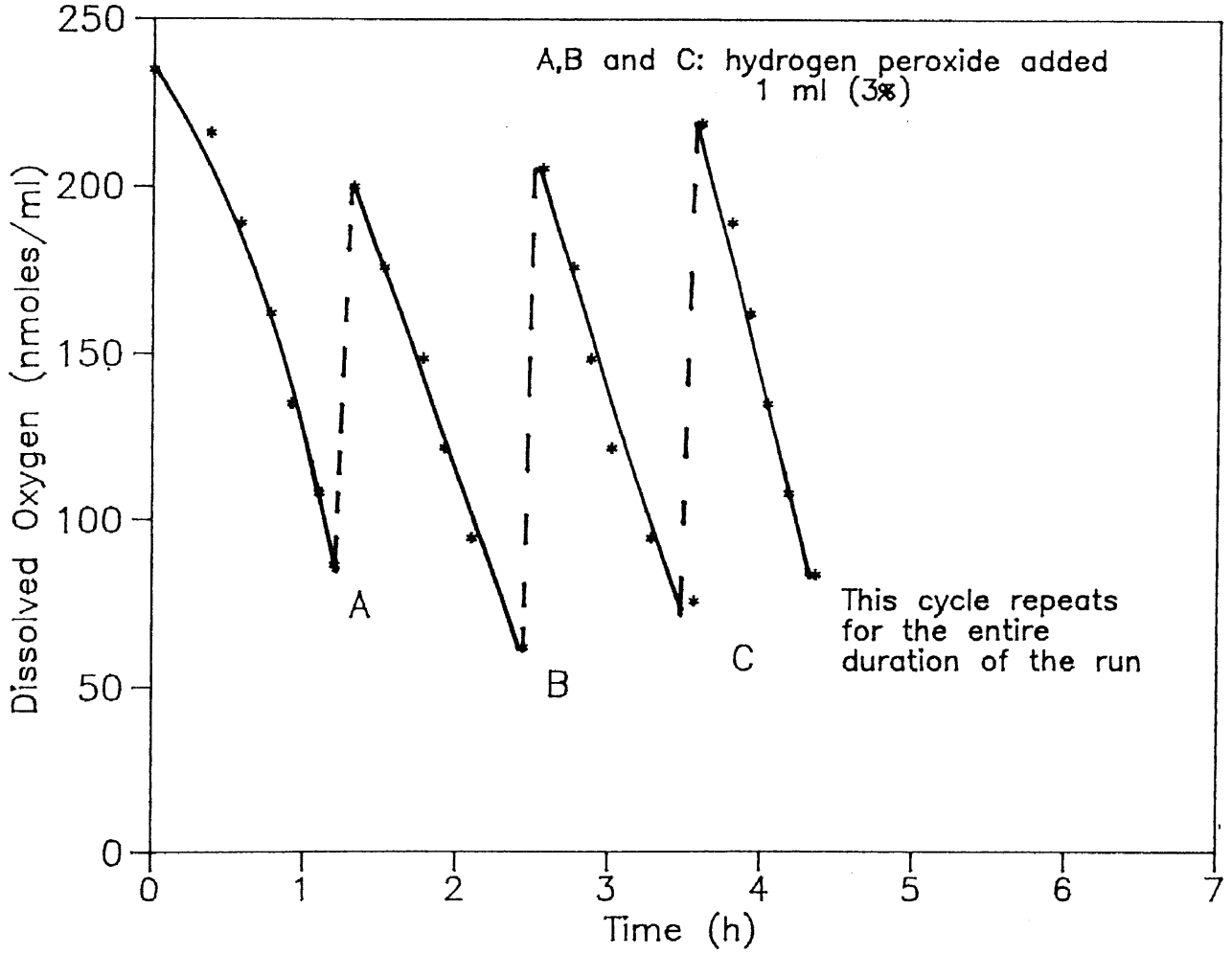


Fig.4.4 Supply of hydrogen peroxide in batch recirculation reactor to maintain dissolved oxygen



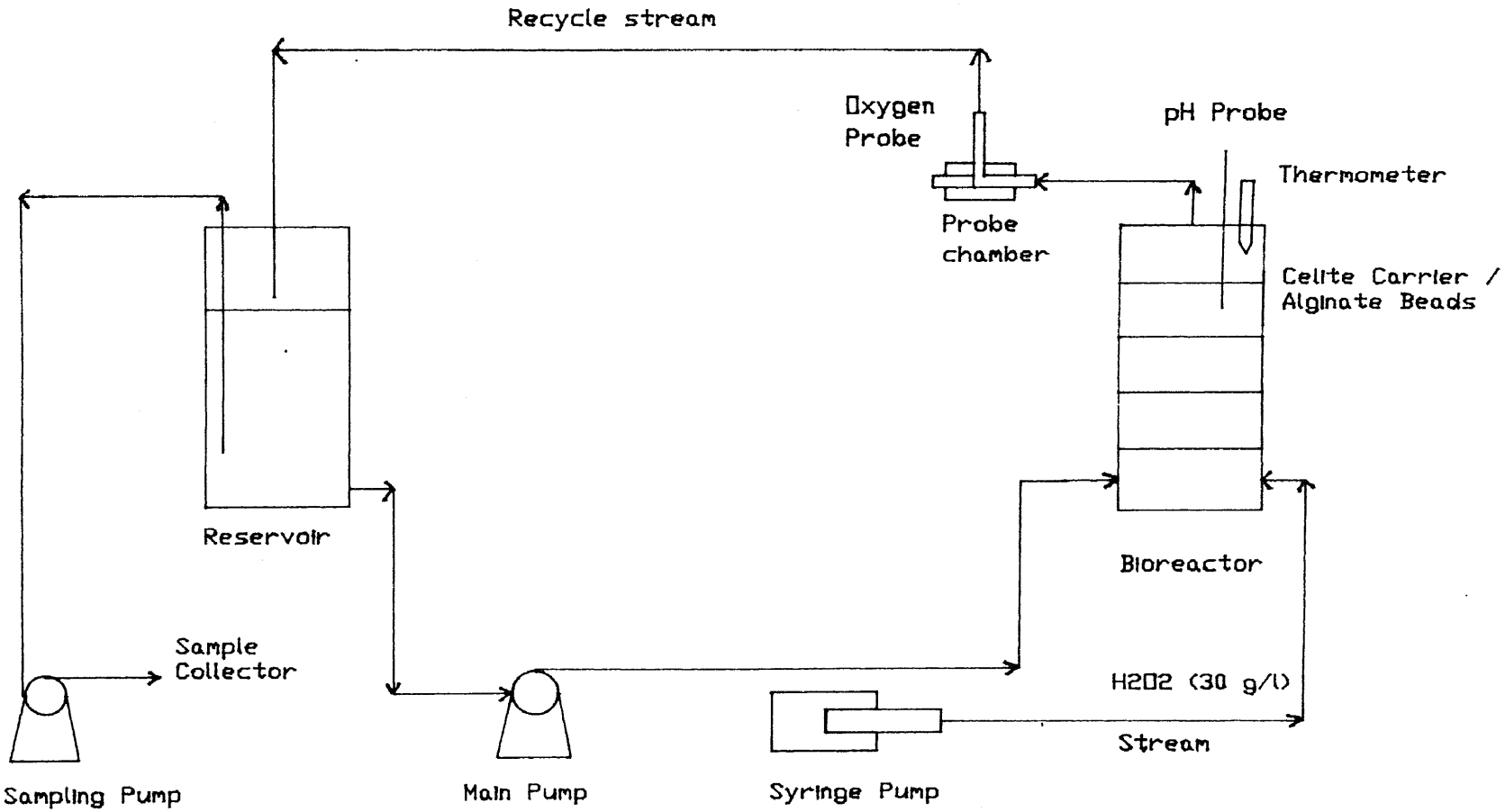


Fig. 4.5 Recirculation Reactor (batch feed mode)

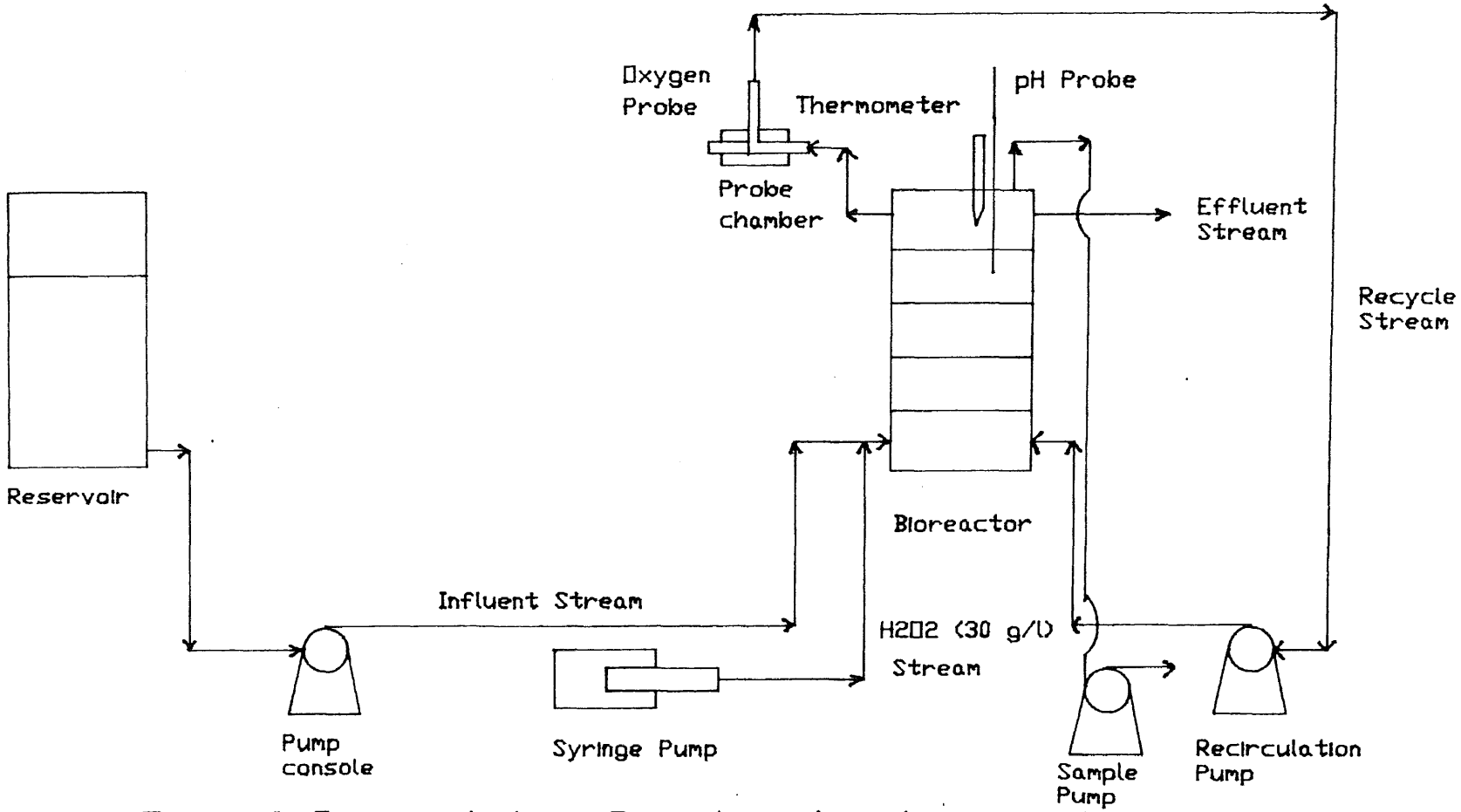


Fig. 4.6 Recirculation Reactor (continuous feed mode)

CHAPTER V

RATE EQUATIONS

The biodegradation rate was modeled using a Monod expression. Given the size of the benzene molecule, it is assumed here that the pore diffusion inside the beads (in the case of alginate) and film diffusion across the biofilm (in the case of celite carrier) are not important.

The Monod expression for substrate utilization rate can be given as (Tchobanoglous, 1972),

$$r_b = \frac{\mu_m * X * S}{Y * (K_s + S)} \qquad (1)$$

where,

r_b = Substrate utilization rate (ppm/h)

μ_m = Maximum specific growth rate (h^{-1})

K_s = Half-velocity coefficient (ppm)

S = Substrate concentration (ppm)

X = Concentration of microorganisms (ppm)

Y = Maximum yield coefficient (mass/mass)

Denoting the ratio μ_m/Y, defined as the maximum rate of substrate utilization per unit mass of microorganisms by k (h^{-1}), the expression for substrate utilization becomes,

$$r_b = \frac{k * X * S}{(K_s + S)} \quad (2)$$

For a reactor configuration operating under essentially constant biomass, the substrate utilization may be rewritten as,

$$r_b = \frac{k_M * S}{K_s + S} \quad (3)$$

$k_M = k * X$ = Maximum substrate utilization rate (ppm/h).

In biodegradation studies of volatile compounds it also is necessary to have a term representing physical removal when attempting to model such a system.

5.1 BATCH STUDIES

For aerobic biodegradation in a batch reactor, removal of substrate can be expressed as,

$$-\frac{dS}{dt} = r_b + r_a \quad (4)$$

Where,

r_a = Rate of physical removal of the substrate.

t = Time (h)

Therefore,

$$-\frac{dS}{dt} = \frac{k_M * S}{K_s + S} + K_3 * S \qquad (5)$$

where

K_3= First order rate constant for air stripping, and

S= Concentration of substrate (ppm).

Integration of equation (1) yields,

$$\left(\frac{K_s}{k_M+K_s*K_3}\right)*\ln\left(\frac{S_0}{S_i}\right)+\left(\frac{k_M}{K_3*(k_M+K_s*K_3)}\right)+\ln\left(\frac{k_M+K_s*K_3+K_3*S_0}{k_M+K_s*K_3+K_3*S_i}\right)=t_i. \qquad (6)$$

Where, the subscript 0 stands for zero time and the subscript i stands for any time t.

