This is to certify that the

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Kinetics of Growth and Catechol Production
Bacillus stearothermophilus

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Ramkumar Subramanian

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Robert Mark Worden

Major professor

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KINETICS OF GROWTH AND CATECHOL PRODUCTION BY *BACILLUS STEAROTHERMOPHILUS*

By

Ramkumar Subramanian

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ABSTRACT

KINETICS OF GROWTH AND CATECHOL PRODUCTION BY *BACILLUS STEAROTHERMOPHILUS*

By

Ramkumar Subramanian

The purpose of this investigation was to study the growth and catechol production kinetics of *Bacillus stearothermophilus*.

Bacillus stearothermophilus BR219, a phenol resistant thermophile, converts phenol to the specialty chemical catechol. In continuous culture studies on a dilute (DP) medium supplemented with 10 mM phenol, steady states were achieved between dilution rates of 0.25 and 1.3 hr^{-1}. Phenol degradation was found to be uncoupled from growth.

Bacillus stearothermophilus BR321, obtained by transposon mutagenesis of BR219, grows on DP medium supplemented with phenol and accumulates catechol. In batch culture, catechol concentrations up to 1 mM were obtained. A mathematical model was developed to describe batch growth for different initial phenol concentrations. In continuous culture experiments conducted at a dilution rate of 0.12 hr^{-1}, steady states were obtained for feed phenol concentrations up to 0.25 mM, resulting in catechol concentrations up to 0.1 mM. Above this phenol concentration, oscillatory dynamics were observed.

To my parents and sisters for their love, affection and support

which make my accomplishments possible

ACKNOWLEDGMENTS

I wish to acknowledge the help of Dr. R. Mark Worden for all his support and guidance throughout the course of this research. I would like to thank Dr. Pat Oriel for all the useful suggestions he gave during the group meetings. I would also like to thank Dr. G. Gurujeyalakshmi and Dr. M. R. Natarajan for helping me learn microbiology laboratory techniques. Mike Bly helped me a lot, especially during the early phase of my research, and I would like to thank him for that. Finally, I would like to thank Bharath Rangarajan for the useful suggestions he gave during the development of the mathematical model.

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CHAPTER 1
INTRODUCTION

The objective of this research was to study the growth and catechol (1,2-dihydroxybenzene) production kinetics of *Bacillus stearothermophilus*. This bioconversion could serve as an alternative method for producing catechol, i.e. bioconversion of phenol, as compared to chemical processes like o-chlorophenol oxidation and phenol hydroxylation using hydrogen peroxide, which are used in industry.

Bacillus stearothermophilus BR219 degrades phenol via the *meta* pathway (Buswell, 1974; Adams and Ribbons, 1988) completely to carbon dioxide and water with catechol as the first intermediate in the process. Addition of tetracycline blocks this pathway by inhibiting catechol 2,3-dioxygenase, the subsequent enzyme in this process, resulting in the accumulation of catechol (Gurujeyalakshmi and Oriel, 1988a).

Bacillus stearothermophilus BR321 is a mutant developed from BR219 that contains transposon Tn916 inserted in the catechol 2,3-dioxygenase gene. This mutation prevents the formation of active catechol 2,3-dioxygenase and thus causes catechol accumulation without the addition of tetracycline (Natarajan and Oriel, 1992). When this research began, only BR219 had been isolated. The research plan was to study and optimize the growth rate of BR219 and subsequently

optimize catechol production after adding tetracycline. Batch experiments had been conducted with BR219 by Dr. Gurujeyalaksmi and Dr. Oriel (1988a-b,1989) and by Stefan Winter and Dr. R. M. Worden (Worden *et al.* 1991).

Once isolated, strain BR321 was substituted for BR219 because BR321 did not require the addition of tetracycline for catechol accumulation. With no antibiotic, it became possible to produce catechol while the cells were growing, thus making continuous reactor operation possible. In addition, the cost of tetracycline was eliminated.

This thesis consists of experiments conducted with both BR219 and BR321. It has been divided into the following chapters:

1) Introduction
2) Background
3) Experimental Methods
4) Continuous Culture Experiments with BR219
5) Batch Experiments with BR321
6) Continuous Culture Experiments with BR321
7) Conclusions and Recommendations

Chapter 2 summarizes the available literature pertinent to this research.

Chapter 3 describes the experimental methods used in the course of this research, including fermentation protocols and techniques for measuring cell density and viability, and catechol and phenol concentrations.

Chapter 4 deals with continuous culture experiments using the

parent strain (BR219). The experimental setup, procedure and data analysis are described in detail.

Chapter 5 describes batch experiments conducted to analyze the catechol producing capabilities of BR321. The experimental setup and procedure are described in detail. A mathematical model was developed to describe changes in concentrations of viable cells, rate-limiting substrate, phenol, and catechol during batch growth. The details of this model are discussed in Chapter 5.

Chapter 6 describes continuous culture experiments conducted using BR321. This experiment reflects the potential industrial feasibility of the bioprocess, as it demonstrates continuous production of catechol.

Finally in Chapter 7, the conclusions drawn from this research are outlined. Ongoing research is also briefly described, and future plans and possibilities are given.

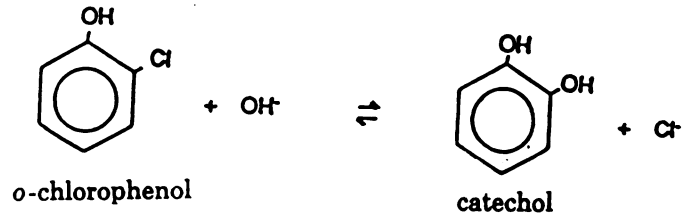
CHAPTER 2
BACKGROUND

In this chapter, the background information pertinent to this research is discussed. Topics include the current methods of production and uses of catechol, the advantages of using thermophilic bacteria, catechol metabolism, and growth studies of BR219 conducted by other researchers.

Catechol

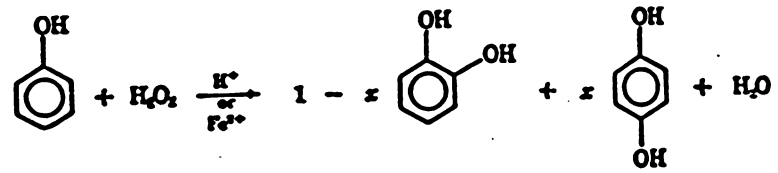
Catechol, also known as pyrocatechol or 1,2-dihydroxybenzene or 1,2-benzenediol, is a specialty chemical used in industry in fur dyeing, leather tanning, photographic applications, and as a polymer intermediate (Kirk and Othmer, 1981). It is a colorless, crystalline compound with a phenolic odor and a sweet and bitter taste (*ibid*). Its main use is in the photography industry, where it is used to reduce the exposed silver halide grains in a photographic emulsion at a faster rate than the unexposed grains and provide a very fine grain and high contrast images (*ibid*).

Catechol was first obtained in 1839 by dry distillation of catechin (*ibid*). In the early 20th century, catechol was manufactured by the hydrolysis of o-chlorophenol, as shown below.



o-chlorophenol catechol

Currently, there are no plants in the world producing catechol by this method. Phenol hydroxylation, using hydrogen peroxide, as shown below, is used by most of the major producers of catechol.



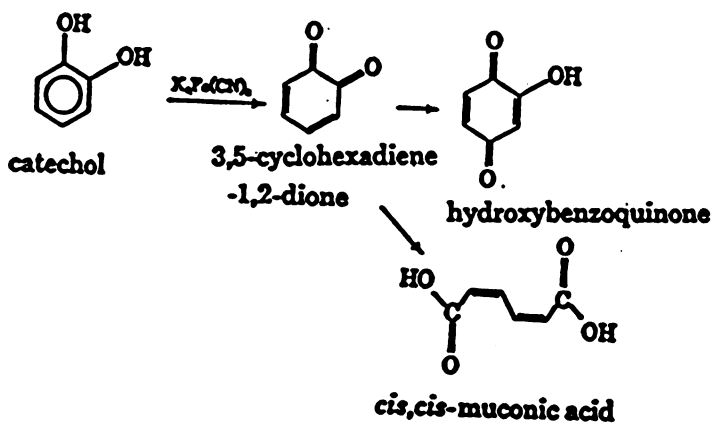
phenol catechol hydroquinone

About 78 % of the phenol is converted to dihydroxybenzenes, with every mole of phenol yielding 0.44 moles of catechol (Varagnat, 1976). Some plants use coal tar distillation to produce catechol. A detailed discussion of these production methods is given in the Encyclopedia of Chemical Technology edited by Kirk and Othmer (1981). Annual production of catechol in the world is around 20,000 tonnes (Kirk and Othmer, 1981). The current price of catechol is $2.80/lb for purchases up to 22,000 pounds and $2.10/lb for more than 22,000 lbs (Chem. Marketing Rep. 1991a). Phenol currently costs $0.28/lb (Chem. Marketing Rep. 1991c). Catechol's utility in industry

is increasing, and its market is expanding.

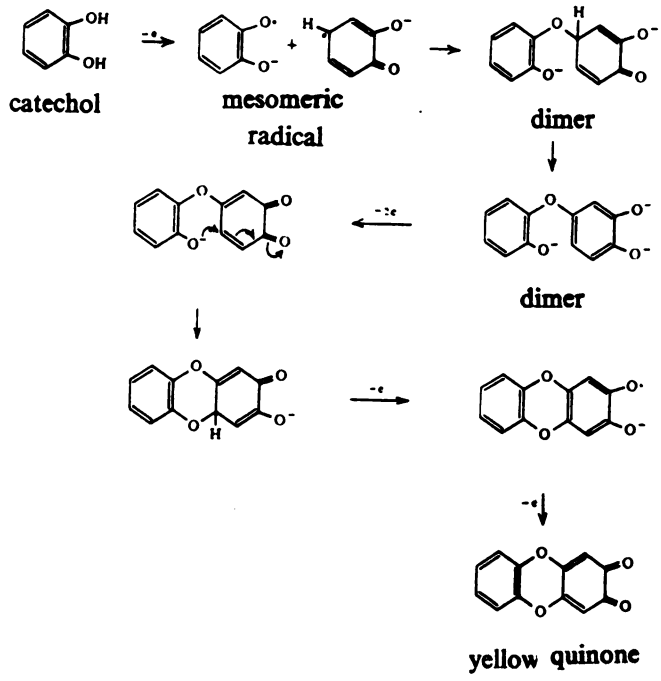
Any new technology for producing catechol will have to compete successfully against the existing technologies. The advantages of biochemical production of catechol are that phenol is a cheap substrate, the catalysts required for the reaction are inexpensive (bacteria), the operating temperature is less than the chemical process (55°C as compared to 80°C), and phenol is the only other aromatic compound in the product stream. In the chemical process, hydroquinone and small amounts of resorcinol are also formed, and successive distillations are required to get crude catechol. Hydrogen peroxide, which costs $0.25/lb (Chem. Marketing Rep., 1991b), has to be used on an equimolar basis in the chemical process for oxidation. These advantages for the biochemical process suggest that it may compete successfully with existing processes.

Catechol is more easily oxidized than phenol (Kirk and Othmer, 1981). It can be converted by most oxidizing agents to its ortho and para benzoquinones, as shown below.



Waters (1964) discusses the the autoxidation of catechol when

exposed to atmospheric oxygen. Catechol first oxidizes to a mesomeric radical, which eventually oxidizes to the stable yellow quinone. The autoxidation process is shown below:



High pH values and high temperatures increase the autoxidation rate of catechol.

Thermophilic bacteria

Bailey and Ollis (1985b) classify thermophiles as those microorganisms that can grow in temperatures ranging from 40 to 80°C, with the optimum temperature between 55 and 75°C. Sonnleitner and Fiechter (1983) describe thermophilic microorganisms as those that can grow and form products at temperatures above 65°C. The advantages of thermophilic bacteria in biotechnology have been discussed by Sonnleitner and Fiechter (1983) and Sonnleitner (1983), and include increased productivity due to increased reaction rates due

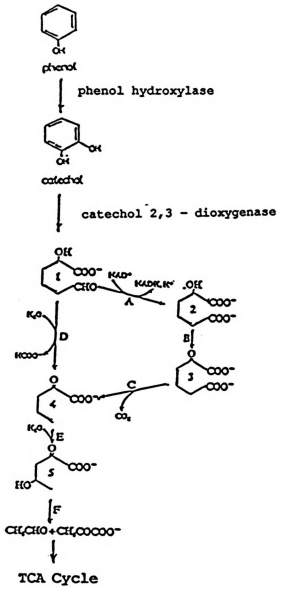
to the higher temperature, reduced microbial contamination problems because of the higher temperature, ability to control the reaction rate by cooling alone, resistance to chemical denaturation and higher tolerance to products.

It has been reported that mesophiles (bacteria that can grow at lower temperatures than thermophiles i.e. around 37°C) can degrade phenol; however, phenol is an inhibitory substrate (Gurujeyalaksmi and Oriel, 1988a). The first thermophile to utilize phenol was reported in 1974 by J. A. Buswell (1974).

Catechol Metabolism

Gurujeyalaksmi and Oriel isolated a strain of *Bacillus stearothermophilus* (BR219) from river sediment that possesses the highest phenol tolerance reported to date (Gurujeyalaksmi and Oriel, 1988a). This strain can grow in phenol concentrations of 15 mM with optimal growth occurring at 10 mM (*ibid*). Phenol degradation was found to occur via the *meta* pathway (Buswell, 1974) (Fig. 2.1), as shown by the formation of catechol and 2-hydroxy muconic semialdehyde, the first two products in this pathway. Buswell and Twomey (1975) had also reported the utilization of phenols and cresols by *Bacillus stearothermophilus*, but their strain was inhibited by phenol at concentrations above 5 mM. The details of the metabolism were given in another paper by Buswell (1975) including the first few steps of the *meta* degradation pathway. Further steps in this pathway are discussed by Adams and Ribbons (1988). Dagley (1971) has discussed the catabolism of aromatic compounds by microorganisms in detail.

Gurujeyalaksmi and Oriel studied the accumulation of catechol



1) 2 hydroxymuconic semialdehyde
2) 4 oxalocrotonate (enol)
3) 4 oxalocrotonate (keto)
4) 2 oxopent 4 enoate
5) 4 hydroxy 2 oxalovalerate

A) 2 hydroxymuconic semialdehyde dehydrogenase
B) 4 oxalocrotonate tautomerase
C) 4 oxalocrotonate decarboxylase
D) 2 hydroxy 6 oxohepta 2,4 dienoate hydrolase
E) 2 oxopent 4 enoate hydratase
F) 4 hydroxy 2 oxovalarate aldolase

Fig 2.1 *Meta* pathway
(From Buswell (1975) and Adams and Ribbons (1988))

by *Bacillus stearothermophilus* BR219. They reported that addition of tetracycline inhibited the enzyme catechol 2,3-dioxygenase, which oxidizes catechol, resulting in the accumulation of catechol (1989). They also reported that the optimal concentration of tetracycline for inhibition to occur was 5 μg/mL at 55°C. They further said that the highest conversion of phenol to catechol occurred at 5 mM phenol (16% phenol conversion), while the highest concentration of catechol was obtained when 10 mM phenol was used as substrate (Figs. 2.2-2.6). For low phenol concentrations, the cells began to die immediately after a key nutrient was depleted, i.e. there was no stationary phase (Fig. 2.2). However, when higher phenol concentrations were used, a small stationary phase occurred before death of cells began. Typical maximum optical densities (650 nm) obtained with the dilute media were around 0.35. Gurujeyalaksmi and Oriel (1988a) measured doubling times of around 100 minutes with the first isolates of the bacterium.

Kinetic studies of this bacteria were conducted by Stefan Winter under the guidance of Dr. R. M. Worden. His research focused on determining the optimal concentration of phenol required for the bioconversion process, the effect of pH on growth and process control of this fermentation. They showed that pH does not affect growth in the range of 6-8, and minimal growth was observed at pH 5.5 (Worden *et al.*, 1991). Typical maximum optical densities (650 nm) obtained in rich media were around 2.8.