Non-linear regression was used to determine k_M and K_s. For this, the sum $\underline{S}$ to be minimized is

$$\underline{S} = \Sigma (S_i - S_i^*)^2 = \Sigma (t_i - t_i^*)^2, \qquad (7)$$

$$\frac{\partial S}{\partial k_M} = 2 * \sum (t_i - t_i^*) * \left(\frac{\partial t_i^*}{\partial k_M}\right) = 0, \text{ and} \quad (8)$$

$$\frac{\partial S}{\partial K_S} = 2 * \sum (t_i - t_i^*) * \left(\frac{\partial t_i^*}{\partial K_S}\right) = 0, \quad (9)$$

where

$$t_i^*(k_M+\Delta k_M,\ K_S+\Delta K_S) = (t_i^*(k_M,\ K_S) + \Delta k_M * \left(\frac{\partial t_i^*}{\partial k_M}\right) + \Delta K_S * \left(\frac{\partial t_i^*}{\partial K_S}\right). \quad (10)$$

On substituting (10) in (8) and (9)

$$\sum \left(t_i^* * \left(\frac{\partial t_i^*}{\partial k_M}\right)\right) - \sum \left(\left(t_i^* + \Delta k_M * \left(\frac{\partial t_i^*}{\partial k_M}\right) + \Delta K_S * \left(\frac{\partial t_i^*}{\partial K_S}\right)\right) * \left(\frac{\partial t_i^*}{\partial k_M}\right)\right) = 0 \quad (11)$$

$$\sum \left(t_i^* * \left(\frac{\partial t_i^*}{\partial K_S}\right)\right) - \sum \left(\left(t_i^* + \Delta k_M * \left(\frac{\partial t_i^*}{\partial k_M}\right) + \Delta K_S * \left(\frac{\partial t_i^*}{\partial K_S}\right)\right) * \left(\frac{\partial t_i^*}{\partial K_S}\right)\right) = 0 \quad (12)$$

Equations (11) and (12) are to be solved for Δk_M and ΔK_S.

A FORTRAN program was written for this purpose (Appendix I). Knowing the constant K_3 (from the control run) and given the experimental data along with initial guesses of k_M and K_S, the program converges to the values of k_M and K_S for which Δk_M and ΔK_S are minimum. It was observed that the initial guesses of k_M and K_S had to be provided within an order of magnitude of the regressed values to obtain convergence.

5.2 CONTINUOUS FEED STUDIES

A general expression for the material balance of substrate in continuous feed studies is,

$$F_o * S_o = F_o * S + \left(\frac{k_M * S}{K_S + S}\right) * V + V * \left(\frac{dS}{dt}\right) \qquad (13)$$

Where,

F_o = Feed rate (ml/h),

S_o = Feed concentration (ppm) of benzene at any time t,

S = Outlet concentration (ppm) of benzene at any time t, and

V = Volume of the reactor (ml).

Since the reservoir is open to the atmosphere, the feed concentration of benzene varies with time such that,

$$S_o = S_{oo} * \exp(-K_3 * t). \qquad (14)$$

Where

S_{oo} = Feed concentration of benzene at the beginning, and

K_3 = First order rate constant for volatilization (h^{-1}).

Using equation (14), equation (13) may be rewritten as,

$$F_o * S_{oo} * \exp(-K_3 * t) = F_o * S + \left(\frac{k_M * S}{K_S + S}\right) * V + V * \left(\frac{dS}{dt}\right). \quad (15)$$

Equation (15) can be solved numerically for S to predict the reactor performance over time, at a given feed rate and a given reactor volume. The values of the kinetic parameters (k_M and K_S) are obtained from batch data while the volatilization rate (K_3) may be determined either experimentally, or estimated using activity coefficient values from the literature.

Also if the profile of S can be expressed as some known function of t, equation (15) reduces to an algebraic equation. This can than be solved iteratively to obtain the values of kinetic parameters, which in turn can be compared with the values obtained from batch experiments. The agreement in the kinetic parameters will reflect the validity of the functional relationship used to express S as a function of t. It is important to note here that if the

concentration of biomass X is different in any of the cases, a volume correction has to be applied before comparing the kinetic parameters.

It was observed that using a sixth order polynomial, 93+% of the residuals about the curve fall within the statistical standard deviation limits. By using polynomials of higher order the fit did not improve any further.

The values of k_M and K_S were estimated by a gradient based minimization technique using Marquardt's method (Ragsdell K.M., 1983). This is a modification of Newton's method and requires second order information. The method uses a gradient search far from the solution and switches to Newton's direction when close to the solution. Given the algebraic nature of the equation, the values of k_M and K_S will depend on the initial guess. Based on the values of k_M and K_S obtained from the batch experiments, a good initial guess could be provided. A FORTRAN program written for this purpose is shown in Appendix II.

CHAPTER VI

RESULTS AND DISCUSSION

6.1 BIODEGRADATION STUDIES IN BATCH MODE

Experiments were conducted using activated sludge immobilized in calcium alginate gel and on celite carrier. Various parameters were studied using the two bioreactors. In a typical experiment (Figure 6.1), a comparison done on the basis of the same volume of waste stream being treated shows that both modes of immobilization are equally effective. On the other hand when a comparison is done on the basis of biodegradation rates achieved per gram of dry catalyst, the values of k_M obtained per gram of dry catalyst are around 0.4 ppm/h (for alginate beads) and 0.067 ppm/h (for celite carrier). This means that effectively speaking, the rates obtained in the case of alginate beads are six times higher than those for the celite carrier when compared on a unit weight of dry catalyst. The reason for this difference is that the two immobilization techniques operate completely differently from each other and therefore, for a given situation the optimum biomass loading need not be the same for the two cases. Whereas the optimum loading for celite carrier was decided based on the work done by Manville Corporation's research group, that for alginate

beads was decided based on results obtained with different biomass loadings.

The total time for removal when starting with 100 ppm of benzene in the presence of biomass (24 hours) when compared to that in the absence of biomass (60 hours), suggests that a very high percentage of benzene is being removed biologically. The physical losses are from the reservoir which is open to the atmosphere. The reactor is completely filled and closed, preventing volatilization.

6.1(i) Use of calcium alginate entrapped activated sludge

The recirculation reactor was used to study parameters such as flow rate of recycle stream, biomass loading and spiking concentration of benzene.

(a) Effect of recirculation rate

The recirculation rate has a dual effect on the system. At low flow rates, the system may be under the limitation of external mass transfer. On the other hand, at very high flow rates, the bead structure can be damaged.

Table 6.1 shows that above a flow of 100 ml/min (cross sectional velocity of 2.5 cm/min) there is no apparent effect of external mass transfer on the rate of biodegradation.

As far as bead stability is concerned, the operation

was limited to cross sectional velocities below 30 cm/min (beyond which the beads disintegrated in only 48 hours). In the present study, most of the experiments were conducted at a cross sectional velocity of 7.5 cm/min, at which the beads remained stable for at least one month. In biodegradation studies on polymer being conducted at this time, beads have remained stable for more than five months.

(b) Effect of biomass loading on rate of biodegradation

The values of specific apparent k_M from Table 6.2 suggest that with an increase in biomass loading the k_M does not increase proportionately, although the K_S values do not change appreciably. Also, if the reaction rate is controlled strictly by enzyme kinetics, then both k_M and K_S are functions of biomass loading only. This points towards the presence of unknown mass transfer effects and needs further study.

(c) Effect of spiking concentration on rate of biodegradation

The effect of the spiking concentration of benzene was studied over a wide range of feed concentrations. As expected, the value of k_M does not vary appreciably with change in benzene concentration (Table 6.2). The one exception was with 50 g of biomass. Although it was not

possible to achieve feed concentrations anywhere near the reported saturation concentration of benzene (1800 ppm) due to its volatile nature, feed concentrations in the range of 800 to 1000 ppm were studied (Figure 6.2). It was possible to remove benzene completely in about 57 hours in the presence of biomass when compared to about 115 hours in the absence of biomass. The pH remained in the range of 6.1 to 6.5 during the entire run.

6.1 (ii) Activated sludge immobilized on celite carrier (R-635)

For this case, mainly the effect of variation in spiking concentration of benzene on the rate of biodegradation was studied (Table 6.3). As in the earlier case, the rate remained essentially unaltered with changes in the feed concentration. For experiments done at feed concentrations in the range of 800 to 1000 ppm (Figure 6.3), the removal profiles observed for benzene are similar to the ones obtained in the case of alginate beads.

6.1 (iii) Biodegradation of BTX mixture

Starting with a mixture containing benzene, toluene, and o, m and p-xylene at concentrations of 150 ppm, 100 ppm, 25 ppm, 23 ppm and 207 ppm respectively (Figures 6.4 and 6.5), it took about 40 hours to remove all

the contaminants in the presence of biomass when compared to about 81 hours in the absence of biomass (not shown). It was observed that both alginate beads and celite carrier performed equally well. While no particular selectivity with respect to a compound was observed, benzene and p-xylene were the last ones to be removed, as they were present at high levels to begin with.

6.1 (iv) Kinetics of biodegradation

The total removal data for benzene were fit to an equation which incorporated both biological and physical removal processes. As seen in Figure 6.6, the predicted equation agrees well with the experimental data.

From Table 6.2 and Table 6.3, it can be seen that the k_M and K_S values are very different in the two cases. Whereas k_M depends on the biomass as well as the mass transfer characteristics of the system, K_S, the half velocity coefficient is primarily dependent upon the type of biomass (enzyme system induced), and has little dependence on the mass transfer characteristics. Although the biomass was the same to begin with in both the cases, it is possible that there is more than one kind of microorganism present in activated sludge that can use benzene as a substrate. If during the preparation of inoculum for the celite carrier, microorganisms that attach themselves and grow on the

carrier surface are different from those prevalent in the alginate bead, the type of biomass active in the two cases is different.

The model does not incorporate any mass transfer effects, which might be playing a role. Whereas in the case of celite carrier it is the transport across the film that is important, in the case of alginate beads it is the pore diffusion through the bead that is important. Also, there is diffusion limitation through the floc or film.

Thus the possiblity of different consortia being operative along with unknown mass transfer effects, will probably account for the difference in the values of k_M and K_S for the two cases.

The kinetic constants thus evaluated (k_M, K_S and K_3) can be used as a starting point to design other immobilized cell reactors.

6.2 BIODEGRADATION STUDIES IN CONTINUOUS FEED MODE

These experiments were conducted using a high speed recirculation pump to assure an operating regime close to a completely mixed reactor. Due to the physical removal of benzene in the reservoir, the influent concentration does not remain constant. In turn, starting from practically no benzene in the reactor, the concentration in the effluent

steadily rises reaching a temporary steady maximum, followed by a steady decrease until it becomes zero. Depending on the flow rate of the influent stream used and the influent concentration at the beginning of an experiment (maintained in the vicinity of 600 ppm for all the runs), the maximum steady state effluent concentration and the total run time varied. For a typical case, where the flow rate of the influent stream was 45 ml/h, Figure 6.7 shows the variation in the effluent concentration with time.

6.2 (i) Retention time and alginate entrapped sludge

Retention time, an important parameter for reactor design, was varied and the resulting changes in the effluent concentration were studied (Table 6.4). The effluent concentrations summarized in Table 6.4 reflect the maximum effluent concentration noted for a given flow rate of the influent stream. Figure 6.8 shows the profiles of benzene in the effluent stream for different flow rates of the influent stream. As can be seen,with a starting concentration of 600 ppm at the inlet, there was essentially no benzene in the effluent for an influent stream flow rate of 35 ml/h which corresponds to a residence time of 17.14 hours.

6.2 (ii) Retention time and sludge immobilized on celite

Parallel experiments performed with the celite carrier suggest that the performance of the two catalysts on volume basis is similar (Table 6.4). Figure 6.9 shows the profiles of benzene in the effluent stream for different flow rates of the influent stream. In this case also, for an influent stream flow rate of 35 ml/h which corresponds to a residence time of 17.14 hours, benzene could not be detected in the effluent stream.

6.2 (iii) Biodegradation of BTX mixture

For experiments conducted in both the cases to study the uptake pattern and the extent of biodegradation achieved when a mixture of benzene, toluene, and o, m and p xylene was used at a flow rate of 60 ml/h, and influent concentrations of 155 ppm, 57.5 ppm, 31 ppm, 35 ppm, and 160 ppm respectively, the maximum steady state concentrations in the effluent were 18 ppm, 1.14 ppm, 0.21 ppm, 0.8 ppm, and 22 ppm for the alginate beads and were 26 ppm, 1.67 ppm, 0.62 ppm, 0.18 ppm, and 25 ppm for the celite carrier (Figures 6.10 and 6.11). For flow rates of 45 ml/h or less, none of them could be detected at the outlet. Here again, all the compounds were taken up simultaneously, as in the batch runs.

6.2 (iv) Kinetics of biodegradation

Values of k_M obtained in the case of alginate beads, after correcting for the volume, ranged between 3.3 and 7.5 ppm/h, and for celite carrier ranged between 3.57 and 7.9 ppm/h. When compared with the corresponding batch values, the agreement is very good in the case of alginate beads, whereas the corresponding values match within an order of magnitude in the case of celite carrier. The values of K_S obtained for the two cases ranged from 3.3 ppm to 40 ppm, and 4.1 ppm to 28 ppm respectively. Although the variation in K_S values is a little high, these values compare within an order of magnitude with the values of K_S obtained from batch experiments for the two cases. This possibly is due to the inability of the polynomial to fit the end data precisely, to which the K_S is very sensitive.

TABLE 6.1: EFFECT OF FLOW RATE ON BIODEGRADATION RATE USING CALCIUM ALGINATE BEADS IN BATCH RECIRCULATION REACTOR

Spiking concentration of benzene: 100 ppm

Biomass loading : 150 g

Reactor volume : 2 l

Reactor diameter : 6.3 cm

Flow rate (ml/min)	Apparent k_M (ppm/h)
25	2.93
50	4.75
100	6.81
300	6.78

TABLE 6.2: EFFECT OF BIOMASS LOADING AND SPIKING CONCENTRATION ON BIODEGRADATION RATE USING CALCIUM ALGINATE BEADS.

Reactor volume: 2 l

Flow rate : 300 ml/min

1 g wet bead = 1.6 mg dry biomass

K_S = 19.5 ppm

Biomass loading (g)	Spiking concentration of benzene (ppm)	Apparent k_M (ppm/h)	Specific Apparent k_M (ppm/h/g dry bead weight)
25	100	3.02	1.21
50	50	9.07	1.81
	100	4.70	0.94
100	50	6.01	0.60
	100	6.84	0.68
	150	5.90	0.59
150	100	6.68	0.45
	200	5.69	0.38

TABLE 6.3: EFFECT OF SPIKING CONCENTRATION ON BIODEGRADATION RATE USING CELITE CARRIER (R-635) IN BATCH RECIRCULATION REACTOR

Celite carrier: 300 g

Reactor volume: 2 l

K_S = 60 ppm

Spiking concentration of benzene (ppm)	Apparent k_M (ppm/h)	Specific Apparent k_M (ppm/h/g dry bead weight)
100	24.85	0.083
150	13.48	0.045
230	19.00	0.063
500	23.80	0.079

TABLE 6.4: EFFECT OF FLOW RATE ON MAXIMUM EFFLUENT CONCENTRATION OF BENZENE IN CONTINUOUS FEED REACTOR

Starting influent concentration : 600 ppm

Biomass loading (alginate) : 150 g beads (15 g dry)

Biomass loading (celite carrier): 300 g carrier (dry)

Reactor volume : 600 ml

Flow rate (ml/h)	Effluent concentration of benzene (ppm)	
	alginate	celite
35	0	0
40	25	26
45	51	53
60	86	105
80	115	125

Fig. 6.1 Comparison between celite carrier and alginate beads in batch recirculation reactor

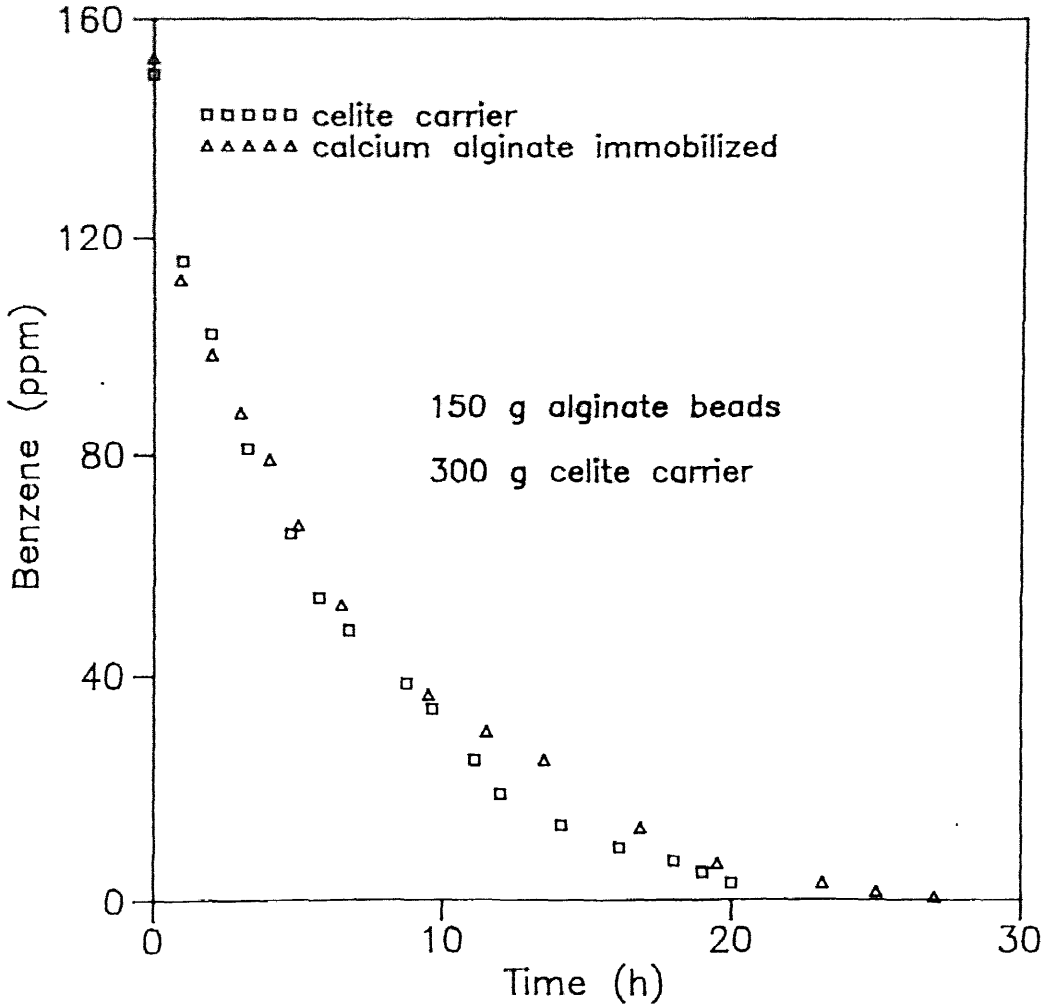


Fig.6.2 Variation in pH and benzene concentration with time (alginate beads) in batch recirculation reactor

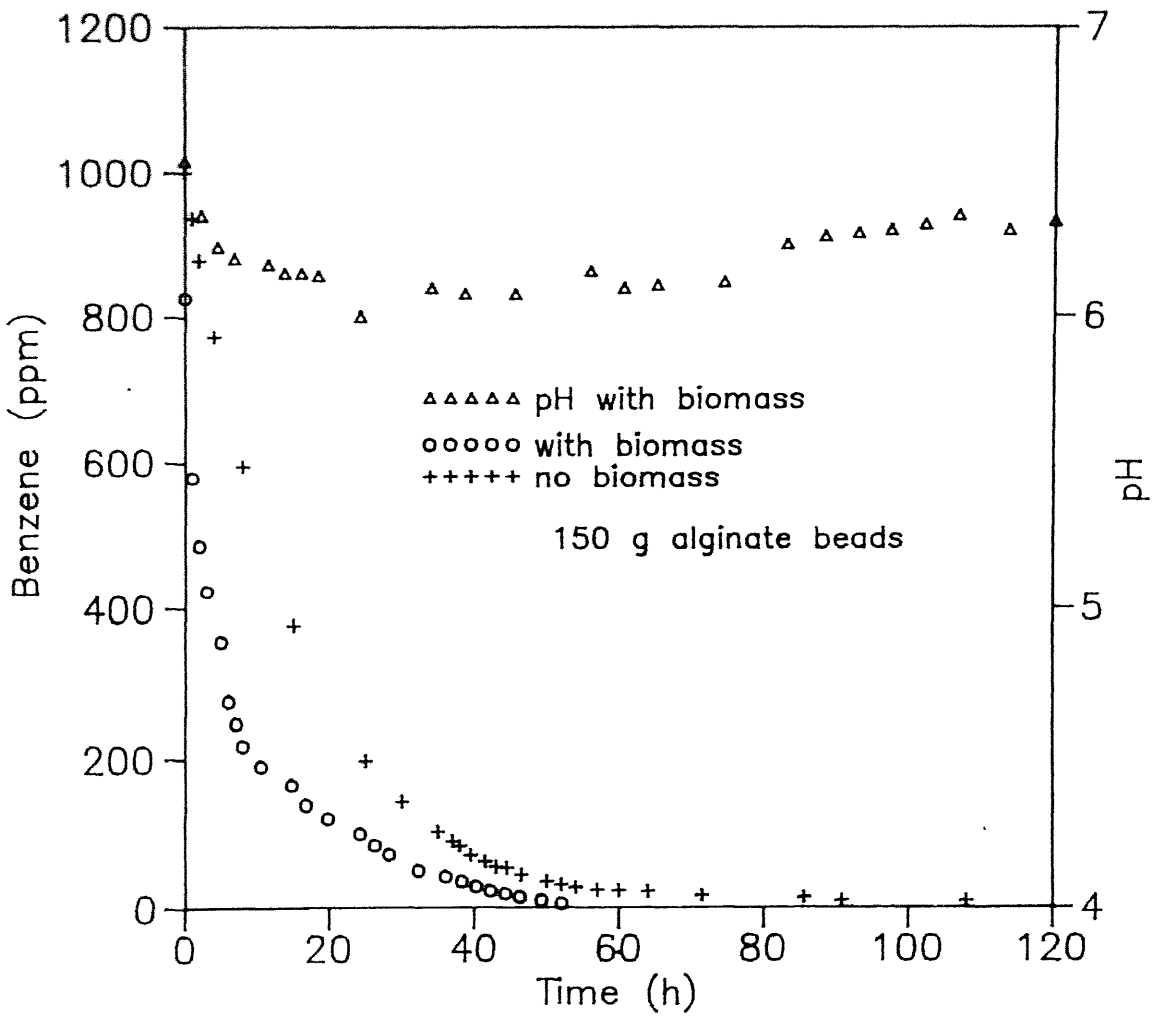


Fig. 6.3 Variation in benzene concentration with time (celite carrier) in batch recirculation reactor

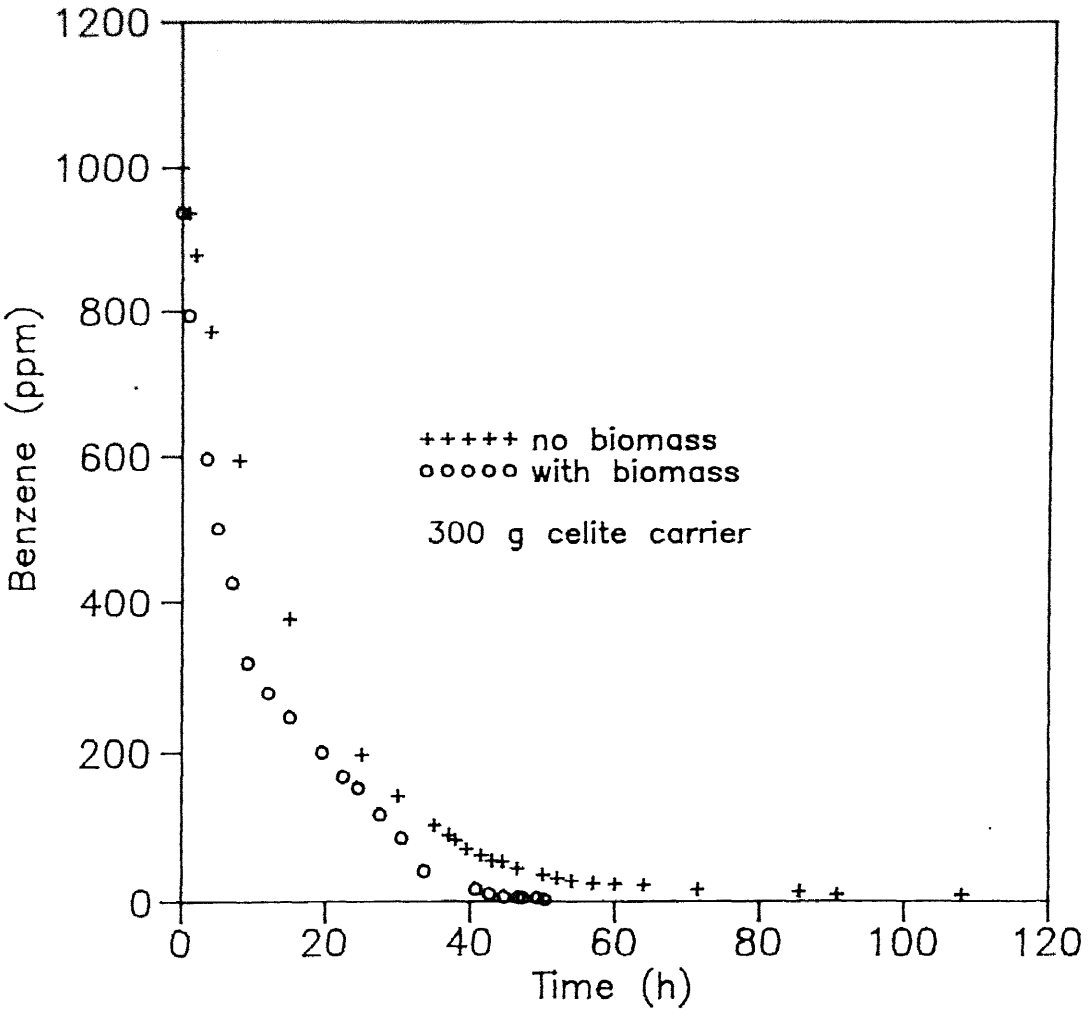


Fig.6.4 Biodegradation of BTX mixture in batch recirculation reactor using alginate beads