Gurujeyalakshmi and Oriel (1988b) reported that when tetracycline inhibited the phenol degradation pathway, the accumulation of catechol was higher for strains of BR219 carrying a conjugating transposon Tn916. Subsequently Drs. Natarajan and Oriel (1992),

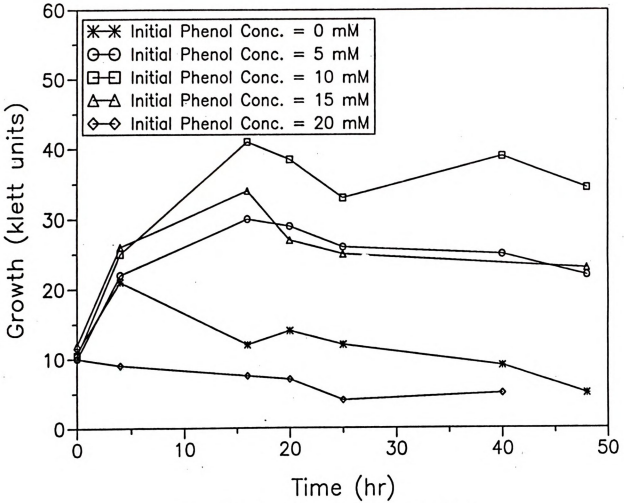


Fig. 2.2 Growth kinetics of BR219
From Gurujeyalakshmi and Oriel (1988a)

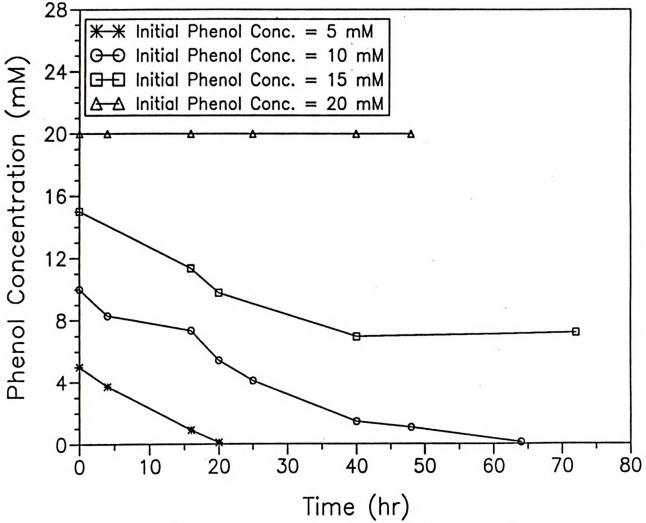


Fig. 2.3 Phenol degradation by BR219
From Gurujeyalakshmi and Oriel (1988a)

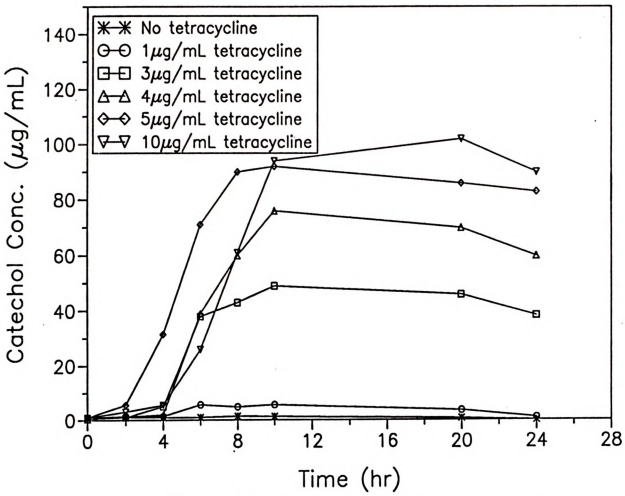


Fig. 2.4 Effect of tetracycline on catechol production in DP – 5 mM phenol
From Gurujeyalaksmi and Oriel (1989)

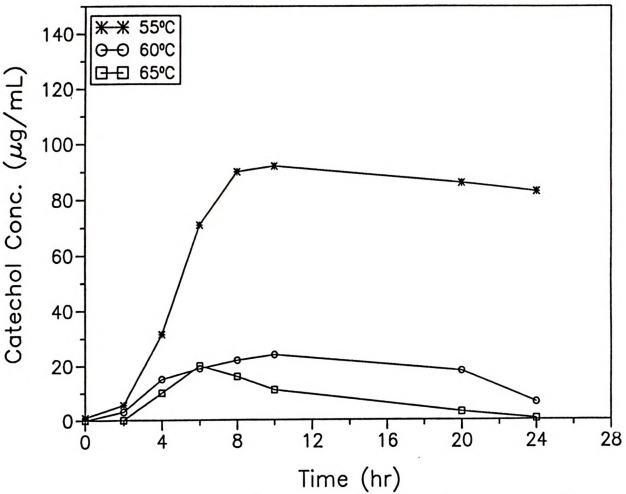


Fig. 2.5 Effect of temperature on catechol production in DP – 5 mM phenol and 5μg/mL tetracycline
From Gurujeyalakshmi and Oriel (1989)

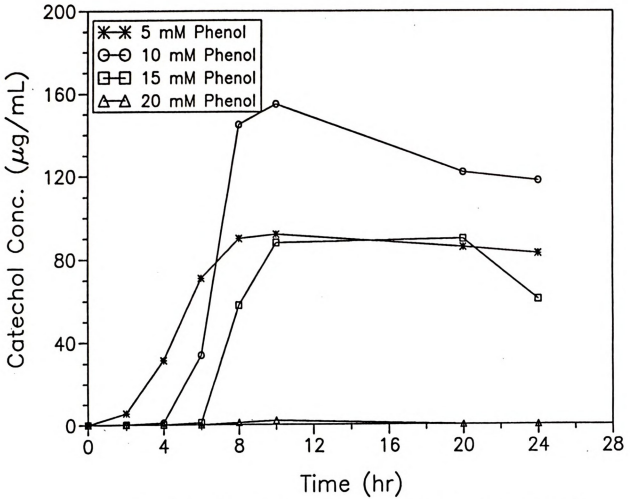


Fig. 2.6 Effect of phenol concentration on catechol production in DP – phenol and 5μg/mL tetracycline From Gurujeyalakshmi and Oriel (1989)

successfully mutagenized BR219 with transposon Tn916 to yield a mutant (BR321) that accumulated catechol throughout the growth phase. Thus, with BR321 the tetracycline addition step could be eliminated in the catechol production process.

CHAPTER 3
EXPERIMENTAL METHODS

Introduction

In this chapter the techniques used in the experiments are described in detail. These techniques include measurements of cell density and viability, catechol concentration, and phenol concentration. The methods for making LB-Broth and DP media, and LB and DP plates are also described.

Media used

1) LB-Broth (Maniatis *et al.*, 1982)

This contains per liter

10 g tryptone (Difco, Detroit, Michigan)
5g yeast extract (Difco, Detroit, Michigan)
5g sodium chloride

2) DP medium (Buswell, 1974)

This contains per liter

1 g ammonium chloride
0.5 g potassium phosphate, dibasic
0.2 g yeast extract (Difco, Detroit, Michigan)

0.1 g casamino acids (Difco, Detroit, Michigan)

0.02 g magnesium sulphate

1 mL trace element solution, which contains per liter

500 µg boric acid

40 µg copper sulphate

100 µg potassium iodide

200 µg ferric chloride

400 µg manganous sulphate

aliquot of phenol (for desired concentration)

For LB or DP media, the different components were added to reverse osmosis (RO) water to a final volume of 1 L and dissolved by constant stirring. The pH was adjusted to 7.2 using small amounts of hydrochloric acid or sodium hydroxide. The resulting medium was then autoclaved as described below and then stored at room temperature.

Sterilization

The media were sterilized at 121°C using 15 psig steam in a Castle sterilizer (Wilmot Castle company, New York) for 15 minutes for small quantities of media (250 mL) and 45 minutes for large quantities of media (12 L).

LB and DP plates

The media (LB or DP) were made up as described earlier. Before adjusting the final volume to 1 L, 10 g of agar or gelrite and 1 g of magnesium chloride were added. The solution was then sterilized as

described earlier. After cooling to 40 - 50°C, the solution was poured into the plates. The plates were left at room temperature for a day to check for contamination and then stored inverted at 4°C.

Cell Density

The cell density was measured by turbidity at 650 nm using a Perkin Elmer (Norwalk, Connecticut) Lambda 3A spectrophotometer with RO water as the blank. When the optical density was above 0.8, the sample was quantitatively diluted with DP or LB medium (as needed) for better accuracy.

Colony Counts

The cell viability was measured by plating a known volume of cell broth on LB plates, after asuitable dilution to give 30 - 300 colonies per plate, and then incubating the plates in an oven at 55°C (Boyd, 1988).

Cell Dry Weight

To measure the cell concentration based on dry weight, 25 mL of the broth was withdrawn from the reactor with a volumetric pipette, and centrifuged for 10 minutes at 5900g. The supernatant was carefully removed using a pipette. Twenty-five mL of sterile water was added, and the cells were resuspended using a vortex machine. The cells were centrifuged again, and once again, the supernatant removed. Finally, the cells were transferred quantitatively to a pre-dried, pre-weighed, aluminum pan and dried for 20 hours at 100°C. The pan was cooled in a desiccator and weighed. The difference in the pan weight with and

without the cells gave the dry weight of cells.

Catechol Assay

Catechol was measured as described by Irie *et al.* (1987) with some minor modifications. A reagent solution was made that contained

0.1 % by weight 4-amino antipyrene
2 % by weight sodium carbonate
0.02 N sodium hydroxide

An appropriate amount of sample (to obtain the desired final concentration) was mixed with 3 mL of the reagent solution. After sitting for 20 minutes, the absorbance was measured at 515 nm with the reagent solution as the blank. The reagent solution is colorless at 515 nm and can be replaced by RO water as the blank.

Fig. 3.1 shows a typical calibration curve for this assay. This assay was repeated many times to verify its reproducibility and accuracy. The catechol assay is highly linear in the range of 0.0001 to 0.012 g/L (coefficient of linearity - 0.9997).

Phenol assay

Phenol was measured using a technique described by R. W. Martin (1949). Eight mL of RO water, 240 µL of a 2 % by weight solution of 4-amino antipyrene, 800 µL of a 2 N ammonium hydroxide solution, 800 µL of a 2% by weight potassium ferricyanide solution, and a small aliquot of sample containing phenol were added to a test tube. The solution was well mixed and allowed to react for 30 minutes,

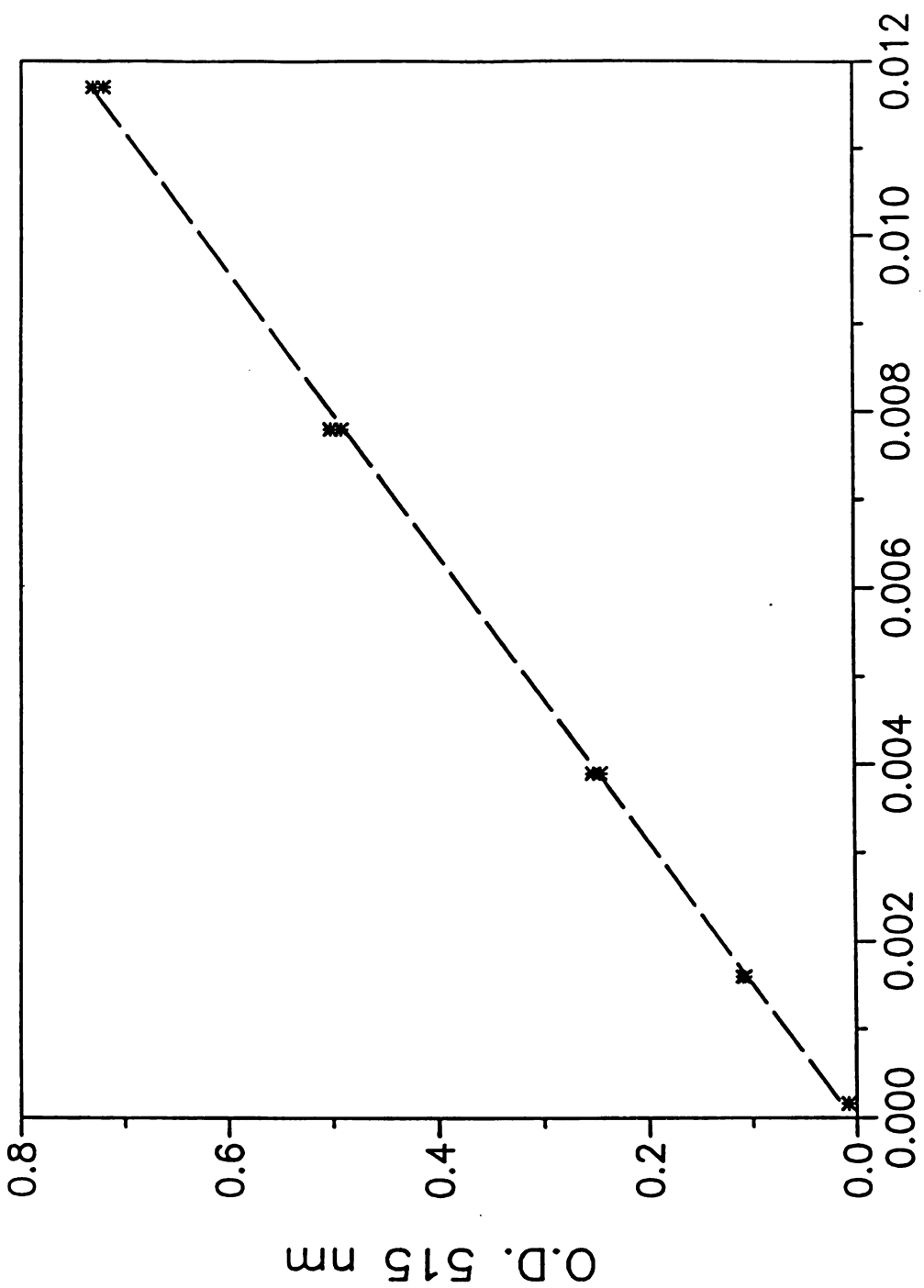


Fig. 3.1 Catechol Assay

before the absorbance was measured at 510 nm, using the same mixture without the sample as the blank.

Figure 3.2 shows a typical calibration curve for this assay. This assay is highly linear up to phenol concentrations of 0.005 g/L.

These assays were calibrated before each set of experiments to verify the existing calibration. The difference in the calibration curves obtained was always less than 2%.

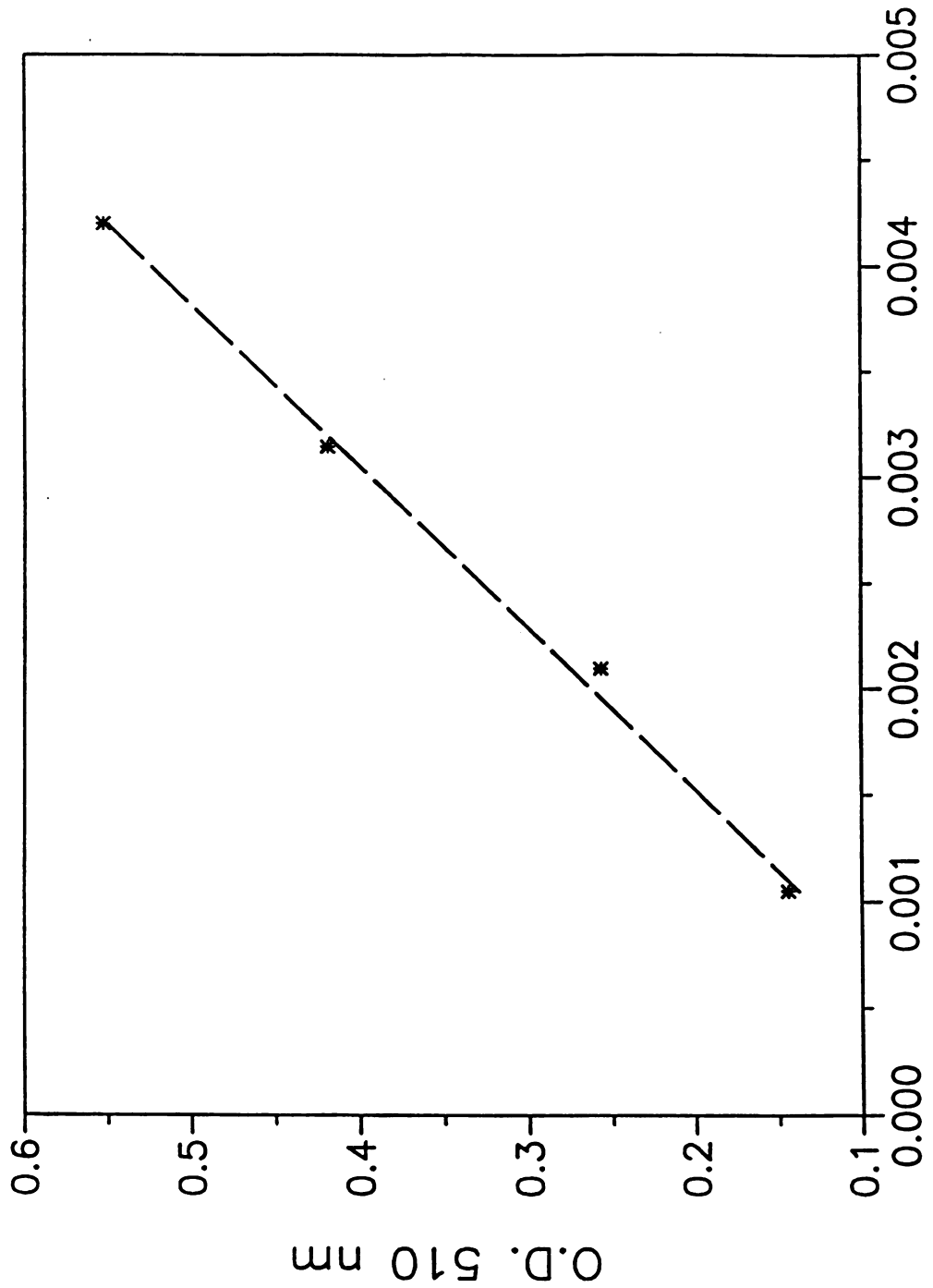


Fig. 3.2 Phenol Assay

CHAPTER 4

CONTINUOUS CULTURE EXPERIMENTS WITH *BACILLUS STEAROTHERMOPHILUS* BR219

Introduction

The main objective of these experiments was to study the growth of *Bacillus stearothermophilus* BR219 in continuous culture. Batch growth studies, conducted in both rich and dilute media (Gurujeyalakshmi and Oriel, 1988a-b,1989; Worden *et al*, 1991), have been discussed in Chapter 2. Continuous culture experiments were performed to better understand the growth and phenol degradation kinetics of this organism .