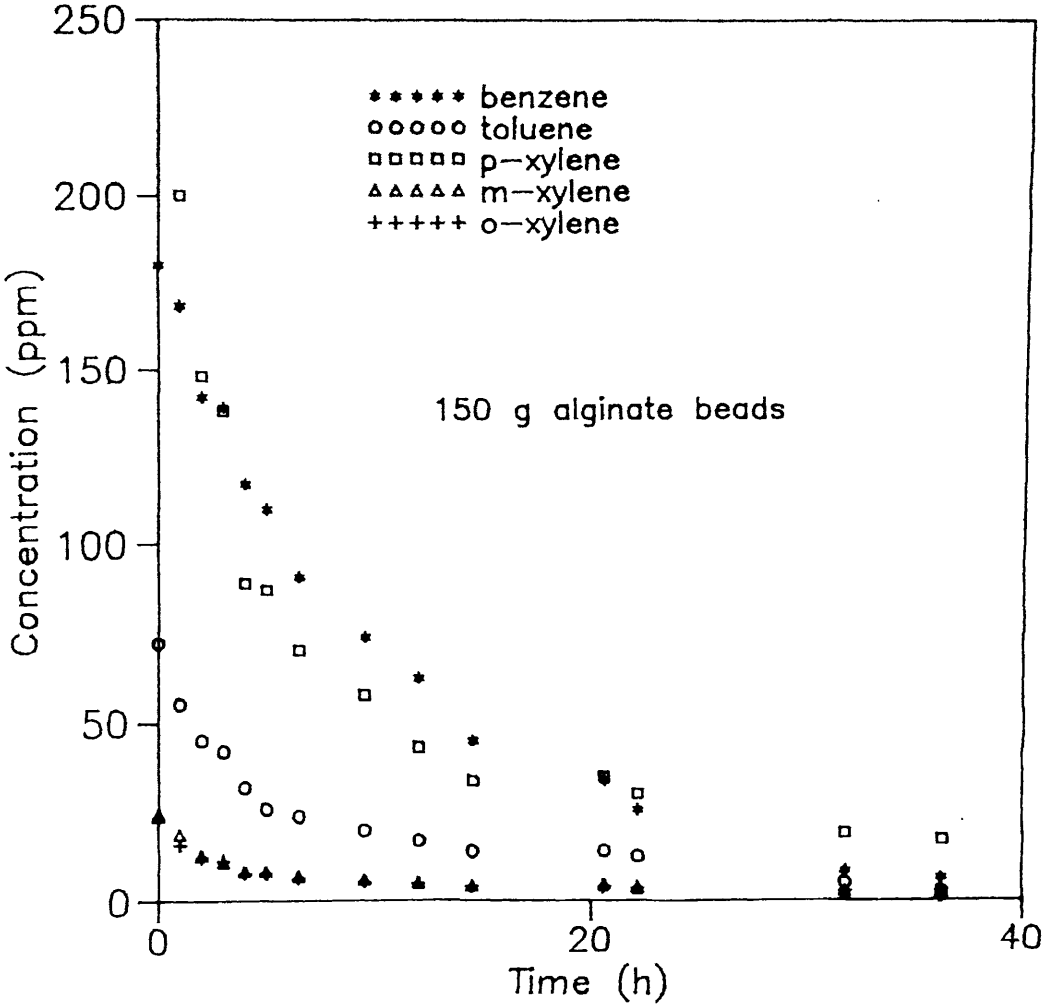


Fig.6.5 Biodegradation of BTX mixture in batch recirculation reactor using celite carrier

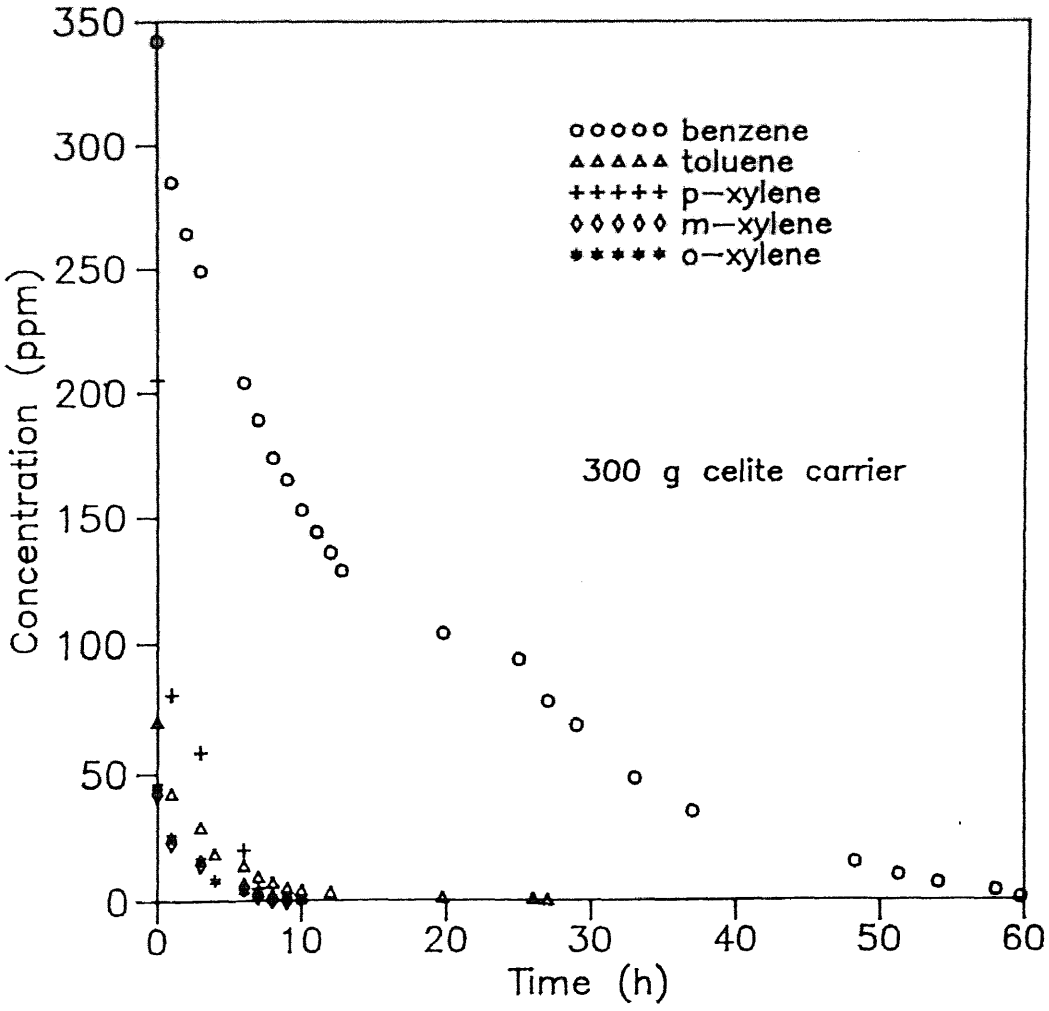


Fig.6.6 Predicted profile and experimental data for alginate beads and celite carrier in batch recirculation reactor

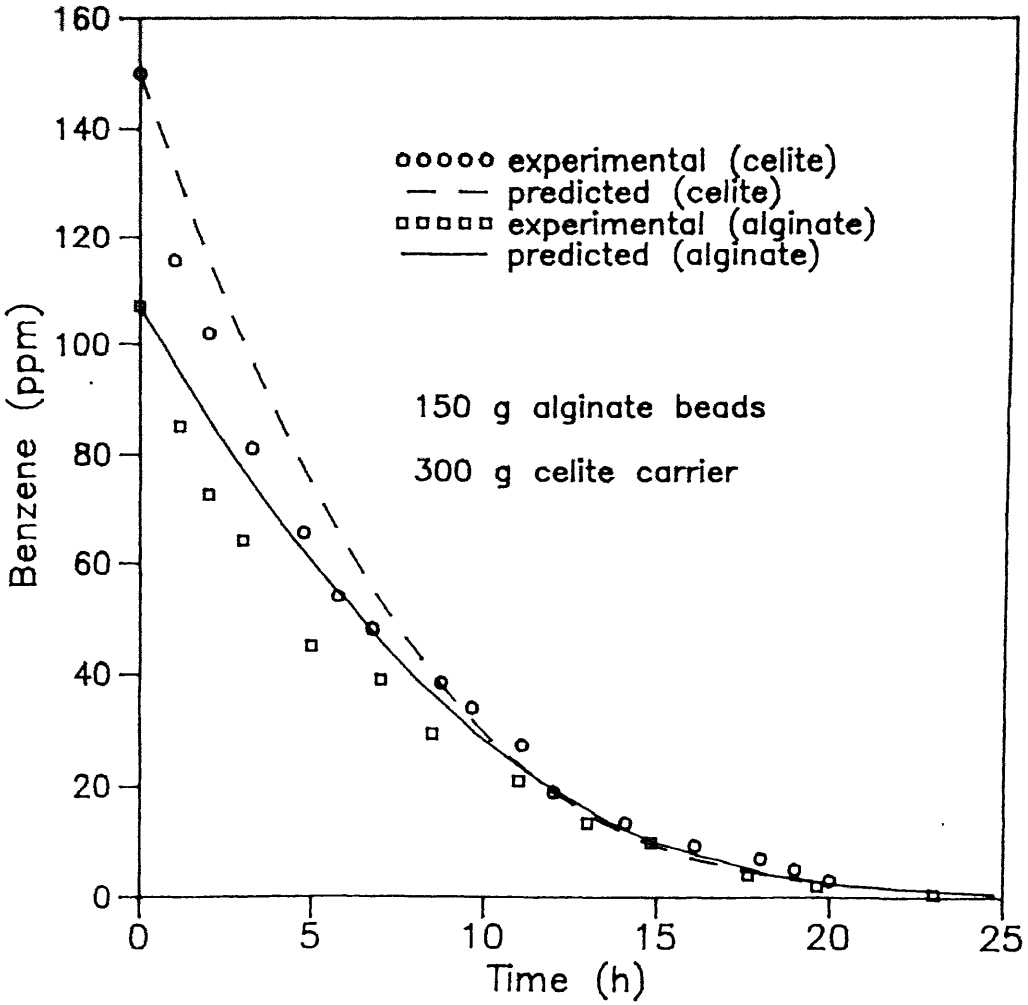


Fig.6.7 Effluent profiles for continuous feed mode reactor

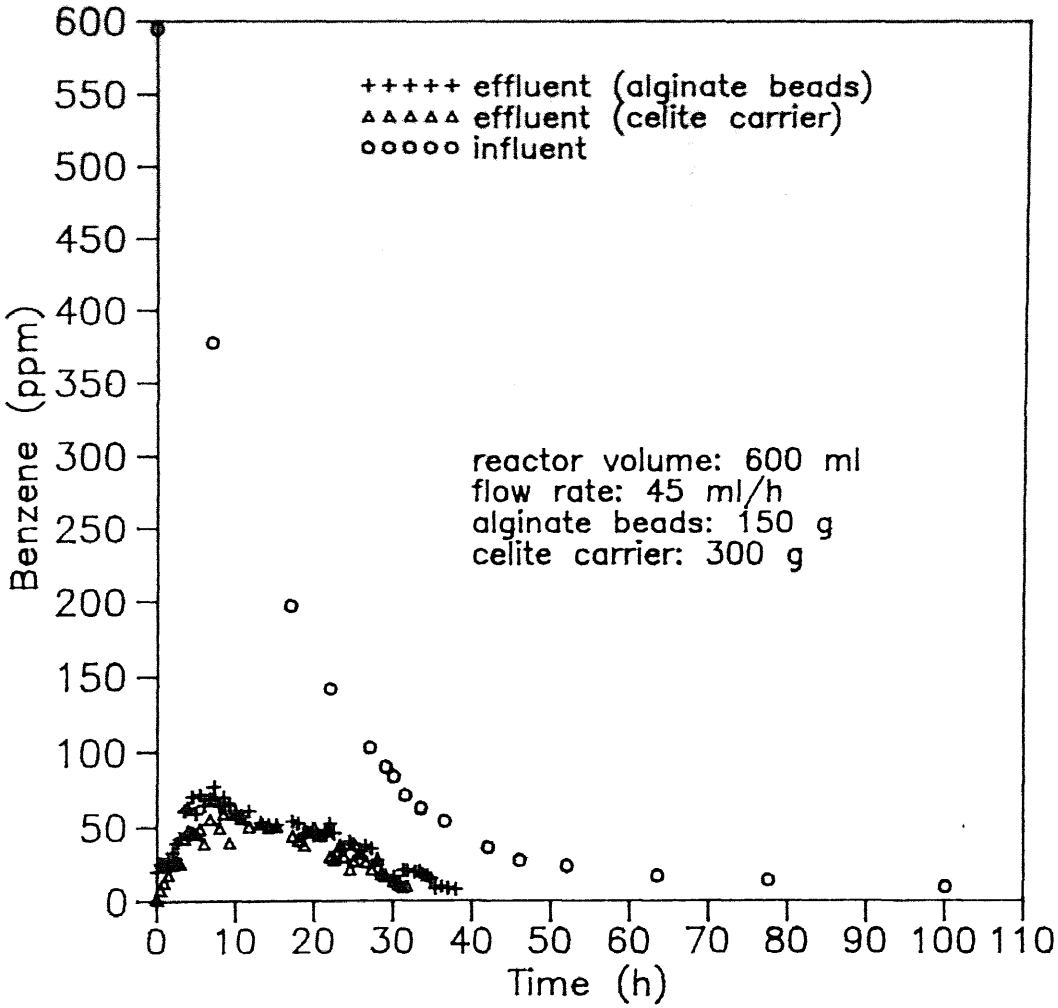


Fig.6.8 Effect of flow rate on effluent concentration (alginate beads)

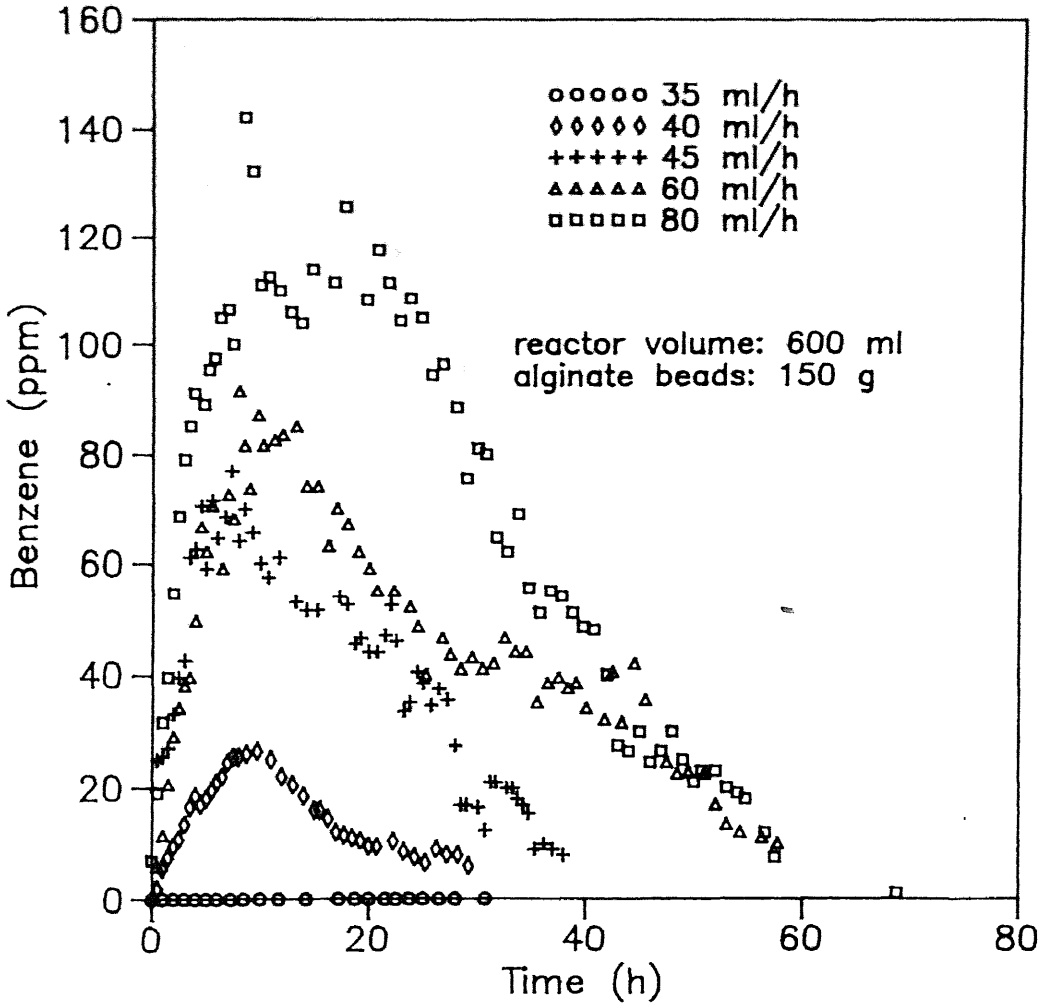


Fig.6.9 Effect of flow rate on effluent concentration (celite carrier)

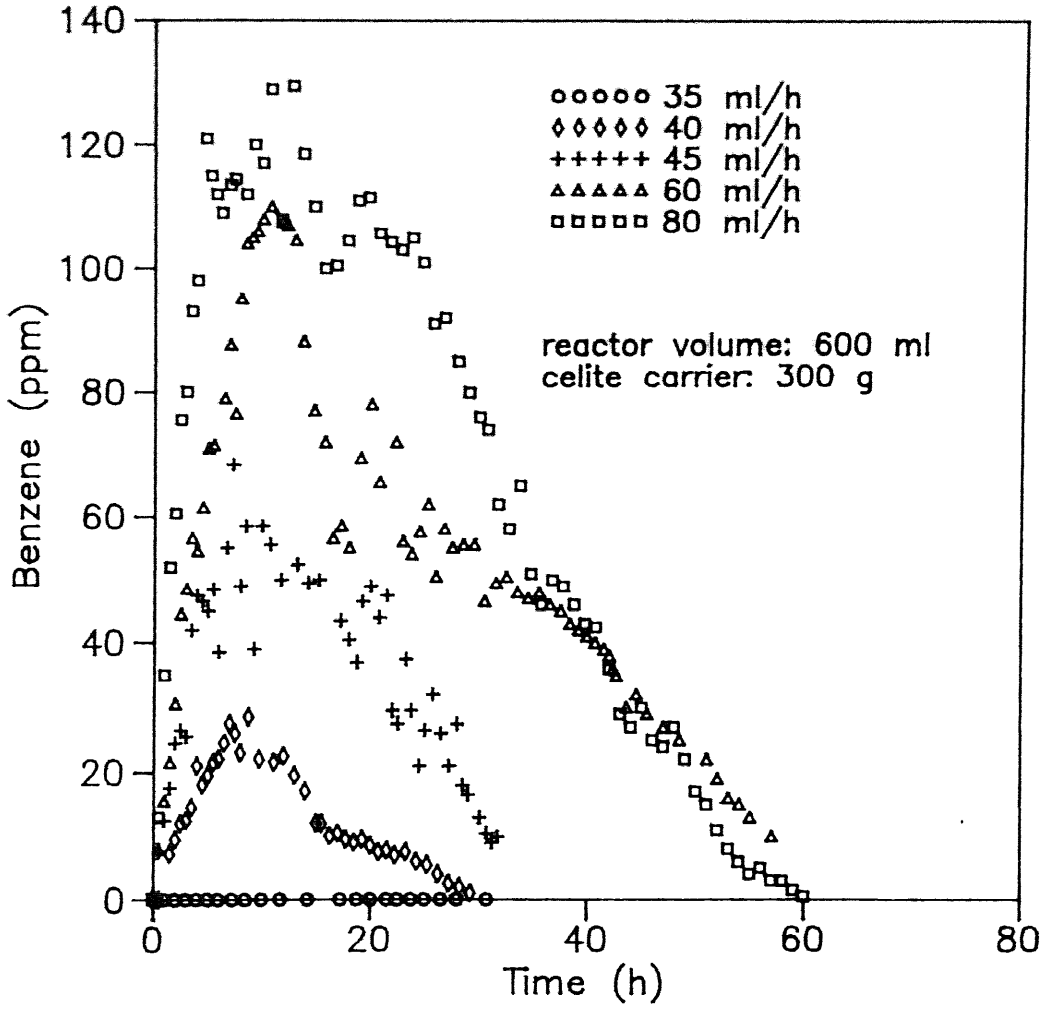


Fig.6.10 Biodegradation of BTX mixture in continuous feed mode reactor using alginate beads

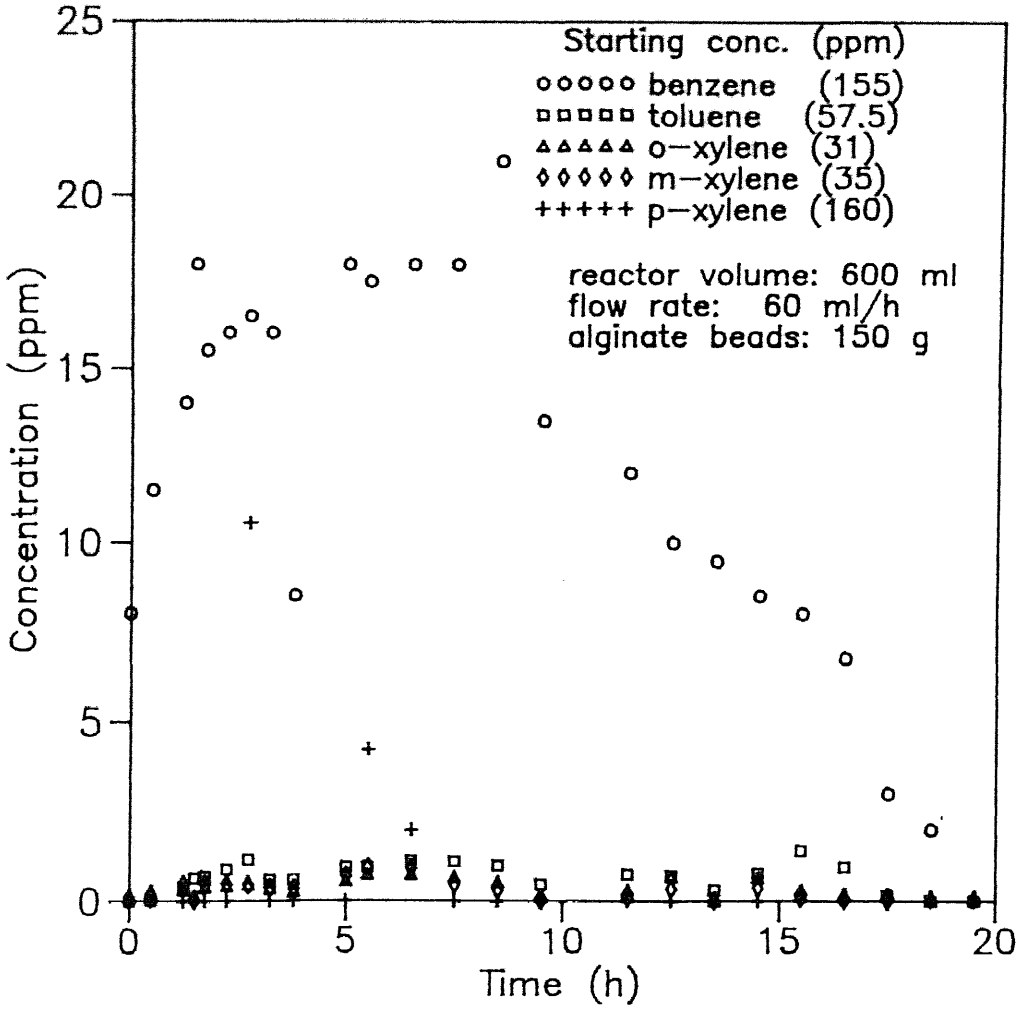
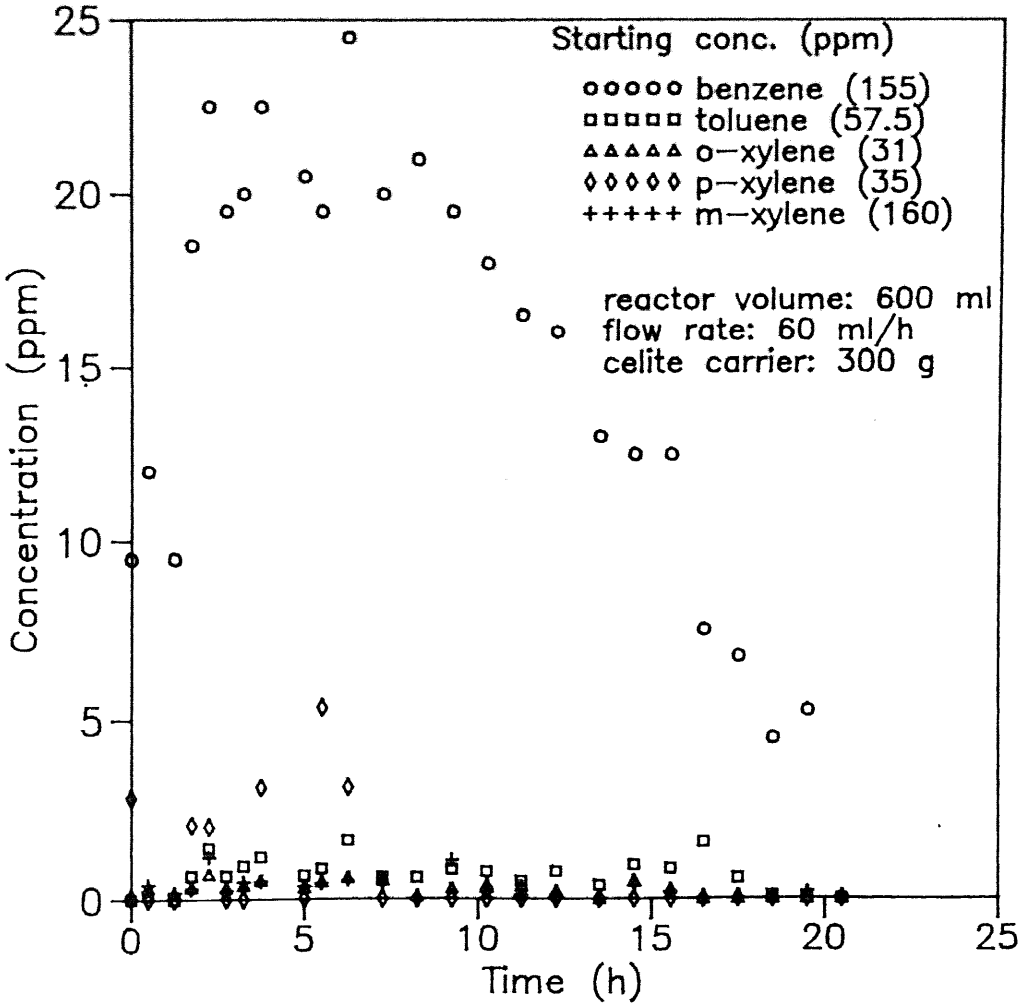


Fig.6.11 Biodegradation of BTX mixture in continuous feed mode reactor using celite carrier



CHAPTER VII

CONCLUSIONS

The results obtained indicate that the technique of immobilization in general, and immobilization of activated sludge in particular, has potential to become a promising technology in hazardous waste treatment. Various advantages of the technique have been exploited, and the weaknesses understood. The fact that there is essentially no cell washout means a much cleaner system to work with. High localized cell density allows high activity for a given volume. On the other hand, diffusional resistances of oxygen have to be overcome by using proper methods of oxygenation and providing for adequate cross sectional velocities. The sensitivity of the alginate matrix towards strong ions like phosphate limits the amount of inorganic salts that can be used in the nutrient medium. In the present work this limitation did not hamper the activity of the catalyst to any appreciable extent.