Materials and Methods

The bacterium *Bacillus stearothermophilus* BR219 was obtained from the laboratory of Dr. P. Oriel. The experiments were conducted in a 1.3 liter Bioflo II - New Brunswick (Edison, New Jersey) fermentor .

The experimental setup is shown in Fig. 4.1. The detailed procedure is given below.

The inoculum was prepared by transferring a single colony from a LB plate of BR219 to a sterile flask containing 100 mL of sterile LB-Broth and incubating for 13 -17 hours at 55°C with shaking at 200 rpm.

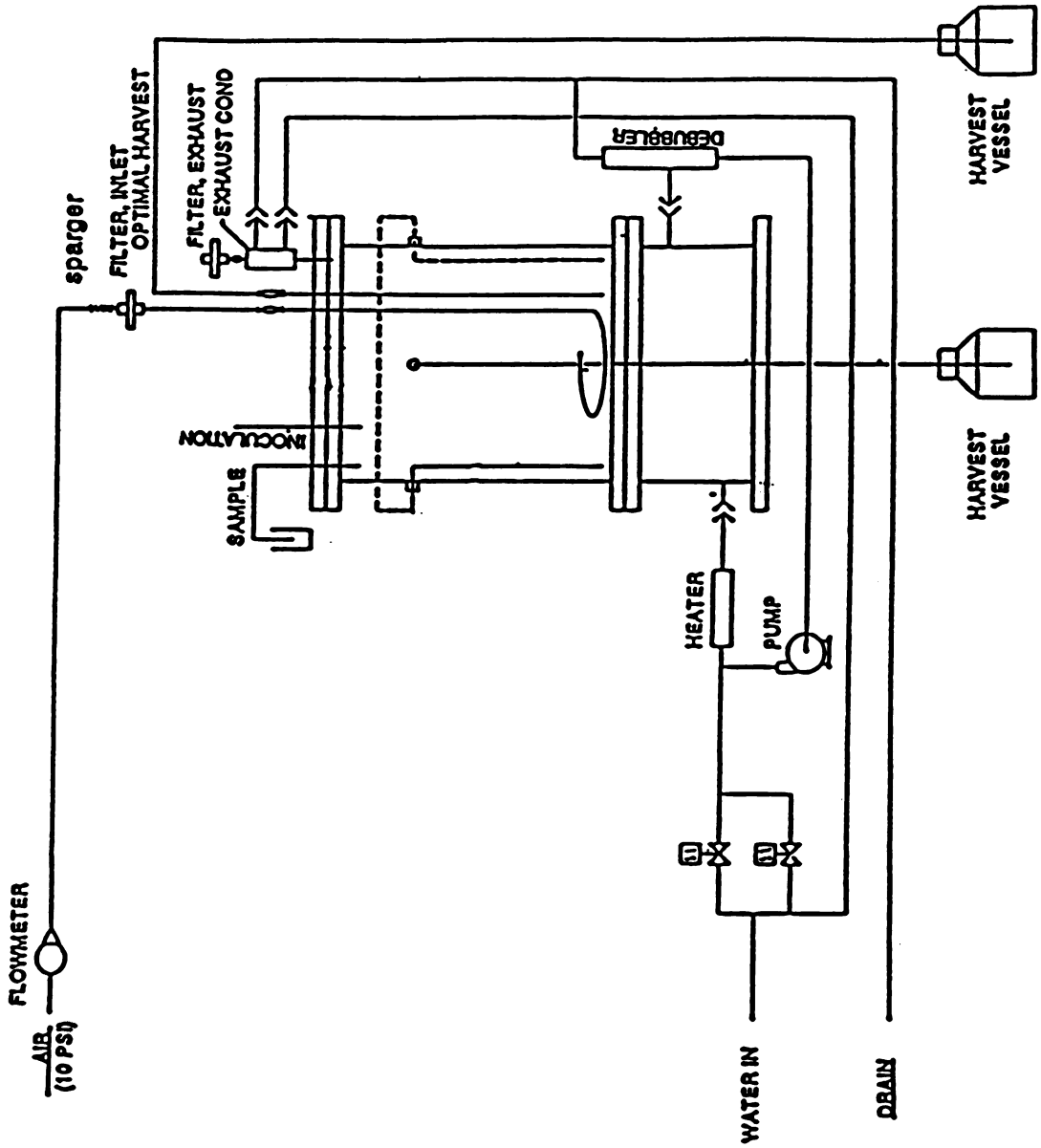


Fig 4.1 Experimental setup

The feed carboy, connecting tubes, overflow vessel, and the fermentor vessel were sterilized and connected aseptically. The reactor was filled using a pump and then heated to 55°C. Air sparging at 3 L/min and agitation at 100 rpm were started. A 5 % by volume inoculum was injected into the reactor, using a sterile syringe and needle.

Once growth began, continuous feed was started. The reactor was monitored occasionally to see if steady state had occurred. This was done by measuring the turbidity at 650 nm as described in Chapter 3. If the cell density remained the same for 3 residence times, steady state was assumed. At steady state, the cell turbidity, the cell dry weight, and the inlet and outlet phenol concentrations were measured. The pump was then adjusted to increase the liquid flow rate, thus increasing the dilution rate.

The dilution rate was varied from 0.25 to 1.5 hr^{-1}. Phenol concentration in the feed was kept steady at 10 mM. Sterile conditions were maintained for the most part; however, due to the high concentration of phenol and the high temperature involved, aseptic operation may not have been necessary.

Results

Cell concentration profiles leading to each of the steady states are plotted in Figs. 4.2-4.5. The steady state cell (X) and phenol (P) concentrations are plotted as a function of the dilution rate (D) in Fig. 4.6. The corresponding steady state data are also tabulated in Table 4.1. The cell concentration was constant between dilution rates of 0.25 to 1.3 hr^{-1}, while the phenol degradation varied from a high of 20 % at a dilution rate of 0.25 hr^{-1} to 5 % at a dilution rate of 1.3 hr^{-1}(Fig. 4.7).

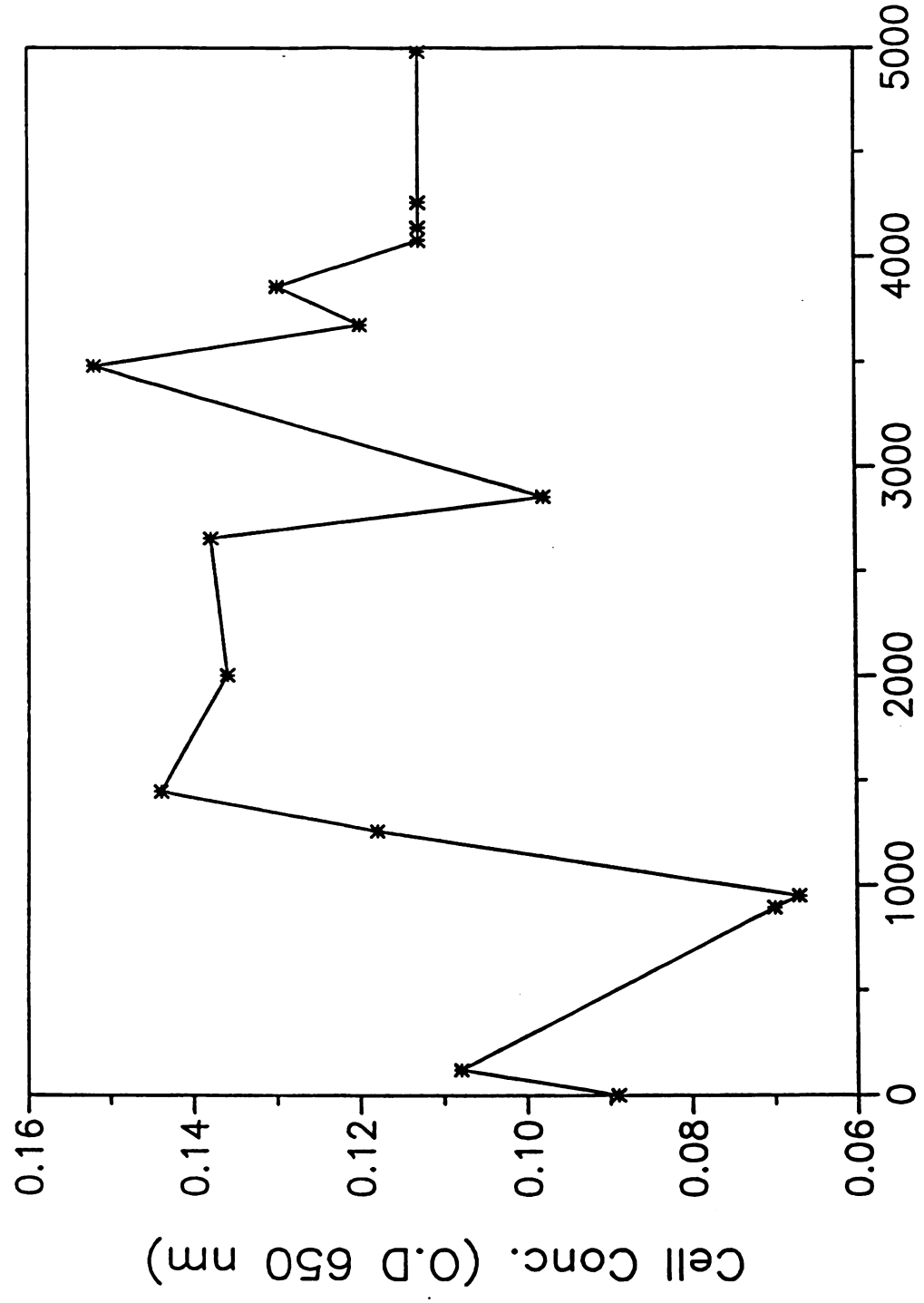


Fig. 4.2 Unsteady state growth D = 0.25

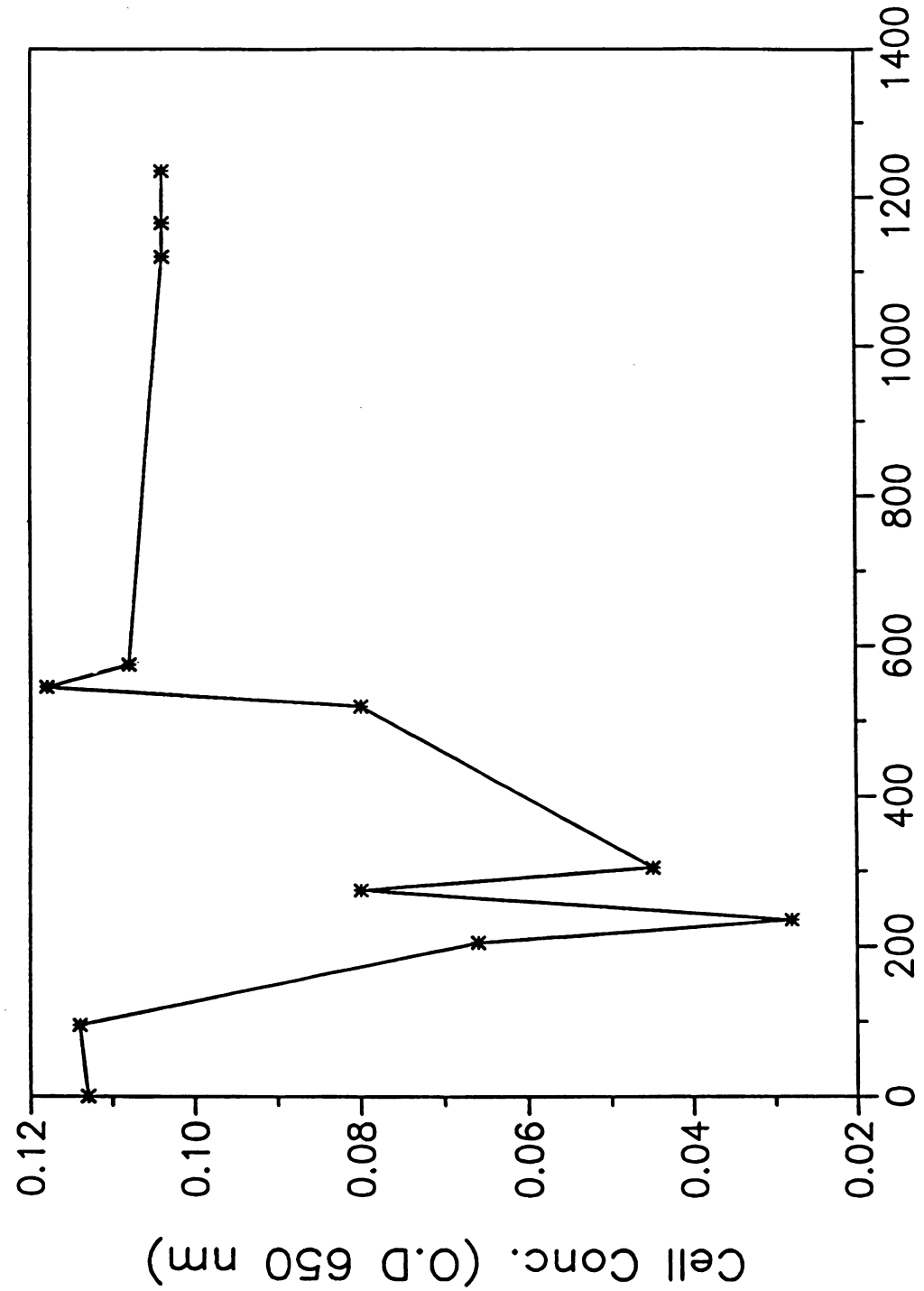


Fig. 4.3 Unsteady state growth D = 0.5

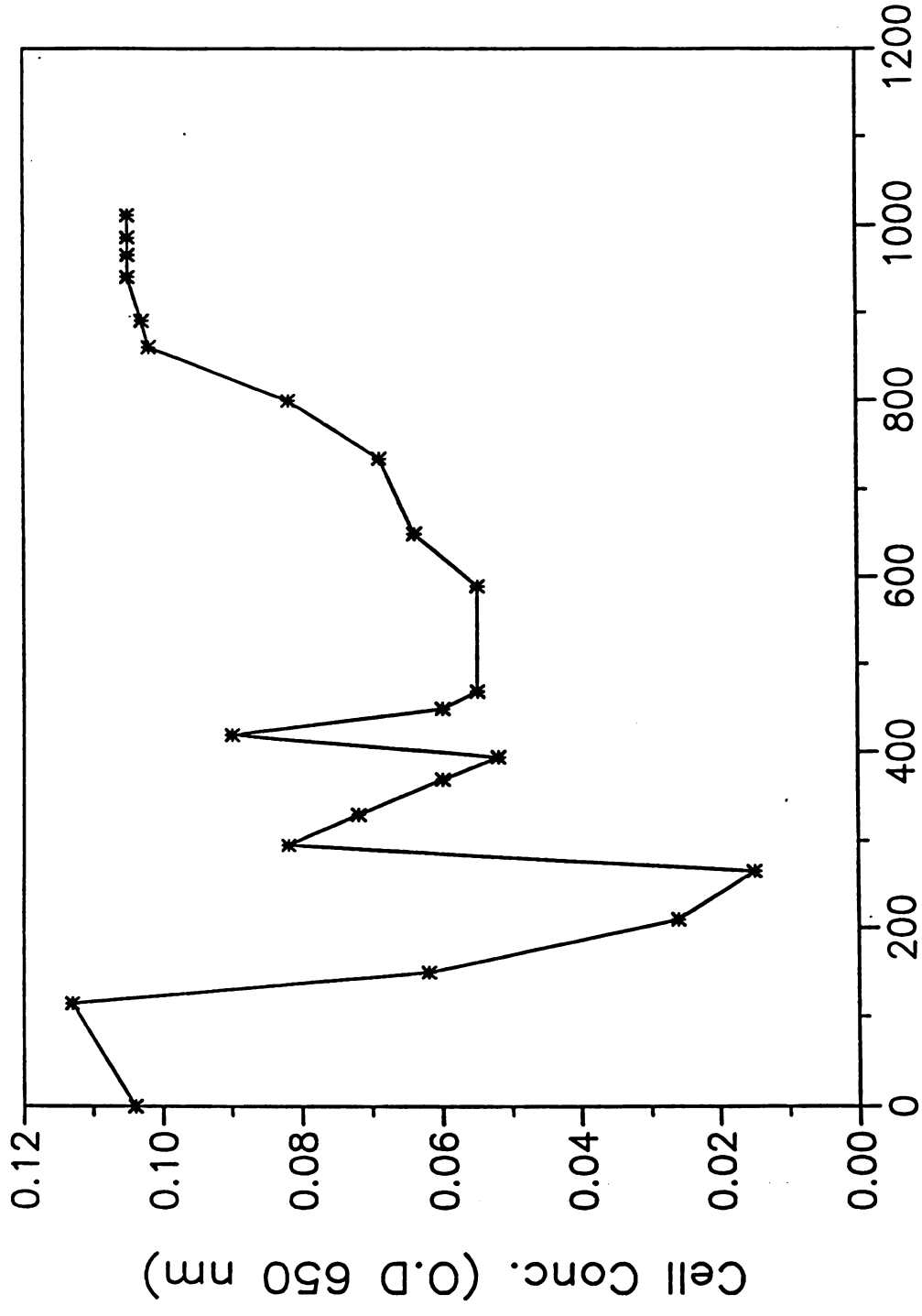


Fig. 4.4 Unsteady state growth D = 1.00

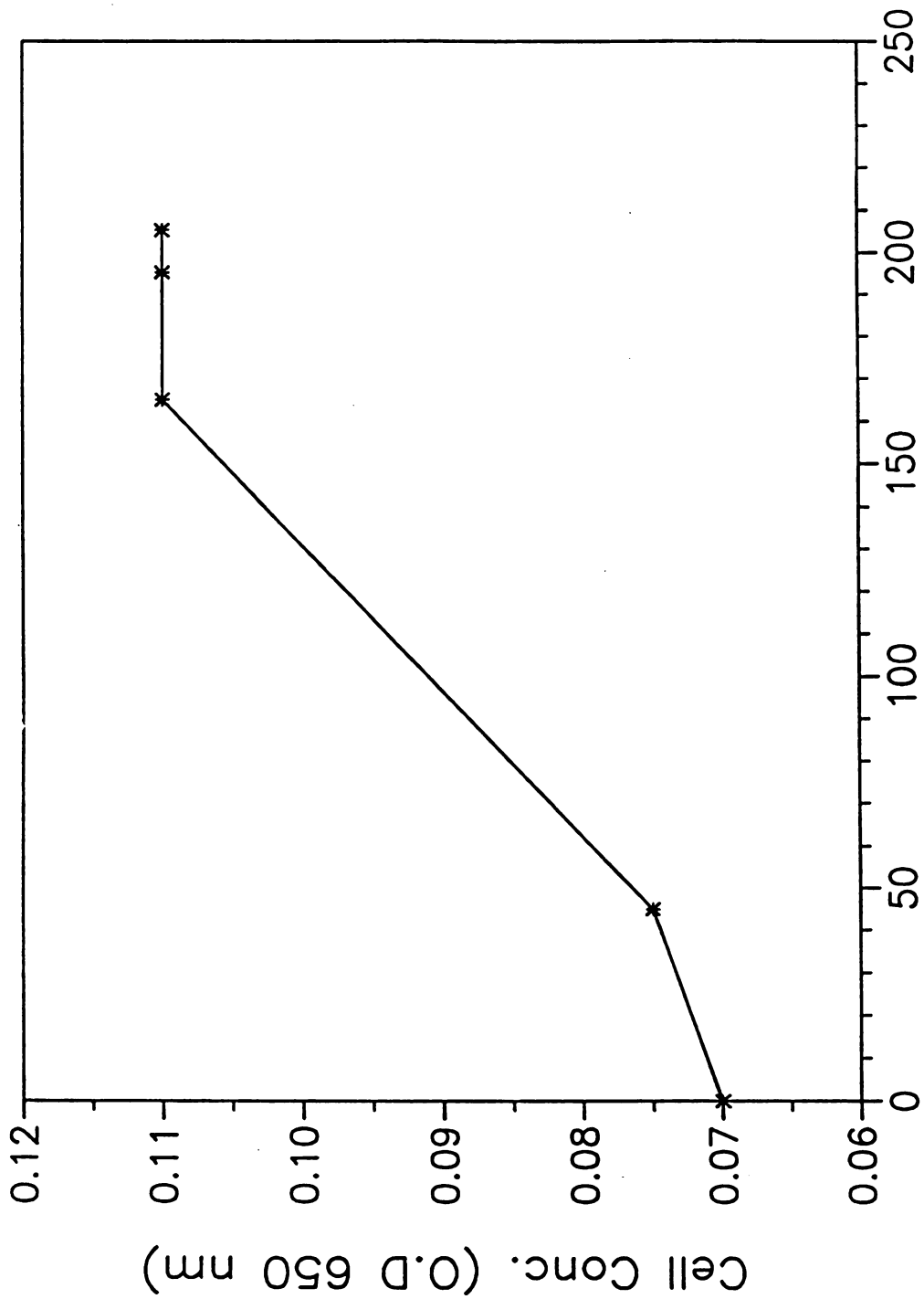


Fig. 4.5 Unsteady state growth D = 1.30

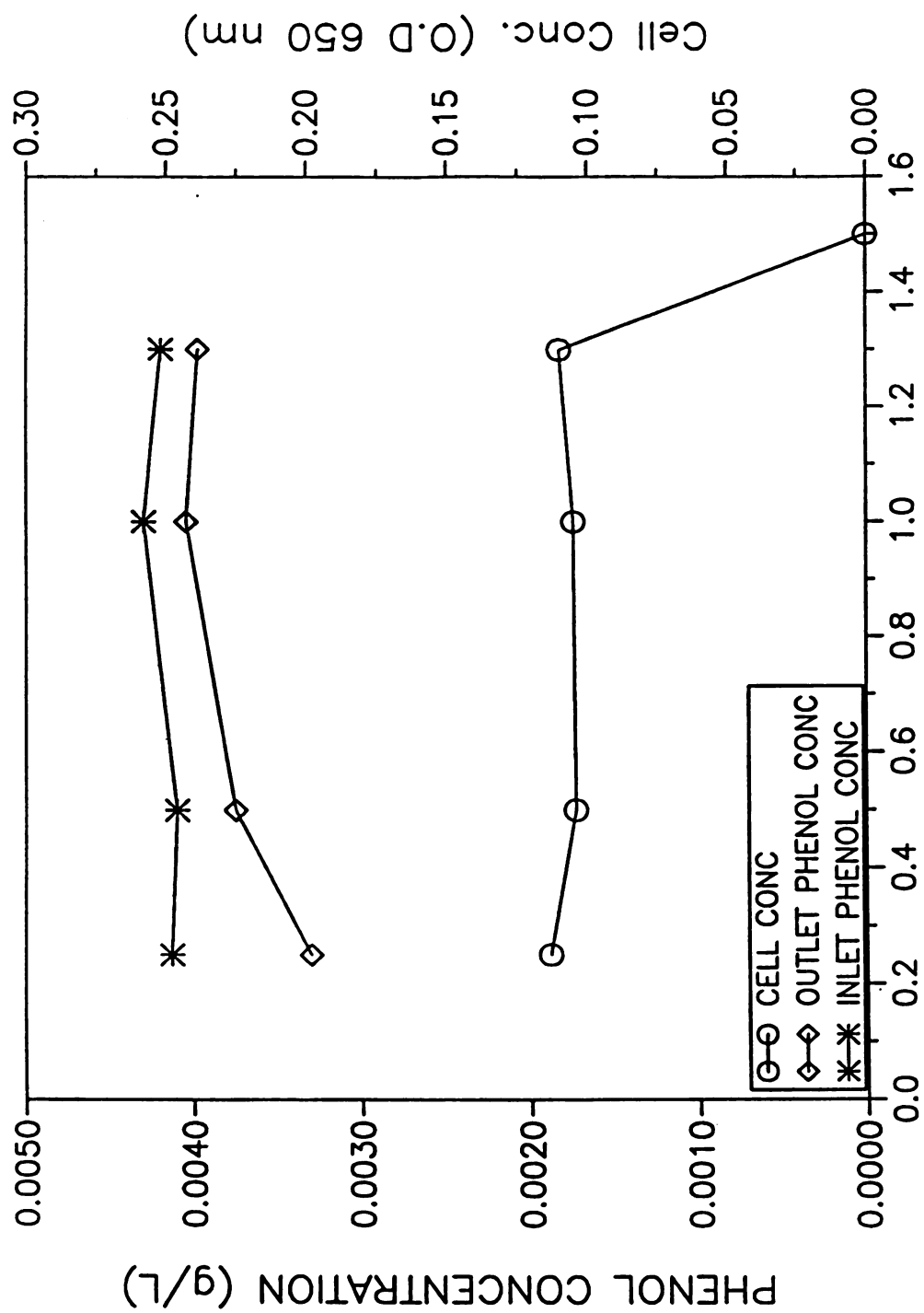


Fig. 4.6 Cell and phenol concentrations as a function of dilution rate

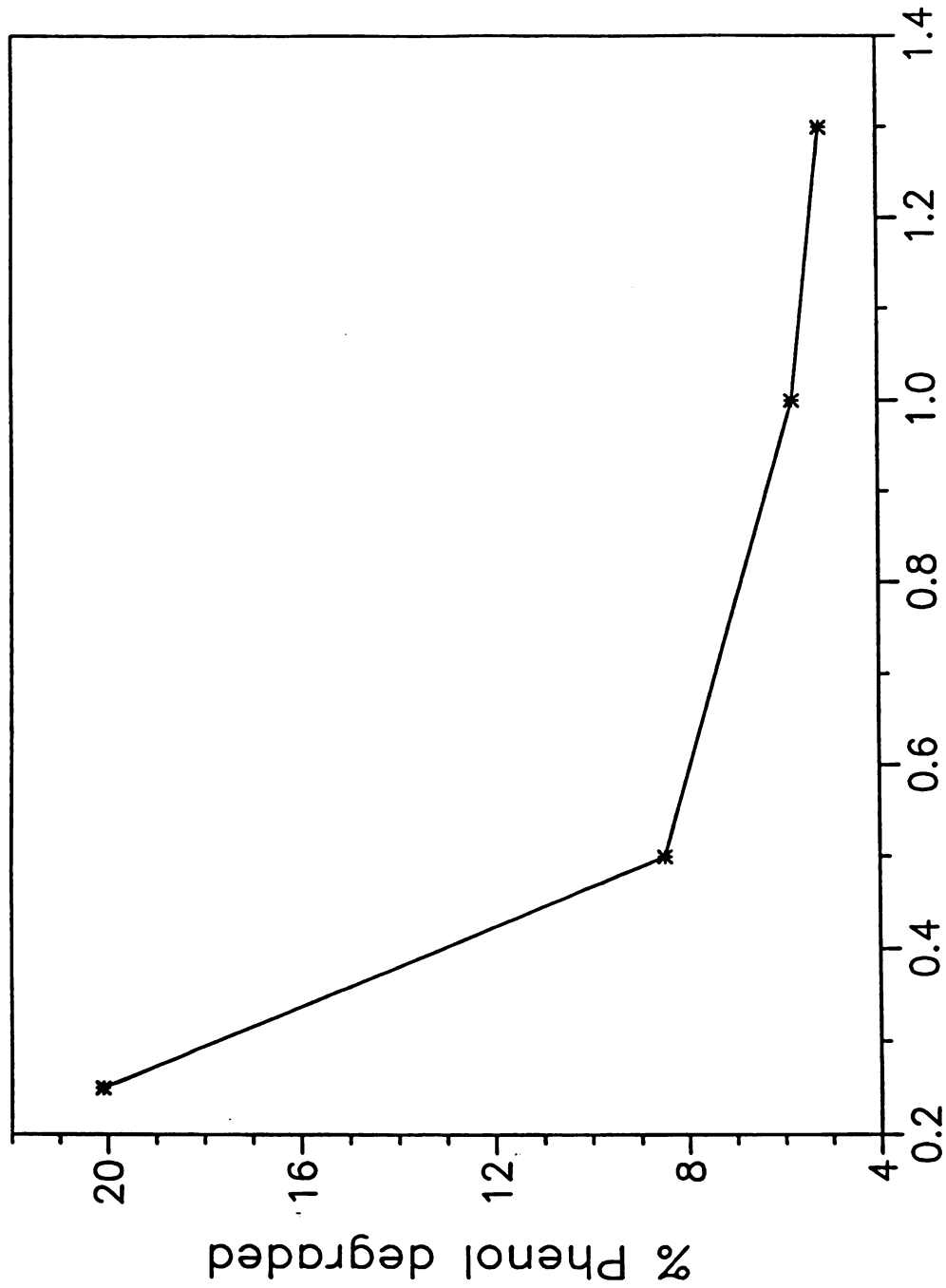


Fig. 4.7 Phenol degradation as a function of dilution rate

Because of the imprecision of the dry weight biomass assay at the low cell concentrations, the standard deviations of the dry weight measurements were very large (7.4×10^{-4} g/25 mL).

Table 4.1: Cell concentration and phenol degradation as a function of dilution rate.

Dilution rate (hr^{-1})	Cell Concentration		% Phenol degraded
	O.D. 650 nm	dry weight (g/25 mL)	
0.25	0.113	0.009	20.1
0.5	0.104	0.007	8.5
1.0	0.105	0.007	5.8
1.3	0.110	-	5.2
1.5	wash out		

At low dilution rates, the reactor was fairly stable. Minor changes in the feed concentrations, i.e. when the carboys were changed, did not affect the cell concentration significantly. However as the dilution rate was increased, the reactor stability decreased. When the pump was switched from a dilution rate of 0.5 to 1 hr^{-1} and from 1 to 1.3 hr^{-1}, sudden lag phases resulted which lasted for several hours. The onset of a lag phase caused the majority of the cells to be washed out within three residence times. When such a lag phase was observed,

the pump was switched off until growth became apparent again.

The theory of chemostats, as discussed by Bailey and Ollis (1985a) and by Marison (1988) show the relation between growth rates and dilution rates. Mass balances on mass and phenol in the reactor assuming sterile feed and negligible cell death rate give

$$dX/dt = -DX + \mu X \qquad (4.1)$$

$$dP_{out}/dt = D(P_{in} - P_{out}) - q_p X \qquad (4.2)$$

At steady state,

$$dX/dt = 0 \text{ and } dP_{out}/dt = 0 \qquad (4.3)$$

Hence,

$$\mu = D \qquad (4.4)$$

$$q_p = D(P_{in} - P_{out})/X \qquad (4.5)$$

where P_{in} and P_{out} are inlet and outlet phenol concentrations.

The specific growth rate (μ), which is the rate of increase in cell concentration per unit of cell concentration, and the specific phenol degradation rate (q_p), which is the rate of phenol consumed per unit of cell concentration, are shown in Fig. 4.8 as a function of the dilution rate. They are also shown in Table 4.2.