H_2O_2 was used as a source of dissolved oxygen. It was with H_2O_2 that it was possible to have biological removal as the primary mode of removal, as opposed to stripping by air bubbling.

For a given volume of treatment in the batch mode, and a given flow rate in the continuous feed mode, for the same range of benzene concentration, it is observed that both celite carrier and alginate beads are comparable in their performance on a volume basis. On the other hand when a comparison is done on the basis of biodegradation rates achieved per gram of dry catalyst, the rates obtained in the case of alginate beads are almost an order of magnitude higher than those for the celite carrier.

Models incorporating physical removal as one of the removal mechanisms have been developed for both batch as well as continuous feed mode. Biological removal was assumed to follow Monod kinetics. The assumption of constant biomass is a logical one knowing the fact that each individual bead represents a very high concentration of biomass.

For batch runs, the agreement was quite good between the predicted profile of benzene concentration and the experimental results. The k_M and K_S values are different with alginate and celite carrier. This could be because the consortia operative in the two cases are different or that it is a reflection of mass transfer characteristics that have not been accounted for and are entirely different for the two cases. The advantage gained by the high k_M values with celite carrier is nullified by the coincidental occurrence of high K_S values. For reactor design, this

information is useful in that, given high input concentrations of benzene to be treated, one may combine the two reactors in series with celite carrier in front and alginate taking the role of finishing reactor.

In the case of alginate beads, the agreement in k_M values obtained from batch runs and continuous runs is excellent, although the K_S values match within an order of magnitude. This possibly is due to the inability of the polynomial to fit the end data precisely, to which the K_S is very sensitive.

In the case of celite carrier, both, the k_M and K_S values from batch runs and continuous runs match within an order of magnitude.

Experiments done with mixtures of benzene, toluene, and o, m and p-xylene suggest that there is no specificity in uptake patterns followed, and benzene and p-xylene which were present at high concentrations compared to other components, are the last to be removed.

CHAPTER VIII

SUGGESTIONS FOR FURTHER WORK

The work presented here has established a technique of microbial immobilization for hazardous waste treatment on the laboratory scale. The next logical step would be to scale it up to an intermediate scale, followed by a possible pilot scale demonstration.

The waste stream treated was made up in the laboratory. For the technique to be of any practical importance, a real waste stream containing BTX and other recalcitrant materials should be treated.

A detailed economic analysis of the system after studying the intermediate scale up will answer the possibility of whether or not a pilot test is needed.

If the system can be modified so as to trap the carbon dioxide that is supposed to be generated during biodegradation of benzene, a double check on the extent of biological removal can be made.

The possible influence of mass transfer resistances needs to be evaluated further and these effects should be included in the model to broaden its applicability.

There is need for developing a control scheme whereby the oxygen consumption by the microorganisms can be

monitored and supplied as needed.

Further work is necessary to assess the weaknesses of the technique more precisely. This will also play an important role towards deciding the possible application of the technique in a real life scenario.

Use of surfactants to form emulsions whereby the solubility of benzene can be increased by several orders of magnitude, will enable the biodegradation studies to be conducted at high concentrations of benzene.

CHAPTER IX

PRELIMINARY ECONOMIC ANALYSIS

A preliminary economic analysis was attempted mainly to set up a protocol for detailed economic analysis to be done on an intermediate-sized reactor, and to assess the material and energy requirements of the process.

Calculations were done for treating a 2 liter stream of benzene from an initial concentration of 100 ppm to a final concentration of 1 ppm in a batch reactor using 150 g of wet beads over a period of 24 hours.

I. Material requirements

(i) Catalyst preparation

ITEM	AMOUNT	FIXED COST $	VARIABLE COST $
1. Mixed liquor	2.34 l	--	NONE[a]
2. Weight of pellets (55 mg dry biomass/g pellets) after centrifuging	62.50 g	--	NONE[a,b]
3. Labor			NONE[a,c]
4. Sodium alginate	4.92 g	--	0.284
5. Sodium chloride	0.47 g	--	0.004
6. Calcium chloride	5.51 g	--	0.24
7. Hydrogen peroxide (30 g/l solution)	5.04 g	--	0.484
SUB-TOTAL			1.01

[a] The items are listed because they will need to be considered in an operating unit.

[b] Once average bead life is determined, a price needs to be calculated for this item.

[c] Labor is to be considered as a function of plant size.

(ii) Equipment requirements

1. Plexiglass tube (13 cm ID x 15 cm OD x 19 cm L)	1	50.00	--
2. Three polycarbonate discs (13 cm D x 0.3 cm L)		5.00	--
3. Recirculation pump	1	500.00	--
4. H_2O_2 pump	1	200.00	--
5. Oxygen polarograph	1	900.00	--
6. DO probes	2	300.00	--
7. Mixer(acclimation)	1	300.00	--
8. Centrifuge	1	27,000.00[a]	--
9. Extrusion pump	1		
10. Blender or Mixer	1	50.00	--
11. Stirrer($CaCl_2$)	1	150.00	--
TOTAL		29,455.00	

[a] Other methods of concentrating the mixed liquor may be considered.

(iii) Nutrient requirements

1. Magnesium chloride	0.20 g	--	0.002
2. Manganese sulfate	0.02 g	--	0.001
3. Ferric chloride	0.001 g	--	0.0001
4. Potassium phosphate	0.02 g	--	0.001
5. Ammonium sulfate	0.02 g	--	0.001
6. Hydrogen peroxide (30 g/l solution)	0.72 g	--	0.07
SUB-TOTAL			0.08

II. Energy requirements.

ITEM	RATING kW	TIME h	ENERGY kW.h	TOTAL COST $
1. Centrifuge	0.373	0.5	0.18	0.0072
2. Bead maker pump	0.093	0.5	0.05	0.002
3. Mixer for acclimation tank.	0.047	168	7.9	0.32
4. Recirculation pump	0.093	24	2.21	0.088
5. Pump for supplying hydrogen peroxide	0.047	24	1.10	0.044
SUB-TOTAL			11.44	0.46
TOTAL (excluding equipment)				1.55

CHAPTER X

INTRODUCTION

From the necessity of purifying wastewater evolved the activated sludge process in the beginning of the twentieth century. Over the years, activated sludge has become one of the major biological wastewater treatment processes, others being stabilization ponds, trickling filters, and rotating biological contactors. It is probably the most widely used method, and usually has been effective.

Activated sludge has been used by researchers to estimate the biodegradability of host of organic compounds (Tabak et al., 1981; Kincannon et al., 1983; Petrasek et al., 1983; Kim and Maier, 1986; Lewandowski et al., 1986; Weber et al., 1987; Andrews et al., 1988 and Lakhwala et al., 1989). Starting with pioneering work of Buswell and Long (1923) and Butterfield (1935), other researchers (Allen, 1944; Mckinney and Weichlein, 1953; Prakasam and Dondero, 1967a and 1967b; Adamse, 1968; Strom and Jenkins, 1984 and Lau et al., 1984a and 1984b) have attempted to characterize the complex microbial structure of activated sludge. These studies were aimed in part at understanding two important phenomena observed in activated sludge processes, namely, floc formation and bulking.

There are no studies to our knowledge where activated sludge was characterized to find the microorganisms responsible for biodegradation of an aromatic hydrocarbon.

In the present work, activated sludge immobilized in calcium alginate gel was used to study biodegradation of benzene and BTX mixture. The mixed cultures were characterized in three groups namely, unacclimated, acclimated and end run. These groups were formed primarily to study the changes in the community structure of activated sludge that might occur as the mixed culture passes through the stages of acclimation, followed by biodegradation experiments.

CHAPTER XI

LITERATURE SURVEY

Activated sludge has been recognized as one of the most versatile methods for wastewater treatment. Beginning from the "gestation period" to the development of "aerated lagoons and ditches", Sawyer (1965) has discussed the complete evolution process in terms of 12 milestones.

In a historic paper, Buswell and Long (1923) noted that the purification achieved in activated sludge processes is accomplished by ingestion and assimilation of the organic matter in the sewage and its resynthesis into the living material of the flocs. They established that activated sludge is heterotrophic in nature and is a flocculated mass composed mainly of bacteria and protozoa.

Butterfield (1935), isolated Zooglea ramigera and showed that a pure culture of Zooglea ramigera produces floc that simulated activated sludge and removes oxidizable material present in the polluted water.

Allen (1944) was also one of the early researchers to work on characterization of activated sludge. He found that homogenization of activated sludge facilitates the isolation of the predominant biota by liberating bacteria from the floc. He tested various media to investigate the

bacteriology of activated sludge and found nutrient agar to be superior. The majority of the bacteria that he isolated were gram negative rods with no action on carbohydrates, and were members of the genera Achromobacterium, Chromobacterium and Pseudomonas.

McKinney and Weichlein (1953) reported isolation of seventy two bacteria from sixteen different samples of activated sludge from four sources. Of these, two were municipal sewage treatment plants treating domestic sewage, one was treating industrial wastewater and one was synthetic activated sludge, produced in the laboratory. Of the 72 isolates, 14 were capable of floc formation in sterile synthetic sewage and 12 in sterile settled sewage.

Prakasam and Dondero (1967a and b) studied different agar media for enumeration and characterization of activated sludge. They found plating media containing solely activated sludge extracts to be the best in terms of obtaining higher viable counts as opposed to the nutrient agar of Allen (1944).

Adamse (1968) compared the bacterial flora of a dairy waste activated sludge with that developed in the laboratory under comparable nutritional conditions and found them to be similar. He concluded that in dairy waste activated sludge, Arthrobacter like bacteria were predominant.

Lighthart and Oglesby (1968) suggested that a wide

variety of characters are desirable to define randomly the genome. They suggested that the wide array of data gathered be processed in a binomial system for final classification.

Benedict and Carlson (1971) compared activated sludge samples from a laboratory unit and a domestic sewage treatment plant. They picked 129 colonies from high dilution countable plates. They reported one type of yeast along with the different bacterial colonies.

Another issue of importance related to the activated sludge process was that of bulking. Eikelboom (1975) did pioneering work in identifying filamentous microorganisms. He identified the microorganisms to type using Microscopic examination coupled with two simple staining procedures.

Strom and Jenkins (1984) studied the correlation between the excessive growth of various types of filamentous organisms and the plant operating conditions associated with sludge bulking. A total of 226 samples from 78 plants were examined. Using Eikelboom's key, filamentous types were found as a major population in one or more samples. The same group developed a model for activated sludge bulking (Lau et al., 1984a), and studied the kinetics of Sphaerotilus natans (a filamentous bacterium) and a floc former in pure and dual continuous culture (Lau et al., 1984b). They concluded that, Sphaerotilus natans predominated at low DO with low to moderate dilution rates, and the floc former at high DO or high dilution rates.

CHAPTER XII

MATERIALS AND EXPERIMENTAL METHODS

12.1 ISOLATION AND COUNTING OF VIABLE COLONIES

Alginate beads (25 g) of unacclimated, acclimated and end run (after running the biodegradation experiments) cultures were separately mixed with 25 ml of sterile distilled water and blended for one minute at high speed. Then 0.5 ml of the resulting homogeneous mixture was added to 4.5 ml of 0.1% (w/v) Tween 80 solution to further break the floc. Following this, serial dilution to 10^{-7} was done and 0.1 ml of each dilution was surface spread on agar plates (in triplicate). The selection of different agars used was based on Prakasam and Dondero (1967) which included, floc agar (FA) (Peptone, 2 g; Yeast Extract, 1 g; Agar, 20 g/l), plate count agar (PCA), MacConkey agar (MCA), trypticase soy agar (TSA), nutrient agar (NA) and casitone glycerol yeast extract (CGY) agar (Casitone, 1 g; Glycerol, 2 g; Yeast Extract, 0.2 g; Agar, 15 g/l). Whereas the other agars were obtained from BBL (Cockeysville, MD), the composition of CGY agar was based on Lin (1984), and the composition of floc agar was based on Benson (1985).

It was found that CGY agar offered maximum number of characteristically different colonies. Following this,

further isolation was done using CGY agar plates. Colonies showing the same characteristics on the agar plate were all considered as being of the same type.

Nutrient broth was used as the storage medium for the isolated colonies. Following the transfer of isolated colonies, a day's time was allowed for growth and subsequently the broth was stored at 4°C.

12.2 IDENTIFICATION OF ISOLATES

Colony characteristics (shape, size, margin elevation and color), morphological characteristics (size, shape and motility) and biochemical tests (Hugh, 1975) were used for identification of isolates. Incubations were performed at room temperature (25 to 27°C) and the inoculum was obtained from nutrient broth unless otherwise noted.

1. Colony characteristics.

Size, shape, margin elevation and color of the colony were observed and noted after ten days of incubation on CGY agar plates.

2. Morphological characteristics.

A young culture (18-24 hours) of each isolate grown in nutrient broth was examined in a wet mount to determine the cell size, shape, and motility. Standard gram stain

procedures were adopted to identify gram positive and gram negative bacteria.

3. Carbohydrate reactions.

Oxidative fermentative (OF) basal medium containing 1% (w/v) glucose was utilized. For determining fermentation, a layer of mineral oil was aseptically added to the surface immediately after inoculation. The cultures were inspected for a positive reaction after 3 to 4 days of incubation.

4. Hydrogen sulfide production.

KIA agar was used to detect the production of hydrogen sulfide. The indication is in the form of a black butt at the base. Four days of incubation was allowed.

5. Indole production.

Indole production was detected by adding Kovac's reagent to cultures grown on SIM medium. Immediate formation of pinkish color indicated a positive reaction. Four days of incubation was allowed.