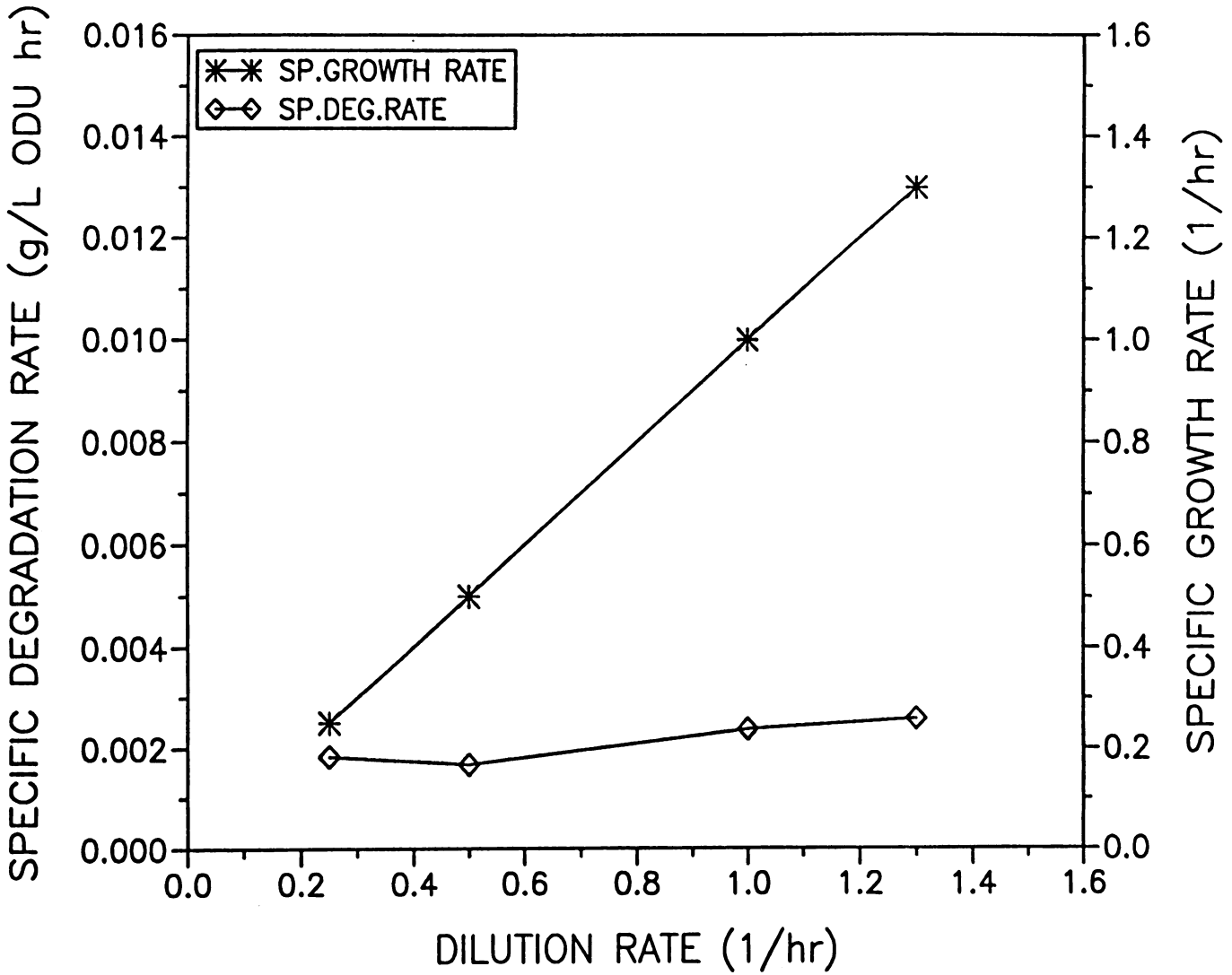


Fig. 4.8 Specific growth rate and specific degradation rate as a function of dilution rate

Table 4.2: Specific growth and phenol degradation rates as a function of dilution rate

Dilution rate (hr^{-1})	Specific growth rate (hr^{-1})	Specific phenol deg. rate (g/(L ODU hr))
0.25	0.25	0.00183
0.5	0.5	0.00167
1.0	1.0	0.00237
1.3	1.3	0.00258

Table 4.3 shows the outlet pH and dilution rate values. The inlet pH was maintained constant at 7.2 .

Table 4.3: Outlet pH as a function of dilution rate

Dilution rate (hr^{-1})	Outlet pH
0.25	7.0
0.5	7.08
1.0	7.12
1.3	7.13

When the dilution rate was increased from 1.3 hr^{-1} to 1.5 hr^{-1} the cells began to be washed out. The pump was switched off until growth became apparent and then turned on again. When three such attempts failed, it was concluded that at this dilution rate a stable, nontrivial steady state could not be reached.

An experiment was conducted to study the potential loss of phenol due to evaporation. In this experiment, DP medium was continuously fed at a dilution rate of 1.2 hr^{-1}. Phenol concentration was measured in the inlet and outlet streams. There were no bacteria present, but all the other conditions were as before (55°C, air sparging at 3 L/min, and agitation at 100 rpm). There was no measurable loss of phenol for more than 3 residence times, and hence, it was concluded that evaporative losses of phenol were negligible.

Discussion

As shown in Fig. 4.8, over a five fold (500%) increase in specific growth rates there was only a 45% increase in the specific phenol degradation rate. This suggests that phenol degradation is uncoupled from growth. It may also suggest that the enzymes needed for this purpose are under tight genetic control (Worden *et al.*, 1991). The small change in the phenol degradation rates may be a result of the adaptation of the culture during the experiment. The steady states were achieved gradually from the lowest to the highest dilution rate, and this may have resulted in strain improvement through natural selection (*ibid*).

Note that even though pH was not controlled in these experiments, the pH did not vary much with dilution rate. It has already

been shown that pH within the range of 6-8 does not significantly affect growth (see Chapter 2). This result suggests that tight pH control may not be needed in a commercial application.

These results show that *Bacillus stearothermophilus* BR219 can grow on 10 mM phenol at 55°C at high specific growth rates (up to 1.3 hr^{-1}). It has already been shown that the organism can grow on 15 mM phenol (Gurujeyalakshmi and Oriel, 1988a), which is the highest known microbial tolerance for phenol. Also, the high growth rates were achieved on a dilute medium such as DP supplemented with phenol. This combination of high growth rates, extreme phenol tolerance, and potential unimportance of pH control and sterile conditions, makes this organism well suited for phenol biodegradation. In addition, catechol is an intermediate in the phenol-degradation pathway. Thus, it may be possible to produce catechol commercially from phenol using BR219. However, at present, tetracycline addition is needed to block unwanted microbial catechol oxidation.

CHAPTER 5

BATCH GROWTH AND CATECHOL PRODUCTION KINETICS OF *BACILLUS STEAROTHERMOPHILUS* BR321

Introduction

The successful transposon mutagenesis of *Bacillus stearothermophilus* BR219 to create a new strain BR321 in the laboratory of Dr. Oriel led to this study. This new strain accumulates catechol in the absence of tetracycline, thus allowing catechol production during the growth phase. Details of the transposon mutagenesis procedure are given by Natarajan and Oriel (1992).

In this research, the kinetics of growth, phenol degradation and catechol production of strain BR321 were examined in batch culture. A simple mathematical model was then developed to explain the batch kinetics of this organism.

Materials and Methods

The experiments were conducted in 250 mL Bellco flasks with stainless steel caps that allow gas exchange while maintaining sterility. An Aquatherm water bath shaker (New Brunswick, Edison, New Jersey) was used to maintain temperature and provide shaking. A Fisher-Scientific (Springfield, New Jersey) Model 235C micro-centrifuge was used to concentrate the cells in preparing the inoculum. The various assays were done as described in Chapter 3.

The inoculum was prepared as described in Chapter 4. The cells, grown in LB-Broth for 13 - 17 hours, were microfuged in Reagiergefabe (Sarstedt, Germany) test tubes for 2 minutes and resuspended in DP medium before being used as an inoculum. This procedure reduced the lag phase in DP medium. One hundred mL of DP medium was used in all experiments; the inoculum was 5 mL (5 %). Samples were taken every hour for the first four hours and afterwards every four hours. The samples were assayed for cell turbidity during the first few hours, and the samples taken every four hours were used to measure cell viability, and concentrations of catechol and phenol.

Experimental Results

Natarajan and Oriel (1992) showed that catechol is produced throughout the growth phase by *Bacillus stearothermophilus* BR321. To understand the kinetics of this organism and to determine the effect of phenol concentration on catechol production, batch growth experiments were conducted at phenol concentrations of 0, 0.8, 1.6 and 2.4 mM. Figures 5.1, 5.2, and 5.3 show the time profiles of growth, phenol concentration and catechol concentration for the different cases. As seen by Fig. 5.1, phenol concentration did not seem to affect growth at the concentrations tested. Table 5.1 shows the percentage of phenol utilized and catechol produced in these different cases.

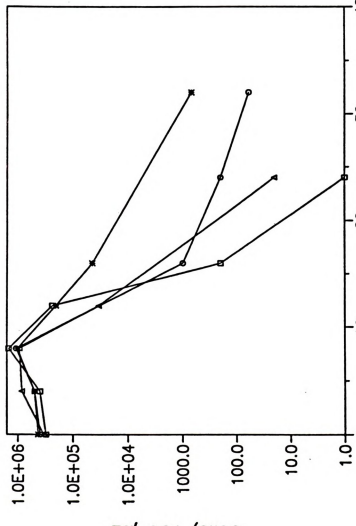


Fig. 5.1 Cell concentration vs time in batch culture

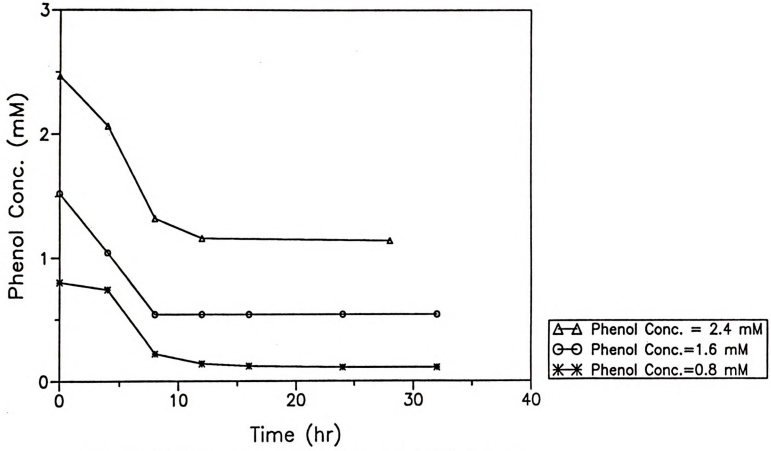


Fig. 5.2 Phenol concentration vs time in batch culture

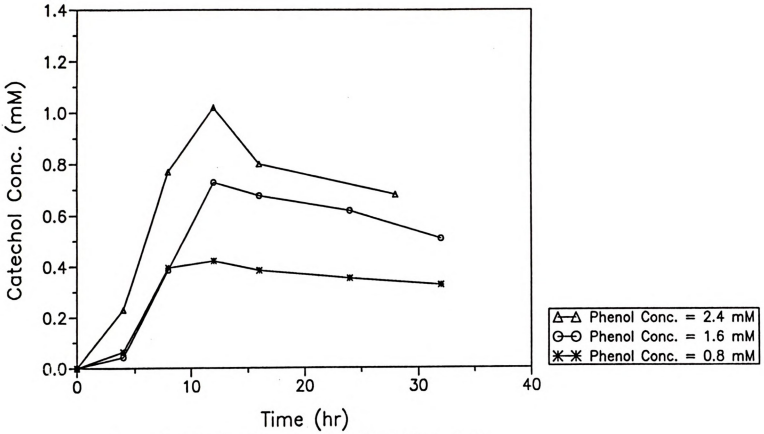


Fig. 5.3 Catechol conc. vs time in batch culture

Table 5.1 Phenol utilization and catechol production as a function of varying initial phenol concentration

Phenol Concentration (mM)		% Phenol degraded	Catechol Produced (mM)
Initial	Final		
0.802	0.14	82.5	0.42
1.52	0.54	64.47	0.73
2.39	1.07	55.23	0.859

Assuming that phenol degradation occurs only by the *meta* pathway, and that catechol 2,3-dioxygenase production is entirely disabled by the transposon insertion, the molar catechol yield should equal the molar phenol consumption. However, Table 5.1 indicates that this equality did not hold. This discrepancy may be due to the autoxidation of catechol as evidenced by the increasing brown color of the flasks with time. The amount of catechol unaccounted for increased with increasing phenol concentrations. The growth rates in the different cases seems to be similar, and the cells died rapidly after reaching a maximum at about 8 hours for all cases. This observation suggests that a lack of substrate may have caused the death of the cells. However, the slopes of the curves in the cell death region are steeper for higher initial phenol concentration, and hence higher catechol concentrations, suggesting that accumulation of catechol may have accelerated cell death.

To investigate this phenomenon further, an experiment was conducted in which catechol was added in different amounts to flasks containing DP medium supplemented with 2.5 mM phenol. Catechol was added to the flasks before inoculating them. The concentration of catechol was varied from 0 to 0.46 mM. Otherwise, the experiment was conducted as before. Figures 5.4, 5.5 and 5.6 show the concentrations of viable cells, catechol and phenol respectively, as a function of time, for these experiments. The results indicate that the addition of 0.135 mM catechol did not affect the growth of the cell; however, higher concentrations of catechol did inhibit cell growth. Note that when there was no external catechol added, or when 0.15 mM or 0.26 mM was added, the maximum catechol concentration exceeded 0.46 mM. However, when 0.46 mM catechol was added initially, the cells did not grow, and no additional catechol was produced. This observation suggested that, through some unknown mechanism, externally added catechol is more inhibitory than catechol produced by the cells.

Model Development

The batch growth experiments showed that the growth rate was relatively unaffected by the phenol concentration in the range of 0 to 2.4 mM during the first 8 hours. After 8 hours cell death occurred. From these results, it was assumed as a first approximation that the specific growth rate was independent of the substrate concentration during the growth phase, and that substrate ran out in 8 hours, initiating cell death. The carbon substrate consisted of complex mixtures of carbon sources (yeast extract and casamino acids) whose concentrations could not be individually measured. The cells were likely to have grown on

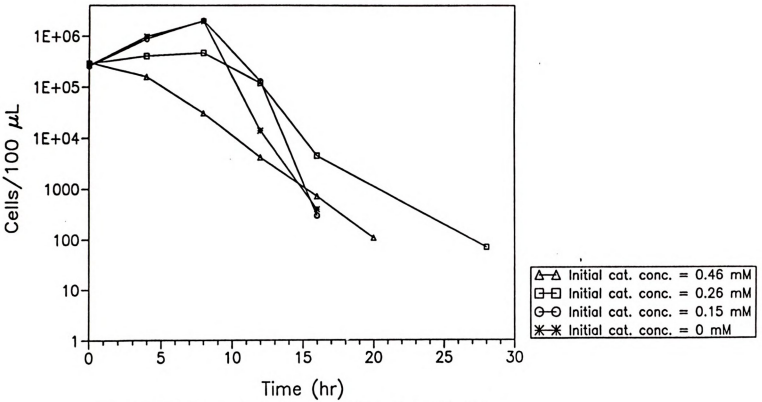


Fig 5.4 Batch growth of BR321 in DP – 2.5 mM phenol with different concentrations of catechol present initially

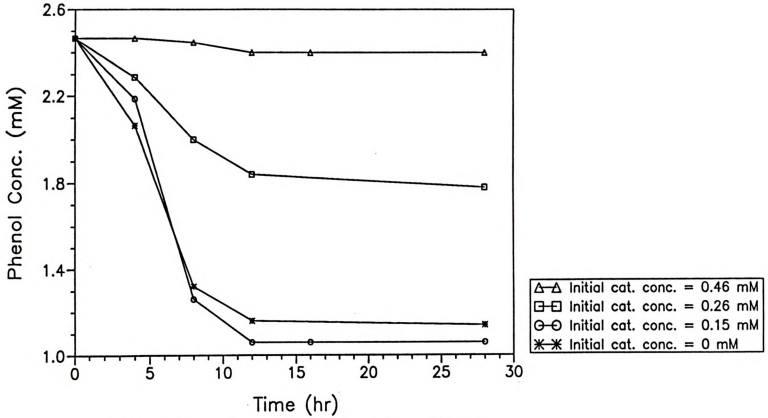


Fig. 5.5 Phenol concentration vs time when different concentrations of catechol are present initially

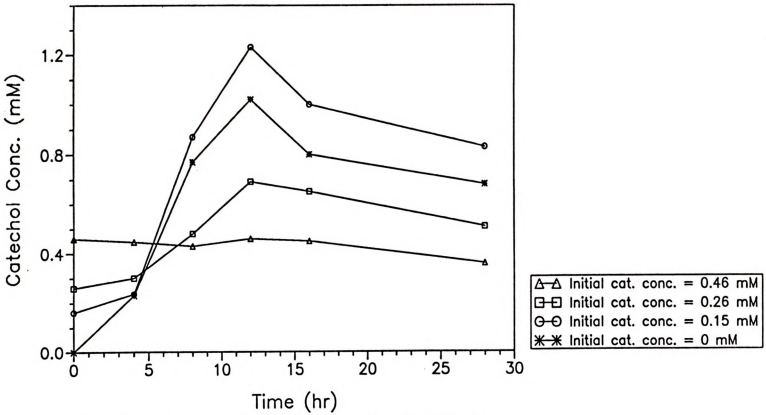


Fig. 5.6 Catechol concentration vs time when different concentrations of catechol are present initially

more preferable substrates first and others later. However, as a first approximation, all substrates were lumped together, for model development purposes.