6. Presence of β-D-galactosidase.

β-D-galactosidase was detected by adding 0.3 ml of o-Nitrophenyl-β-D-galactoside (ONPG) reagent, after

incubating the one day old culture in CGY broth, at 37°C for ten minutes. A yellow color is a positive reaction.

7. Esculin hydrolysis.

Esculin hydrolysis was detected by observing for a black precipitate around the growth after 3 days on a TSA agar plate supplemented with 0.1% (w/v) esculin and 0.05% (w/v) ferric citrate.

8. Test for presence of catalase.

Catalase presence was evidenced by the formation of bubbles when a drop of hydrogen peroxide solution (30 g/l) was added to a smear on a slide. The inoculum was obtained from CGY agar plate after 1 week of incubation.

9. Nitrate reduction.

Nutrient broth supplemented with 0.1% (w/v) potassium nitrate was used to detect the reduction of nitrate to nitrite. A pronounced red color upon addition of two drops of 0.8% (w/v) sulfanilic acid and two drops of 0.5% (w/v) N,N dimethyl-alpha-naphthyl amine to the culture broth after seven days of incubation indicates presence of nitrite. Reduction of nitrite to N_2 gas was detected by bubble formation in the tube prior to chemical addition. Nitrate reduction to nitrogen gas can also be detected by adding a

speck of zinc powder to the broth. Formation of red color only after addition of zinc indicates the presence of nitrate, whereas no color development will indicate that the reduction is complete.

10. Oxidase test.

The oxidase test was performed to determine the presence of cytochrome c. If present it oxidizes the oxidase reagent (N,N,N'tetramethyl-p-phenylenediamine). Formation of blue color is a positive reaction. The inoculum was obtained from CGY agar plate after 1 week of incubation.

11. Urease production.

Urea agar was used to detect the production of urease. A change in color of the indicator phenol red, from yellow to red because of the production of ammonia in 3 days, was considered as a positive test.

12. Growth on MacConkey agar.

Growth on MCA agar was also tested. For this purpose, MCA agar plates were made. Five days of incubation was allowed.

13. Test for spore formation.

For identifying the spore formers, soil extract agar was used (Gordon et al., 1973). Slants of the agar were streaked and the smears of culture were air dried and

stained with safranin for 30 seconds. Unstained spores within and outside the sporangia were easily recognized. Isolates were checked for spore formation at the end of the 1st, 2nd, 4th, 6th and 8th day. Whereas most of the spore formers showed positive results by the 2nd day, a few of them showed positive results as late as the 6th day.

14. Flagellar morphology.

The fuchsin-tannic acid method, described by Leifson (1960) was used. Instead of transferring the growth on solid medium to distilled water for preparing the slide, young cultures (18-24 hours) grown in nutrient broth were used.

12.3 SHAKER FLASK EXPERIMENTS

The end run isolates were tested individually and collectively for biodegradation of benzene.

Sterile Erlenmeyer flasks each containing 150 ml nutrient medium 1 (Materials and Experimental Methods-Section I), were inoculated using 0.1 ml of nutrient broth stock cultures. Benzene was added as the only carbon source at a concentration of around 150 ppm. A control flask containing 150 ml of nutrient medium and benzene at the same levels, was also mounted on the shaker (Lab-Line, Model 3528, reciprocating type) along with the others.

Periodic samples were taken and analyzed on GC for the removal of benzene.

CHAPTER XIII

RESULTS AND DISCUSSION

13.1 COMPARISON OF THE MEDIA

Different agar media were used with a view to choose the agar that offered the maximum number of characteristically different colonies. As can be seen from Table 13.1, the maximum number of different colony types were seen on CGY agar. Thus, CGY agar was chosen as the isolation medium.

13.2 PLATE COUNT OF VIABLE COLONIES

Plate counts were carried out for each of the three groups. When expressed on a unit gram of wet bead basis, the counts were, 2.1×10^8, 9.8×10^6 and 12×10^6 cells/ml for unacclimated, acclimated and end run groups, respectively.

The reduction in viable count by more than an order of magnitude as a result of acclimation could be due to competition between different microorganisms, or it may be that benzene is toxic to some microorganisms, or that some of the microorganisms are unable to use benzene as a carbon source.

The plate counts were primarily done to observe the

relative change in the viable count among unacclimated, acclimated and end run cultures and to serve as a source of cultures for isolation. The actual numbers may be different since the blending time was not optimized.

13.3 IDENTIFICATION OF THE ISOLATES

In order to facilitate analysis, the tests upon which assignments to genus usually would be made using Skerman's key (Bergey's Manual 8th edition, 1974) were expressed as a 4 digit number (Table 13.2). This method of expression has been used by Lin (1984), and is based on the concept used by API (Analytab Products Co., Plainview, NY) for identification of Enterobacteriaceae. The individual tests used for this purpose were different. The results of other biochemical tests performed are recorded (Tables 13.3, 13.4 and 13.5). These results were used for additional confirmation of the genera and possibly for species identification. An exception to the use of Skerman's key was identification of Bacillus. Any isolate showing endospore formation was identified as Bacillus, irrespective of whether it was gram positive or gram negative (Gordon et al., 1973).

13.3 (i) Identification of bacteria in the unacclimated group

Seven of the 13 different isolates obtained under this heading were assigned to five different genera namely, Bacillus (3), Lactobacillus (1), Microbacterium (1), Plesiomonas (1) and Pseudomonas (1). The remaining six isolates could not be assigned a genus name.

An unusual feature noted here when compared to earlier studies done with activated sludge identification was that over half (7/13) of the isolates were gram positive. A majority of isolates were rods and produced acid from glucose. Motility was also observed to be prevalent. Spore formation was observed in 3 isolates. Table 13.3 shows the results of other biochemical tests, the coding factor, and the genus assignment if any obtained for each isolate.

13.3 (ii) Identification of bacteria in the acclimated group

In this group 11 different isolates were obtained. Of these, 9 could be assigned to a genus. The genera identified were Bacillus (4), Microbacterium (1), Plesiomonas (1), Kurthia (1), Klebsiella (1) and Pseudomonas (1).

Here again, more than 50% of the isolates were gram positive, all of them being rods. Spore formation was observed in 4, and a high percentage were found to be motile. Table 13.4 lists the results.

13.3 (iii) Identification of bacteria in the end run group

Of the 15 different isolates obtined, 10 were assigned a genus name. These were, Bacillus (3), Microbacterium (1), Corynebacterium (1), Plesiomonas (1), and Pseudomonas (4).

Here also, all the isolates were rods, with 9 gram positive. Spore formation was observed in 3, and 10 were motile. Table 13.5 lists the results.

13.3 (iv) Difficulties in identification of cultures

Identification of bacteria in activated sludge flocs is difficult and extremely complex. This has been noted by earlier workers also. Lack of a systematic approach and use of contradictory names for the same bacteria makes the task of identification doubly difficult. This is reflected in the number of unidentified isolates (13) from a total of 39 isolates obtained for the three different groups.

Because of its poor success, use of Skerman's (1974) key (Bergey's Manual, 1974) to the genera may not be an ideal method to identify isolates from samples such as activated sludge. Furthermore, if the bacteria in activated sludge were not found before in other environments, they would not be included in Bergey's Manual.

13.4 SHAKER FLASK EXPERIMENTS

Isolates from the end run group were tested individually and collectively for benzene consumption in shaker flasks.

As seen from Table 13.6, ER_1 which was identified as a Pseudomonas, shows distinct removal of benzene. This establishes a Pseudomonas as the primary degrader of benzene among the isolates present.

TABLE 13.1 COMPARISON OF THE ISOLATION MEDIA

Inoculum: Unacclimated group

Dilutions plated: 4^{th}, 5^{th} and 6^{th}

TYPE OF AGAR	NUMBER OF DIFFERENT COLONY TYPES
TSA	1
FA	2
PCA	3
MCA	3
NA	7
CGY	13

TABLE 13.2: CODING OF TEST RESULTS

Test	Coding Factor	Example Response	Octonary Code
Gram Stain	1	+	
OF-O	2	–	1
OF-F	4	–	
Motility	1	+	
Polar Flagella	2	+	3
Peritrichous Flagella	4	–	
Rod Shape	1	+	
Size (≥1μm)	2	–	1
Size (≤0.5μm)	4	–	
Spore Formation	1	+	
Oxidase	2	–	5
Catalase	4	+	
Coded Result 1315			

Each positive response is considered a one, and each negative zero. Each binary coded response is multiplied by its coding factor, and the results for each triplet are summed. This procedure is repeated for all isolates.

TABLE 13.3: TEST RESULTS FOR THE UNACCLIMATED GROUP OF ISOLATES

Unacclimated (UNA)		1	2	3	4	5	6	7	8	9	10	11	12	13
Coded Result from the 12 primary tests		1331	6315	5330	5024	6350	4354	5310	5314	6310	7016	1512	0316	0015
Identified Genus		G1	G1	G5	G6	G7	–	–	–	–	–	–	G8	G1
Colony Color		–	–	–	Y	–	–	–	–	–	R/V	–	–	Y
Urease		–	+	+	–	–	–	+	–	+	+	+	–	+
Esculin		+	+	–	+	–	–	–	–	–	+	–	+	–
Nitrate Reduction		+	+	–	+	–	+	–	–	–	+	+	+	–
Growth on MacConkey		–	+	–	–	+	–	+	+	+	–	+	+	–
Indole Production		+	+	+	–	+	+	–	+	+	–	+	–	–
H_2S Production		+	+	+	+	–	–	–	–	+	–	+	+	–
β–D–Galactosidase		–	+	+	+	–	–	–	–	–	–	–	–	–
Colony Characteristics	Configuration	CC	LF	CM	CM	FM	IS	RS	CM	LF	CC	W	IS	RA
	Margin	WA	WA	WA	S	BR	LO	S	LO	S	S	WA	IR	WA
	Elevation	HI	CN	HI	CN	CN	UM	CN	HI	UM	CN	HI	F	UM

BR = Branching
CI = Ciliate
CC = Concentric
CM = Complex
CN = Convex
CR = Crateriform
DL = Drop-Like
F = Flat
FF = Filiform
FM = Filamentous
+ = Positive Response
HI = Hilly
HL = Hair-Lock Like
IR = Irregular
IS = Irregular and Spreading
– = Negative Response
LF = L-Form
LO = Lobate
R = Round
RH = Rhizoid
RI = Raised
RR = Round with Raised Margin
RA = Round with Radiating Margin
RS = Round with Scalloped Margin
S = Smooth
TL = Thread-Like
W = Wrinkled
WA = Wavy
WO = Wooly
UM = Umbonate
Y = Yellow
R/V = Red/Violet
G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 13.4: TEST RESULTS FOR THE ACCLIMATED GROUP OF ISOLATES

Acclimated (AA)		1	2	3	4	5	6	7	8	9	10	11
Coded Result from the 12 primary tests		1315	6314	0314	5054	0315	1017	6316	1514	1515	6014	1554
Identified Genus		G1	G7	G8	G6	G1	G1	–	G4	G1	G3	–
Colony Color		Y	R/V	–	Y	Y	–	–	Y	R/V	–	–
Urease		+	+	–	+	+	–	+	–	–	–	–
Esculin		+	–	–	+	+	–	–	+	–	+	–
Nitrate Reduction		+	–	–	–	+	–	+	–	+	+	+
Growth on MacConkey		–	+	+	–	–	+	+	+	+	+	–
Indole Production		–	–	–	–	–	–	–	–	–	–	–
H_2S Production		–	–	–	–	–	–	+	–	–	–	–
β–D–Galactosidase		–	–	–	–	–	–	–	–	–	+	–
Colony Characteristics	Configuration	RA	R	R	R	R	FF	RS	CC	RA	LF	R
	Margin	BR	WA	S	S	S	HL	WA	S	BR	WA	S
	Elevation	UM	CN	UM	CN	CN	HI	F	UM	CN	F	DL

BR = Branching CI = Ciliate CC = Concentric CM = Complex CN = Convex

CR = Crateriform DL = Drop-Like F = Flat FF = Filiform FM = Filamentous

+ = Positive Response HI = Hilly HL = Hair-Lock Like IR = Irregular IS = Irregular and Spreading

– = Negative Response LF = L-Form LO = Lobate R = Round RH = Rhizoid

RI = Raised RR = Round with Raised Margin RA = Round with Radiating Margin RS = Round with Scalloped Margin S = Smooth

TL = Thread-Like W = Wrinkled WA = Wavy WO = Wooly UM = Umbonate

Y = Yellow R/V = Red/Violet G1 = Bacillus G2 = Corynebacterium G3 = Klebsiella

G4 = Kurthia G5 = Lactobacillus G6 = Microbacterium G7 = Plesiomonas G8 = Pseudomonas

TABLE 13.5: TEST RESULTS FOR THE END-RUN GROUP OF ISOLATES

End Run (ER)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Coded Result from the 12 primary tests		6316	1315	1314	1117	1054	7316	6314	5316	0356	0354	1014	6517	1017	0316	1014
Identified Genus		G8	–	G2	G1	–	–	G7	G6	G8	G8	–	G1	G1	G8	–
Colony Color		Y	–	Y	–	–	–	–	–	Y	–	–	–	–	Y	–
Urease		–	–	–	–	–	+	+	+	–	–	+	+	+	+	+
Esculin		+	–	+	–	+	–	+	–	+	+	+	+	+	+	+
Nitrate Reduction		+	+	+	+	+	+	+	+	–	–	+	+	+	+	+
Growth on MacConkey		+	–	–	+	–	+	+	+	–	–	–	+	+	–	–
Indole Production		–	–	–	–	–	–	–	–	+	–	–	+	–	–	–
H_2S Production		–	–	–	–	–	+	–	–	+	–	–	–	–	–	–
β–D–Galactosidase		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Colony Characteristics	Configuration	R	R	R	RR	RA	RA	RR	R	R	R	CN	R	CN	R	RA
	Margin	RS	S	S	S	BR	S	S	S	S	S	WA	S	WA	S	IR
	Elevation	UM	CN	CN	CR	RI	DL	CR	CN	DL	UM	CN	RI	F	CN	RI