As seen in Fig. 5.4, the cell death rates seemed to increase with increasing catechol concentration. Hence, catechol was assumed to be toxic to the cells.

The model consists of two sets of equations: one for the growth phase and another for the death phase. Uptake of both phenol and the critical carbon substrate, as well as catechol production were assumed to occur only during the growth phase. The specific growth and death rates were related by a Dirac Delta type term, whereby, when the substrate concentration reaches zero, specific growth rate is zero, and as long as substrate concentration is greater than zero the specific death rate is zero. The model assumptions are stated below.

Model Assumptions

1) The specific growth rate and specific phenol degradation rate are constant, when substrate is present in the reactor.

2) When the substrate is completely consumed, phenol uptake and cell growth cease, and cell death begins.

3) Catechol is toxic to the cells, but phenol is not toxic in the concentration range used.

4) Substrate is utilized for both cell growth and maintenance.

5) There is a molar equivalence between phenol utilized and catechol produced.

Model

Based on the above assumptions the following model was developed:

Growth phase :

$$\frac{dX}{dt} = \mu X - k_c C^n X \qquad (5.1)$$

$$\frac{dS}{dt} = -y_{s/x}\mu X - mX \qquad (5.2)$$

$$\frac{dP}{dt} = -k_p X \qquad (5.3)$$

$$\frac{dC}{dt} = -\frac{dP}{dt} \qquad (5.4)$$

Where;

μ is the specific growth rate (hr^{-1})

X is the cell concentration (cells/100 µL)

C is the catechol concentration (mM)

k_c ($mM^{-2.3}$ hr^{-1}) and n are the catechol inhibition constants

k_p is the specific phenol degradation rate (mM phenol 100 µL/hr cells)

S is the substrate concentration (g/L)

$y_{s/x}$ is the substrate yield coefficient (g substrate 100 µL/ L cells)

m is the maintenance term (g substrate 100 µL/ L hr cells)

Death Phase

$$\frac{dX}{dt} = -k_d X - k_c C^n X \qquad (5.5)$$

$$\frac{dS}{dt} = 0 \qquad (5.6)$$

$$\frac{dC}{dt} = 0 \qquad (5.7)$$

$$\frac{dP}{dt} = 0 \qquad (5.8)$$

where, k_d is the specific death rate (hr^{-1})

Evaluation of model constants

1) The specific growth rate (μ)

The cell turbidity data for the case where there was no catechol inhibition were used to tabulate μ. Integrating the cell mass balance equation for batch growth,

$$\frac{dX}{dt} = \mu X \qquad (5.9)$$

$$\int_{Xo}^{X} \frac{dX'}{X'} = \mu \int_{0}^{t} dt$$

$$ln\left(\frac{X}{X_o}\right) = \mu t \qquad (5.10)$$

The best fit of Eqn. 5.10 to Fig. 5.7 yielded $\mu = 0.32\ hr^{-1}$, with the coefficient of linearity = 0.958. When S is zero, μ is set to zero.

2) The specific death rate (k_d)

The rate of cell death in the absence of substrate was calculated by fitting a straight line to the cell concentration data shown in Fig. 5.1, using that part of the curve where cell concentration is decreasing with time. Integrating the cell death equation for batch growth,

$$\frac{dX}{dt} = -k_d X \qquad (5.11)$$

$$\int_{Xmax}^{X} \frac{dX'}{X'} = -k_d \int_0^t dt'$$

$$ln\left(\frac{X}{X_{max}}\right) = -k_d t \qquad (5.12)$$

The best fit of Eqn. 5.12 to Fig. 5.8 yielded $k_d = -0.30\ hr^{-1}$ with a correlation of linearity of 0.995. When S is greater than zero, k_d is set to zero.

3) The catechol inhibition constants (k_c and n)

The difference in the death rates, shown by the negative slopes in Fig. 5.9, is attributed to the different levels of catechol. A log plot of the death rates vs catechol concentration in the reactor, as shown in Fig. 5.10 yielded values for the constants k_c (slope) and n (antilog of the intercept) from Eqn. 5.1 as follows

$k_c = 0.63$ (catechol concentration is in mM)

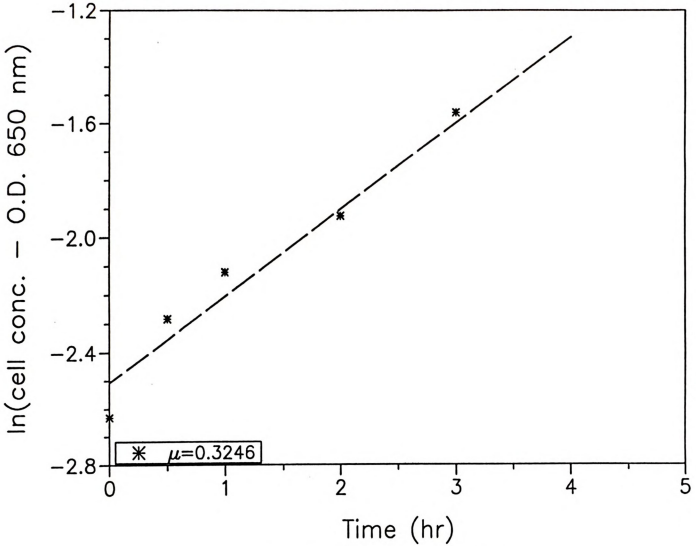


Fig. 5.7 Determination of μ

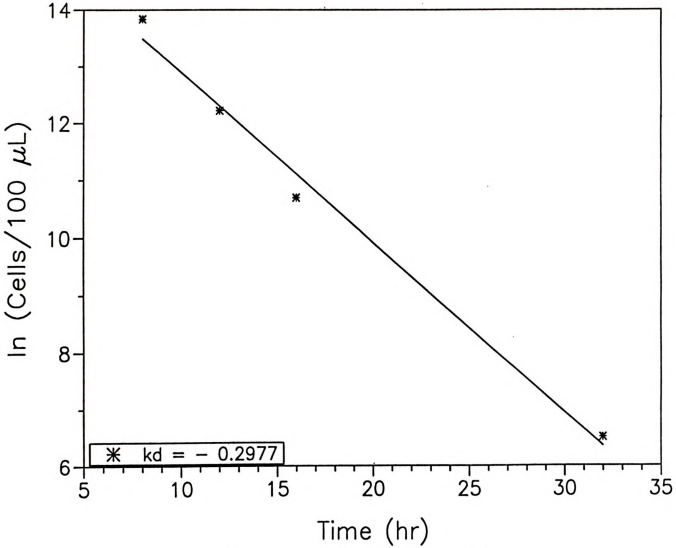


Fig. 5.8 Determination of k_d

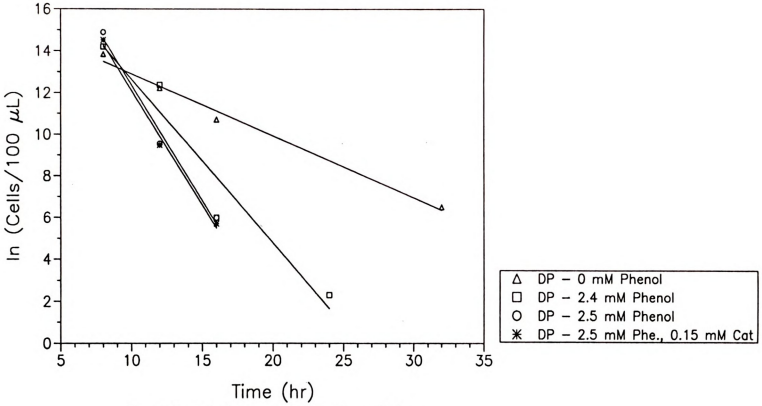


Fig. 5.9 Cell death as a function of time for varying phenol concentrations

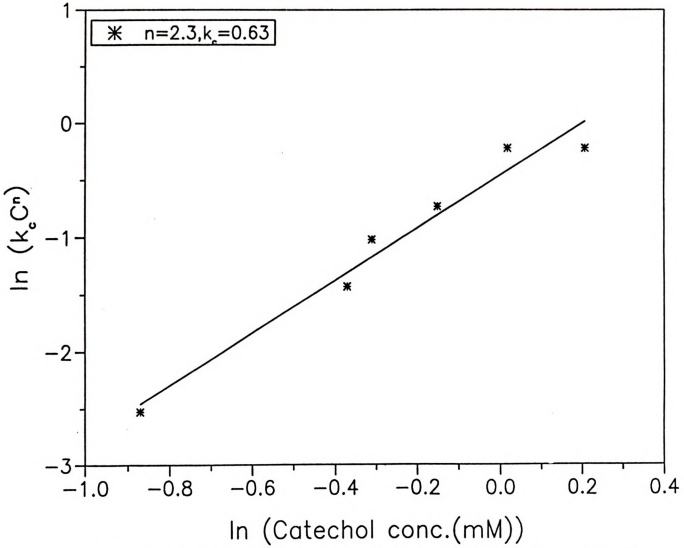


Fig. 5.10 Determination of catechol inhibition constants

n = 2.3

The coefficient of linearity was 0.981

4) The substrate yield coefficient ($Y_{s/x}$)

The value of $Y_{s/x}$ was evaluated as the change in substrate concentration divided by the corresponding change in cell concentration. The value was calculated from the data in Fig. 5.1, using the initial time and the time of maximum cell concentration. The value of ΔS was taken to be S_0, the initial substrate concentration, because, when the cell concentration peaked and cell death began, the substrate was assumed to have run out (S=0).

$Y_{s/x}$ was calculated to be 2.4 x 10^{-7} (g substrate 100 µL/ L cells).

5) The specific phenol degradation coefficient (k_p)

The value of k_p was evaluated as the change in phenol concentration divided by the corresponding change in cell concentration. The value was tabulated from the data in Fig. 5.5.

k_p was calculated to be 3 x 10^{-7} (mM phenol 100 µL/hr cells).

As seen by the coefficients of linearity of the lines, shown in Figs. 5.7, 5.8 and 5.10, the data were well represented by a linear relationship. These constants were incorporated into the model, which was then solved using the IMSL subroutine 'IVPRK', which uses a combination of the 5th and 6th order Runge-Kutta-Verner method to solve the simultaneous differential equations. The IMSL subroutine

also allows the use of very small step sizes (as small as 1×10^{-4} hrs).

Model Results

Figs. 5.11-5.14 show the model predictions as compared to the experimental data for batch growth at different phenol concentrations. Quantitative agreement between the model predictions and the data is good for cell growth and phenol degradation curves. However, the catechol concentration is overpredicted in the model, probably because it does not account for the autoxidation of catechol.

The model was applied to the experiment where catechol was added externally (Figs. 5.15-5.17). Here the model predicts that the final catechol concentration should not be substantially affected by initial catechol concentration. The actual data, however, show that catechol production was strongly repressed, when more than 0.135 mM catechol was present initially. The model also predicts considerable cell growth even when 0.46 mM catechol was present initially, but in reality, this concentration totally repressed growth. The model, therefore, does not accurately predict the results of the catechol-challenge experiment.

Discussion

As seen in Figs. 5.11-5.14, the model does a reasonable job of predicting the growth rate and phenol conversion for different initial phenol concentrations. The model assumes a molar equivalence between phenol utilized and catechol produced and does not take into account the catechol lost to autoxidation. However, catechol loss via autoxidation is significant, as evidenced by the increasing brown

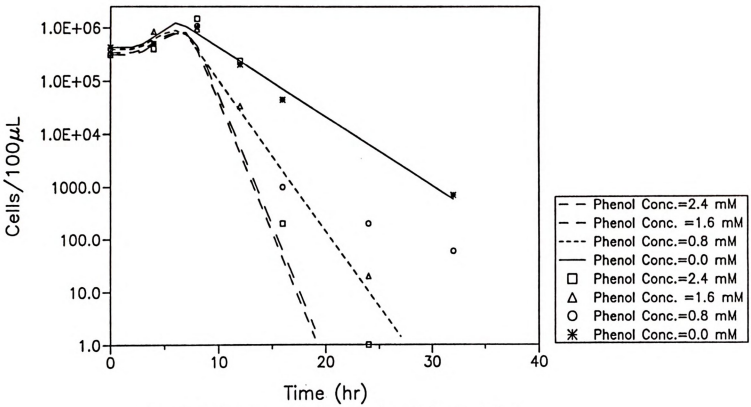


Fig. 5.11 Cell concentration vs time in batch culture

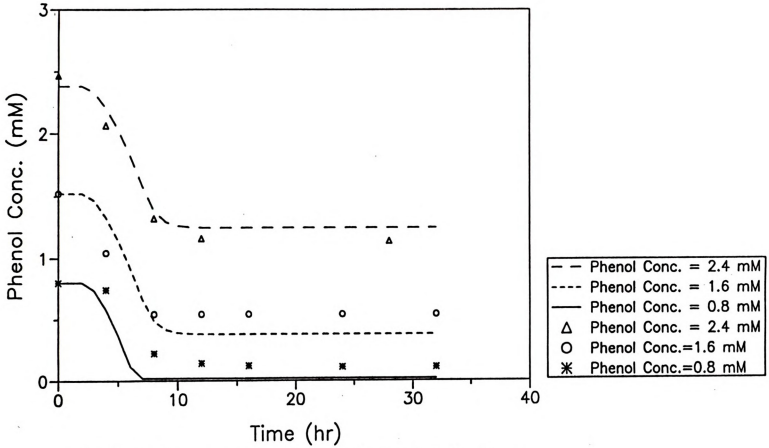


Fig 5.12 Phenol concentration vs time in batch culture

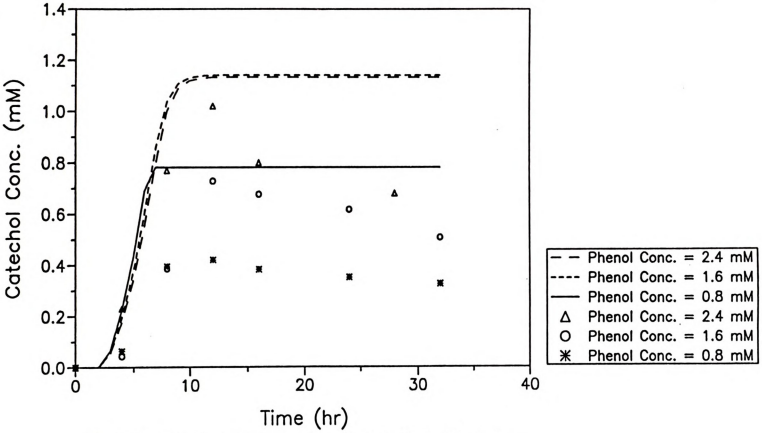


Fig 5.13 Catechol concentration vs time in batch culture

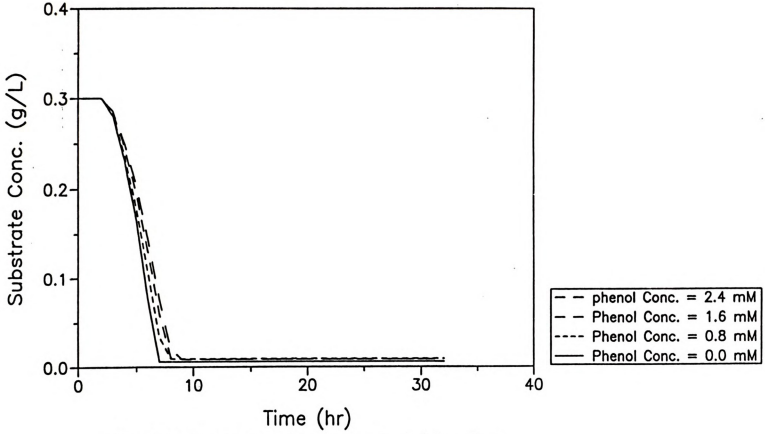


Fig. 5.14 Substrate conc. vs time in batch culture

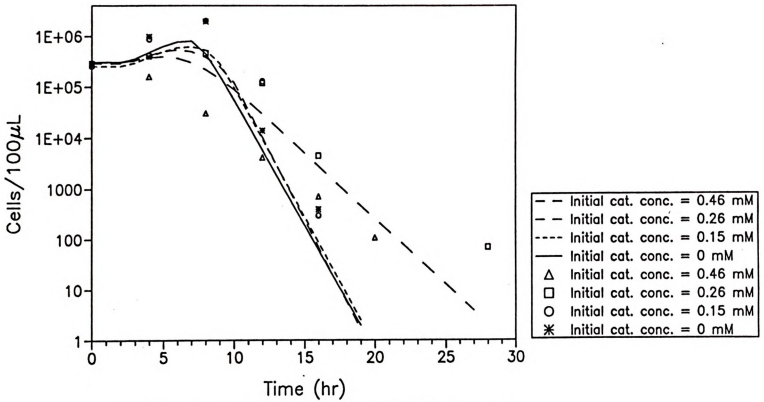


Fig. 5.15 Batch growth of BR321 in DP−2.5 mM phenol with different concentrations of catechol present initially

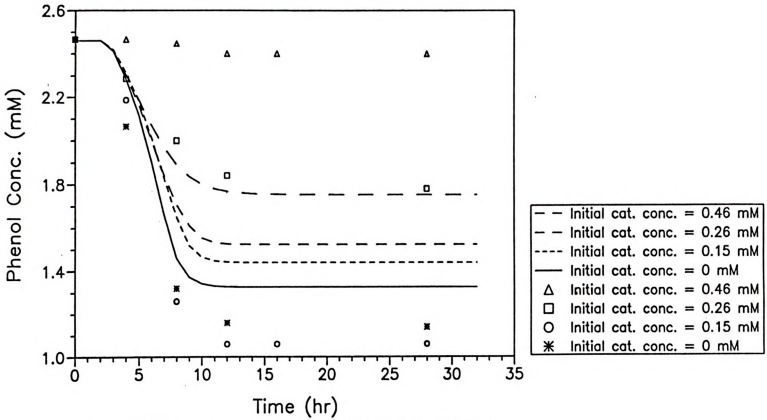


Fig 5.16 Phenol concentration vs time when different concentrations of catechol are present initially

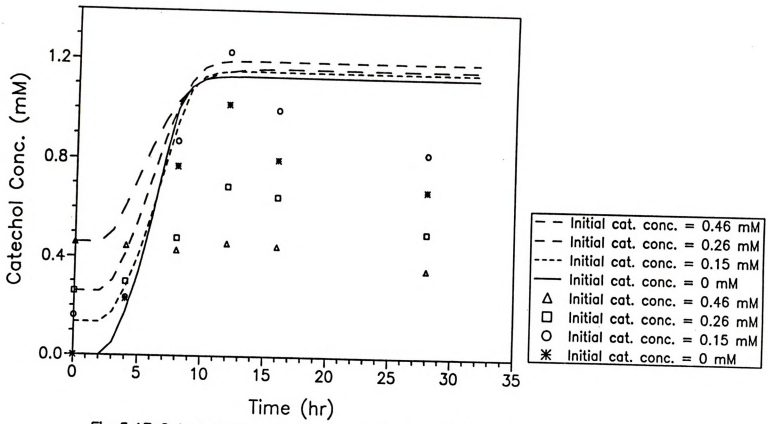


Fig 5.17 Catechol concentration vs time when different concentrations of catechol are present initially

coloration of the flasks with time. Hence, the model overpredicts the catechol concentration. Also, some of the catechol could be present within the cell and may come out as cell death occurs. This catechol would not be measured, and the data shown would not account for it. The model, however, assumes that all the catechol diffuses out as soon as it is produced. This could be another reason, although not as significant, why the model overpredicts the catechol concentration.

The disagreement between the model and the data in the catechol-challenge experiments suggests that the catechol added externally exerts a stronger inhibitory effect than catechol produced by the cells. The catechol concentrations added were much lower (maximum added concentration = 0.46 mM) than the maximum concentrations achieved (1 mM) when no catechol was added. A likely explanation for this trend is that added catechol has a longer residence time in the reactor, and hence oxidizes to a greater extent than the catechol produced by the cells. The oxidation products may be more toxic than the catechol itself. Also, added catechol is present during the growth phase, and this catechol may have a toxic effect on the enzyme induction; it may repress either enzyme production or enzyme activity. Catechol added externally may also induce a lag phase from which the cells might not recover.

Catechol produced by the cells also oxidizes. However, the majority of the catechol is produced late in the growth cycle, even as substrate runs out. By the time this catechol oxidizes, cell death due to lack of substrate would have already begun.

The experimental data, as seen in Figs. 5.4 and 5.11, show that when 0.135 mM of catechol was added, the growth kinetics were not

affected. This is significant because it shows that catechol concentrations in this range do not affect growth. Continuous catechol removal methods could conceivably be used to keep the concentration below this level during the fermentation.

One of the major difficulties of this research was that the substrate concentration could not be measured directly during the fermentation. A better understanding of this process would be possible if the substrate concentration could be measured and if studies on the enzymatic reactions were carried out. This model, however, seems to explain the kinetics of batch growth fairly well, and the fact that it is simple to use is a clear advantage.

Bacillus stearothermophilus BR321 grows at a high rate on a dilute medium such as DP and converts phenol to the specialty chemical catechol. Catechol concentrations as high as 1 mM have been achieved in this process; however, both externally added catechol and catechol produced by the cells affect the kinetics of this organism. A simple model has been suggested and used to understand the kinetics of this organism, and it fits the growth and phenol-uptake data well. The ability to measure the substrate concentrations will further help in understanding the system. The high growth rates and ability to grow on dilute, inexpensive media seem to indicate that this process, in due course, may become a suitable alternative for catechol production.

CHAPTER 6

CONTINUOUS CULTURE EXPERIMENTS WITH *BACILLUS STEAROTHERMOPHILUS* BR321

Introduction

The main objective of the continuous culture experiments was to evaluate the feasibility of this process for making catechol continuously and to understand the growth kinetics of BR321 in continuous culture. As seen in the previous chapters, BR321 grows in DP medium supplemented with phenol and accumulates catechol throughout the growth phase. Continuous production of catechol could make this process more industrially viable, so it was decided to investigate the growth kinetics and catechol production in continuous culture.

Materials and Methods

The experimental setup and procedure were similar to the one in Chapter 4, except catechol concentration was monitored. The turbidity assay, which was used as the indicator for steady state in the experiments with BR219, could not be used in this experiment since oxidized catechol gives the broth a brownish color, thus masking the turbidity. Also, sterility was more important in this set of experiments than with BR219, since lower phenol concentrations were involved. All experiments were done at a dilution rate of 0.12 hr^{-1}. Catechol was assayed periodically, and it served as the indicator for reaching steady

state. At steady state the cell viability and dry weight were measured.

Results

Cell concentration profiles leading to steady states are plotted in Figs. 6.1-6.4. The steady state cell dry weight and viability are plotted as a function of the initial phenol concentration in Fig. 6.5. The steady state results are summarized in Table 6.1. The cell dry weight seemed to remain constant with initial phenol concentration; however, the results have high standard deviations as seen in Fig. 6.5. The steady state catechol concentration is plotted as a function of the feed phenol concentration in Fig. 6.6. When the feed phenol concentration was changed to 0.36 mM from 0.24 mM, the catechol concentration began to oscillate (Fig. 6.7). This experiment was run for more than 50 residence times, and in this period the catechol concentration went through three cycles. Some interesting characteristics were observed during this experiment. The period of the oscillations increased from one cycle to the next. Between cycles there was a lag period which seemed to increase from one cycle to the next, i.e. 10 hours between the first and second cycles to about 100 hours between the second and third cycles. The feed carboy had to be changed more than ten times during the course of this experiment, and these changes might have caused small perturbations in the feed concentration. The maximum steady state catechol concentration achieved was 0.08 mM. The reactor liquid turned increasingly brown as the feed phenol concentration was increased, suggesting increasing concentrations of catechol oxidation products. Autoxidation of catechol is also suggested by the results given in Table 6.1, where the moles of phenol consumed are greater

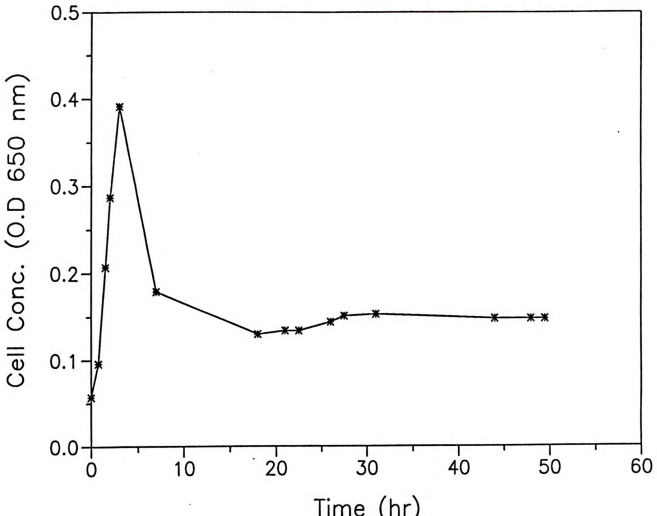


Fig. 6.1 Unsteady state growth of BR321 with initial phenol concentration = 0.0 mM

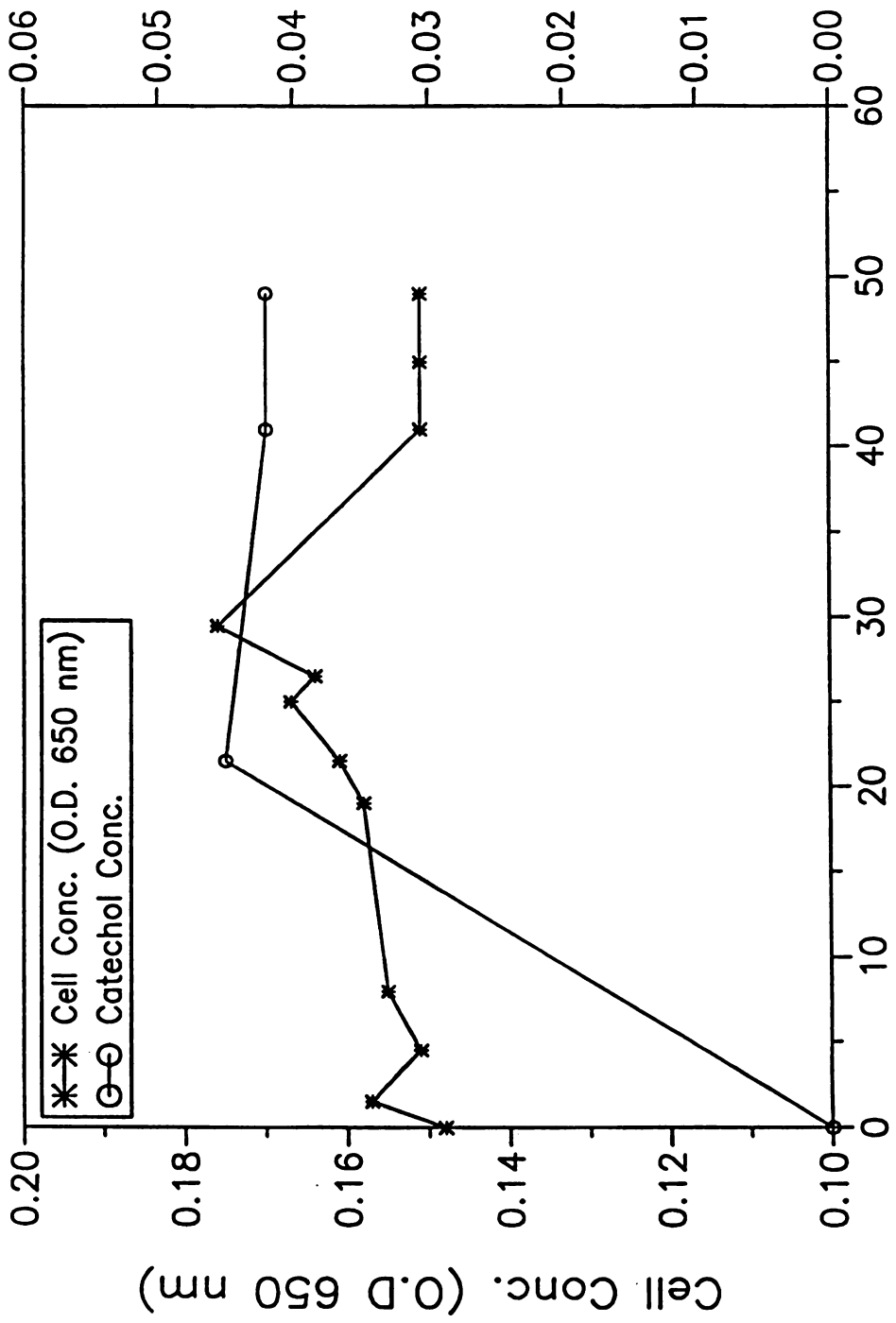


Fig. 6.2 Unsteady state growth of BR321 with initial phenol concnetration = 0.065 mM

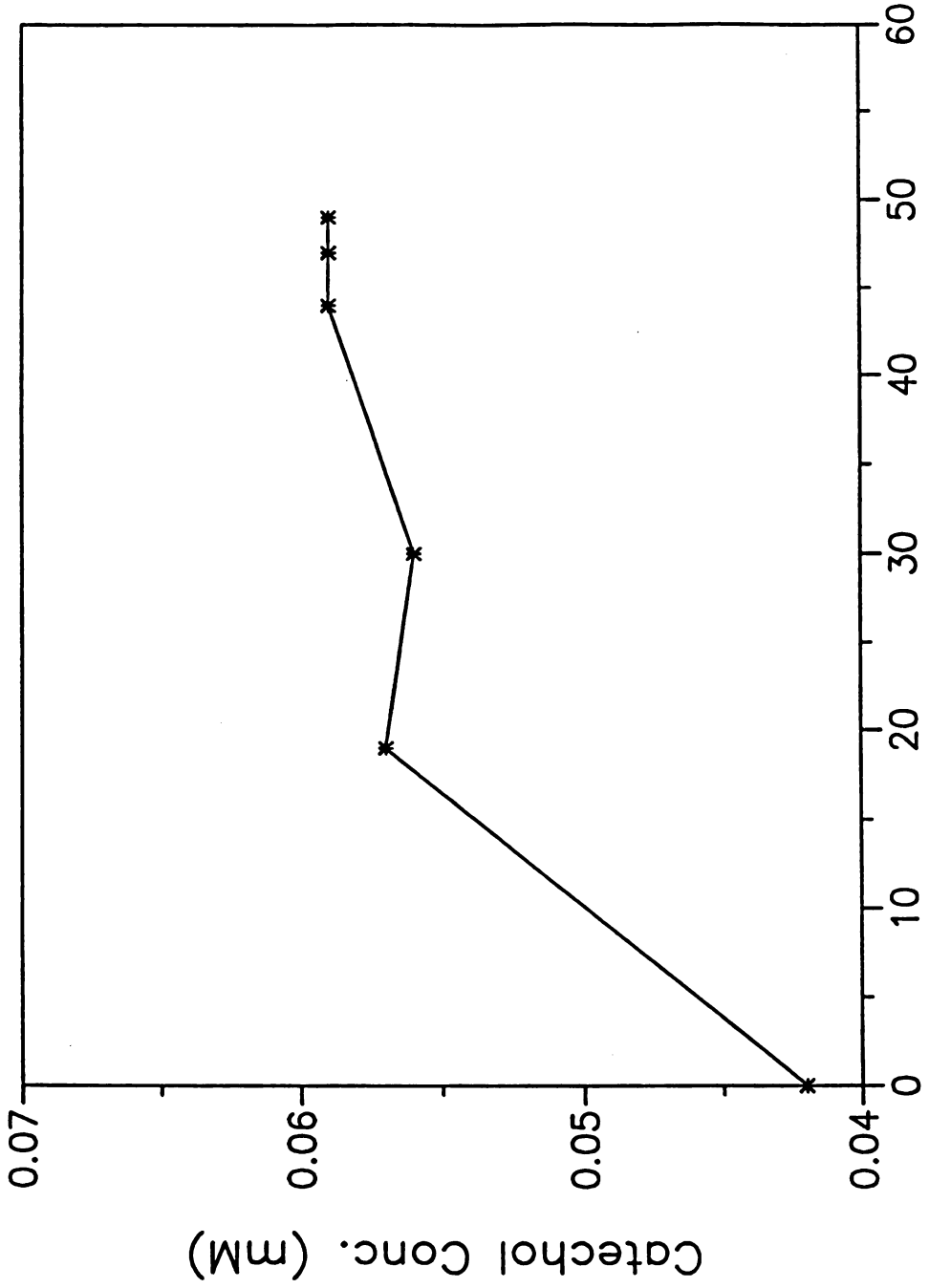


Fig. 6.3 Unsteady state growth of BR321 with initial phenol concentration = 0. 123 mM