BR = Branching
CI = Ciliate
CC = Concentric
CM = Complex
CN = Convex
CR = Crateriform
DL = Drop-Like
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R/V = Red/Violet
G1 = Bacillus
G2 = Corynebacterium
G3 = Klebsiella
G4 = Kurthia
G5 = Lactobacillus
G6 = Microbacterium
G7 = Plesiomonas
G8 = Pseudomonas

TABLE 13.6: SHAKER FLASK EXPERIMENTS FOR REMOVAL OF BENZENE BY INDIVIDUAL END RUN ISOLATES

Temperature : 25°C
Shaker speed : 60 rpm
Reaction volume: 150 ml
Sampling period: 6 h

SPIKE #	1	2	3	4
SAMPLE	(mg of benzene removed)			
CONTROL	17.78	15.31	16.51	15.35
ER_1	20.18	20.41	24.31	28.57
ER_2	17.02	14.70	15.12	15.60
ER_3	16.05	15.52	15.15	16.05
ER_4	17.27	13.57	16.34	16.35
ER_5	16.05	15.90	15.45	15.45
ER_6	18.00	14.12	16.02	15.75
ER_7	15.83	15.45	15.95	14.70
ER_8	14.59	16.20	16.10	16.50
ER_9	17.37	16.35	15.00	13.45
ER_{10}	16.75	13.85	15.00	16.65
ER_{11}	14.76	14.32	15.15	13.35
ER_{12}	18.13	13.56	14.50	14.48
ER_{13}	14.96	14.85	16.25	15.30
ER_{14}	16.88	15.90	14.85	14.40
ER_{15}	15.97	14.25	14.35	14.40
MIXTURE	18.59	19.56	23.34	25.89

CHAPTER XIV

CONCLUSIONS

The bacterial community structure in activated sludge was studied with respect to biodegradation of benzene. Based on the results obtained, the following conclusions were derived.

CGY agar was found to be the best isolation medium.

Both gram positive and gram negative rods were found. A high percentage of the isolates were motile. Both, glucose fermentors and non-fermenting types were common. About 67% of the isolates could be assigned to a genus. These were Bacillus, Microbacterium, Plesiomonas, Kurthia, Klebsiella, Lactobacillus, and Pseudomonas. The inability to identify all of the isolates is common in the literature.

When the results are compared on the basis of a 4 digit code representing 12 factors of primary importance, similarity is observed among the 3 groups, and to an extent also within a group, viz; the end run group, where 4 isolates belonged to the genus Pseudomonas and the acclimated group where 4 isolates belonged to the genus Bacillus. On the other hand if the results of all the tests are considered, there is little similarity. This is expected, as the colonies were picked up selectively to

begin with.

From among the fifteen isolates found in the end run group, ER_1, identified as a Pseudomonas, was established as a primary degrader of benzene.

CHAPTER XV

SUGGESTIONS FOR FURTHER WORK

The importance of understanding the activated sludge community structure and its effect on biodegradation of organics has been demonstrated--in particular for benzene biodegradation. Some suggestions that fall out from the study are discussed below.

Activated sludge was used in the present work. It will be worthwhile to compare the removal rates obtained when the primary degrader is used instead of the mixed culture.

In view of the different values of k_M and K_S obtained for the two immobilization techniques, it will be interesting to characterize the consortia operative on the celite carrier and compare the two.

APPENDIX I.

A FORTRAN PROGRAM FOR CALCULATION OF KINETIC CONSTANTS IN BATCH FEED MODE EXPERIMENTS

```
      DIMENSION S(20),T(20)


 OPEN (10,FILE='FAYAZ',STATUS='unknown')
 OPEN (100, FILE='S3.OUT', STATUS='unknown')
C
      WRITE(*,*)'INPUT AK1'
      READ(*,*)AK1
      WRITE(*,*)'INPUT AK2'
      READ(*,*)AK2
      WRITE(*,*)'INPUT AK3'
      READ(*,*)AK3
      WRITE(*,*)'INPUT S0'
      READ(*,*)S0
      WRITE(*,*)'INPUT NUMBER OF SAMPLES'
      READ(*,*)N
      DO 1 I=1,N
```

```
      READ(10,*) T(I),S(I)
1 CONTINUE
5     SUM1=0
      SUM2=0
      SUM3=0
      SUM4=0
      SUM5=0
      SUM6=0
      SUM7=0
      DO 25 I=1,N
 CALL DEL1(DELAK1,AK1,AK2,AK3,S0,S(I))
      SUM11=T(I)*DELAK1
 CALL TI(TISTAR,AK1,AK2,AK3,S0,S(I))
      SUM22=TISTAR*DELAK1
      SUM33=DELAK1**2
 CALL DEL2(DELAK2,AK1,AK2,AK3,S0,S(I))
      SUM44=DELAK2*DELAK1
      SUM55=T(I)*DELAK2
      SUM66=TISTAR*DELAK2
      SUM77=DELAK2**2
      SUM1=SUM1+SUM11
      SUM2=SUM2+SUM22
      SUM3=SUM3+SUM33
      SUM4=SUM4+SUM44
```

```
      SUM5=SUM5+SUM55
      SUM6=SUM6+SUM66
      SUM7=SUM7+SUM77
25    CONTINUE
      deltk2=(SUM5-SUM6-(SUM4*(SUM1-SUM2)/SUM3))
      deltk2=deltk2/(SUM7-((SUM4*SUM4)/SUM3))
      deltk1=(SUM1-SUM2-deltk2*SUM4)/SUM3
      IF  (ABS(deltk1).GE..001.AND.ABS(deltk2).GE..001)THEN
      AK1=AK1+deltk1
      AK2=AK2+deltk2
      GOTO 5
      ELSE
      WRITE(100,80)AK1,AK2
80    FORMAT(1X,'FINAL VALUE OF AK1=',F9.3,2X,
     &       'FINAL VALUE OF AK2=',F9.3)
      ENDIF
      STOP
      END
C
      SUBROUTINE TI(TISTAR,AK1,AK2,AK3,S0,SI)

      TISTAR=ALOG((AK1+(AK2*AK3)+(AK3*S0))/(AK1+(AK2*AK3)
     1   +(AK3*SI)))
      TISTAR=TISTAR*(AK1/(AK3*(AK1+(AK2*AK3))))
```

```
      TISTAR=TISTAR+((AK2/(AK1+(AK2*AK3)))*ALOG(S0/SI))
      RETURN
      END
C
      SUBROUTINE DEL1(DELAK1,AK1,AK2,AK3,S0,SN)
      DELAK1=ALOG((AK1+(AK2*AK3)+(AK3*S0))/(AK1+(AK2*AK3)
     1  +(AK3*SN)))
      DELAK1=DELAK1*(AK2/((AK1+(AK2*AK3))**2))
      CALL DELL(DELL1,AK1,AK2,AK3,S0,SN)
      DELAK1=DELAK1+DELL1
      DELAK1=DELAK1-((AK2*ALOG(S0/SN))/((AK1+(AK2*AK3))**2))
      RETURN
      END
C
      SUBROUTINE DELL(DELL1,AK1,AK2,AK3,S0,SN)
      DELL1=((AK1+(AK2*AK3))*(AK1+(AK2*AK3)+(AK3*S0)))
      DELL1=DELL1*(AK1+(AK2*AK3)+(AK3*SN))
      DELL1=(AK1*(SN-S0)/DELL1)
      RETURN
      END
C
      SUBROUTINE DELLL(DELL2,AK1,AK2,AK3,S0,SN)
      CALL DELL(DELL1,AK1,AK2,AK3,S0,SN)
      DELL2=DELL1*AK3
```

```
      RETURN
      END
C
      SUBROUTINE DEL2(DELAK2,AK1,AK2,AK3,S0,SN)
      DELAK2=ALOG((AK1+(AK2*AK3)+(AK3*S0))/(AK1+(AK2*AK3)
     1  +(AK3*SN)))*AK1/(ak1 + ak2*ak3)**2
      DELAK2=((AK1*ALOG(S0/SN))/((AK1+(AK2*AK3))**2))-DELAK2
      CALL DELLL(DELL2,AK1,AK2,AK3,S0,SN)
      DELAK2=DELAK2+DELL2
      RETURN
      END
```

APPENDIX II.

A FORTRAN PROGRAM FOR CALCULATION OF KINETIC CONSTANTS IN CONTINUOUS FEED MODE EXPERIMENTS

```
**************************************************************
c     *
*
c     *       program name: main
*
c     *
*
c     *       purpose: This program plays the part of a driver for
c             the subroutine MARQ and its associated routines
c     *       to apply the method of Marquardt to minimize
c     *       an unconstrained function of N variables.
c     *       The subroutine is part of a large optimization
c     *       library developed by K.M. Ragsdell of the
c     *       University of Missouri - Columbia.
c     *
*
c     *
*
c     *       MARQ arguments:
*
c     *
*
c     *           INPUT: XO     - STARTING POINT OF VARIABLES
*
c     *                  N      - NUMBER OF VARIABLES
*
c     *                  LAMDA  - STEP LENGTH PARAMETER
*
c     *                  CRIT   - CONVERGENCE CRITERIA
*
c     *                  MAXI   - MAXIMUM ITERATIONS ALLOWED
*
c     *           OUTPUT: LAMDA - STEP LENGTH PARAMETER
*
c     *                   WX,G,H,P,A,HI - WORKING ARRAYS

*     *                   Biotechnology Laboratory
*                    New Jersey Institute of Technology
**************************************************************
```

```
      program main
      implicit real*8 (a-h, o-z)
      character*14 infile, outfile
      logical dotcheck
      dimension xo(2),wx(2),g(2),p(2),h(2,2),a(2,2),hi(2,2)
      real*8 cnst(0:9), v, k3, f0, ca00, time, step
      common /cnst/ cnst, v, k3, f0, ca00, time, step

     PARAMETER(lin=5, lout=6, dot='.', out='OUT', in='IN', blank='

c     prompt for input file name

      WRITE(*,10)
 10   FORMAT(' Please supply an input filename: '\)
      READ(*,'(A14)') infile

      OPEN (unit=LIN, file=INFILE, status='OLD')
      read(lin,*) (cnst(i),i=0,9), v, k3, f0, ca00, time, step

c     define the system constants
      n = 2
      maxi = 100
c     x0: N*1 dimensional array for initial values of km and ks
```

```
      write(*,'(a\)')' Km = '
      read(*,*) xo(1)
      write(*,'(a\)')' Ks = '
      read(*,*) xo(2)
c     tolerance limits
      crit = 1.0d-5
      dif = 1.d-5
      rlamda = 1.d6
      call MARQ(xo,wx,g,h,p,a,hi,n,rlamda,crit,dif,maxi)
      stop
      end


c     optimization objective function
      real*8 function f(x)
      implicit real*8 (a-h, o-z)
      real*8 x(1), t, left, right
      real*8 cnst(0:9), v, k3, f0, ca00, time, step

      common /cnst/ cnst, v, k3, f0, ca00, time, step
      polyc(t) = (((((((cnst(9)*t + cnst(8))*t + cnst(7))*t
     &            + cnst(6))*t + cnst(5))*t + cnst(4))*t
     &            + cnst(3))*t + cnst(2))*t + cnst(1))*t
     &            + cnst(0)
```

```
      polyd(t) =  ((((((((9.*cnst(9)*t + 8.*cnst(8))*t
     &            + 7.*cnst(7))*t + 6.*cnst(6))*t + 5.*cnst(5))*t
     &            + 4.*cnst(4))*t + 3.*cnst(3))*t + 2.*cnst(2))*t
     &            + cnst(1)

      f = 0.0
      t = 0.0

      do 10 t = 0.0, time/step, step
           left = f0*ca00*exp(-k3*t)
           right = f0*polyc(t) + v*x(1)*polyc(t)/(x(2) + polyc(t))
     &             + v*polyd(t)
           f = f + (left - right)**2
   10 continue
      return
      end


c     dummy function to satisfy the linker
      function CONST()
      implicit real*8 (a-h, o-z)
      const = 0.0
      return
      end
```

```
c     gradient of the objective function
      subroutine GRAD(X,G,F,N)
      implicit real*8 (a-h, o-z)
      real*8 x(n), g(n), t, left, right
      real*8 cnst(0:9), v, k3, f0, ca00, time, step
      common /cnst/ cnst, v, k3, f0, ca00, time, step

      polyc(t) = (((((((cnst(9)*t + cnst(8))*t + cnst(7))*t
     &            + cnst(6))*t + cnst(5))*t + cnst(4))*t
     &            + cnst(3))*t + cnst(2))*t + cnst(1))*t
     &            + cnst(0)
      polyd(t) =  ((((((9.*cnst(9)*t + 8.*cnst(8))*t
     &            + 7.*cnst(7))*t + 6.*cnst(6))*t + 5.*cnst(5))*t
     &            + 4.*cnst(4))*t + 3.*cnst(3))*t + 2.*cnst(2))*t
     &            + cnst(1)

      g(1) = 0.0
      g(2) = 0.0
      t = 0.0

      do 10 t = 0.0, time/step, step
          left = f0*ca00*exp(-k3*t)
          right = f0*polyc(t) + v*x(1)*polyc(t)/(x(2) + polyc(t))
     &            + v*polyd(t)
```

```
      g(1) = g(1) - 2.*(left - right)*v*polyc(t)/(x(2)
     &            + polyc(t))
      g(2) = g(2) + 2.*(left - right)
     &            *v*x(1)*polyc(t)/((x(2) + polyc(t))**2)
   10 continue
      return
      end
```

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