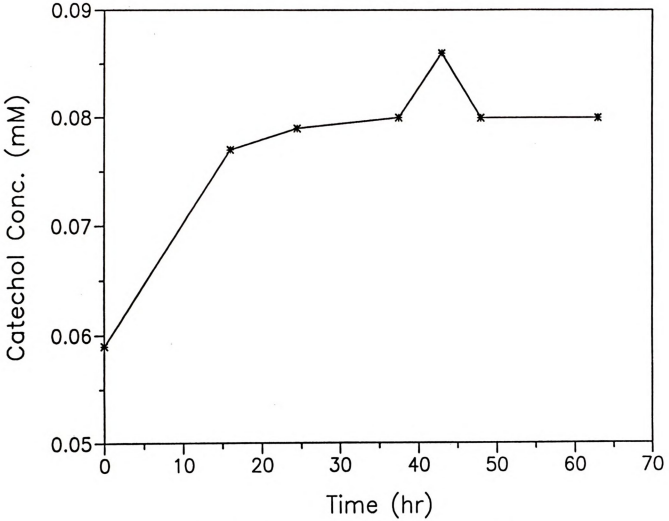


Fig. 6.4 Unsteady state growth of BR321 with initial phenol concentration = 0.239 mM

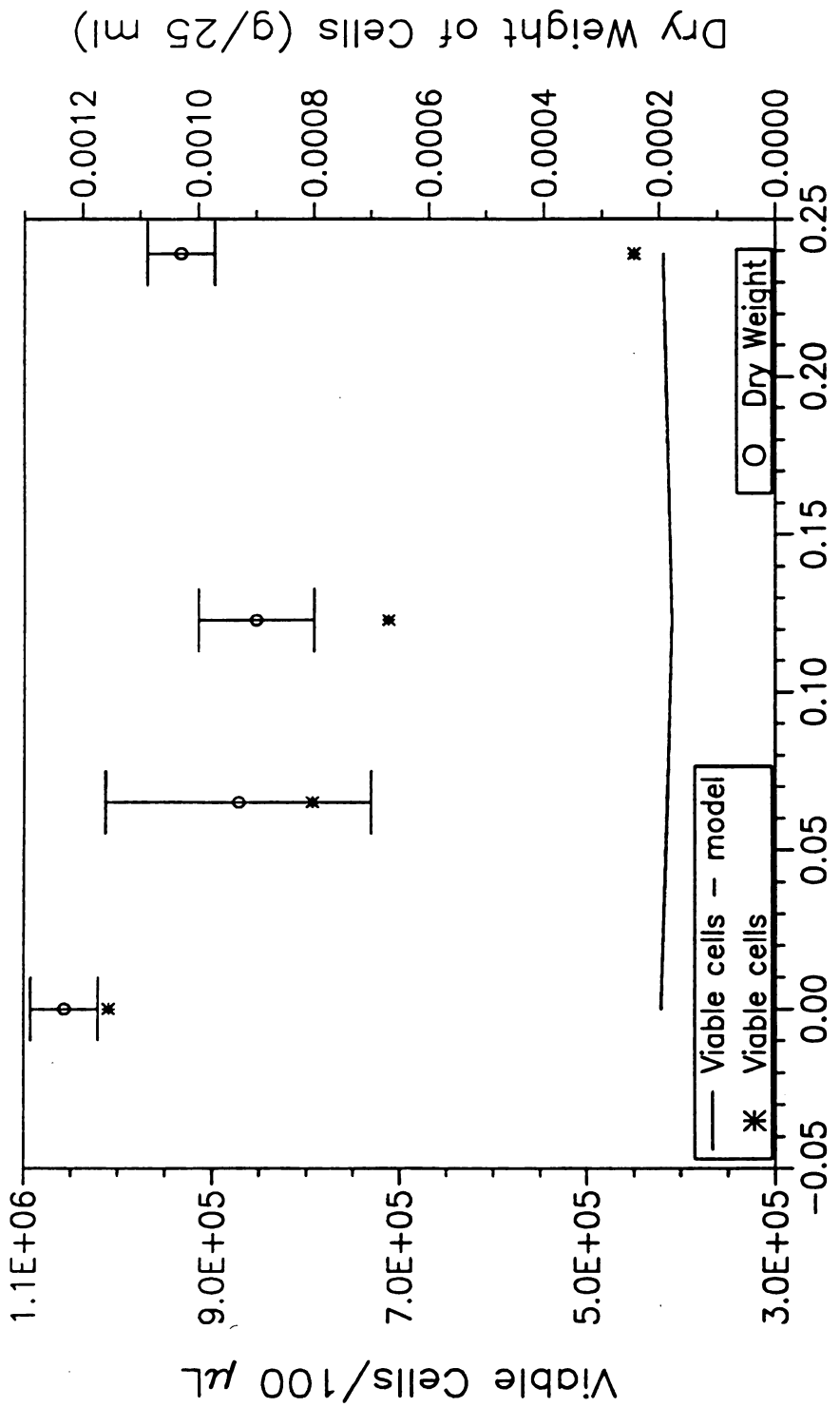


Fig. 6.5 Steady state cell concentration vs initial phenol concentration in continuous culture with BR321

Table 6.1: Summary of steady state results from continuous culture with BR321

Initial Phenol Conc. (mM)	Cell Dry Wt. (g/25 mL)	Viable Cells (Colonies/100 µL)	Final Phenol Concentration (mM)	Catechol Produced (mM)
0.0	0.00123	1.01×10^6	0.0	0.0
0.065	0.00093	7.94×10^5	0.016	0.042
0.123	0.0009	7.12×10^5	0.034	0.0621
0.239	0.00103	4.5×10^5	0.545	0.0805
0.364	----------did not reach steady state----------			

All experiments were conducted at a dilution rate of 0.12

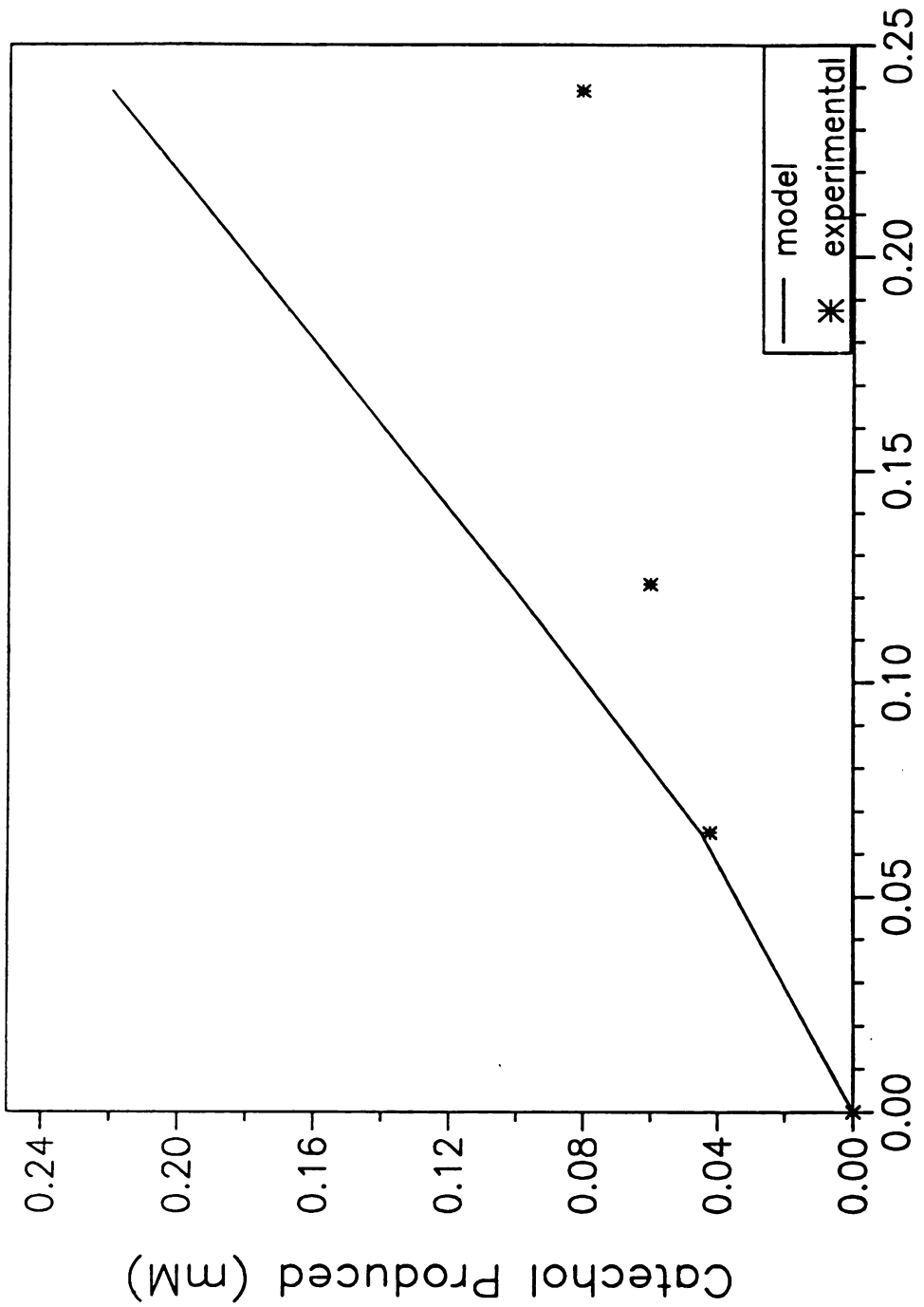


Fig. 6.6 Catechol concentration vs initial phenol concentration in continuous culture with BR321

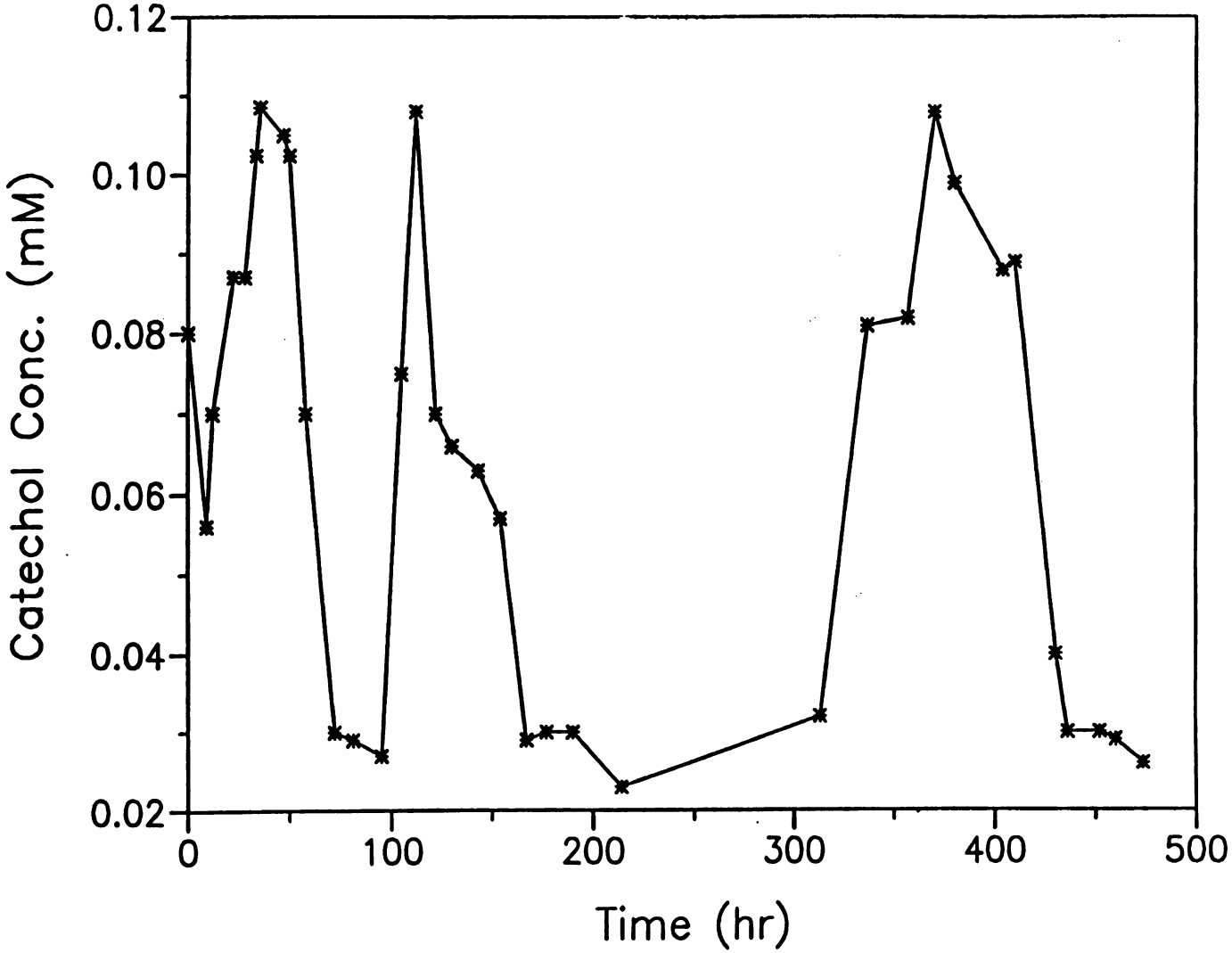


Fig. 6.7 Unsteady State growth of BR321 with initial phenol concentration = 0.36 mM

than the moles of catechol produced.

Model

The model, described in Chapter 5, was extended to the continuous process by including a flow term in each differential equation.

Growth Phase

$$\frac{dX}{dt} = \mu X - k_c C^n X + DX_f - DX \qquad (6.1)$$

$$\frac{dS}{dt} = -y_{s/x}\mu X - mX + DS_f - DS \qquad (6.2)$$

$$\frac{dP}{dt} = -k_p X + DP_f - DP \qquad (6.3)$$

$$\frac{dC}{dt} = -DC + k_p X \qquad (6.4)$$

D is the dilution rate (hr^{-1}), the subscript f refers to the feed, and all other symbols have the same meaning as in Chapter 5.

Death Phase

$$\frac{dX}{dt} = -k_d X - k_c C^n X + DX_f - DX \qquad (6.5)$$

$$\frac{dS}{dt} = DS_f - DS \qquad (6.6)$$

$$\frac{dP}{dt} = DP_f - DP \tag{6.7}$$

$$\frac{dC}{dt} = -DC \tag{6.8}$$

Discussion

The model predictions for the cell and catechol concentrations are shown in Figs. 6.5 and 6.6 respectively. The model predicts that the substrate concentration approaches zero at steady state. The cell concentration predicted by the model is lower than the experimental value. This error may be because the modeling constants were evaluated from batch-growth experiments in Erlenmeyer flasks, while the continuous experiments were conducted in a New Brunswick Bioflo II fermentor, which has better control, mixing properties and aeration. Thus, the constants used in the model might not be appropriate for the continuous culture studies. The catechol concentrations predicted by the model are higher than the experimental values, due in part to autoxidation of catechol, which is not taken into account in the model. Also, the catechol concentration remains constant at the steady state value rather than peaking late in the experiment, as in the batch experiments. Long term exposure to catechol was found to strongly inhibit the catechol production kinetics, as discussed in Chapter 5. The catechol concentrations obtained in these experiments were low compared to those obtained in the batch experiments. The model predicts that such low concentrations should not affect the viability of the cells. However, the catechol oxidation by-products may be more toxic to the cells than the catechol itself. This hypothesis is consistent

with the decreasing cell viability with increasing feed phenol concentrations i.e. increasing product catechol concentrations. Catechol is colorless, but, as the experiment progressed, the reactor became slightly brown owing to catechol oxidation. This brown color became more evident as the product concentration increased in the reactor.

The biochemistry of this fermentation is complicated. The difficulty in measuring substrate consumption makes the modeling of this system harder. The mathematical model can predict the kinetics of this organism in batch culture when catechol is not present initially in the reactor. However, it fails to accurately predict continuous culture data. It underpredicts the cell concentration at low feed phenol concentrations and does not predict decreasing cell viability with increasing feed phenol concentration. The model also predicts steady catechol production when the feed phenol concentration was raised to 0.36 mM, while the actual data show oscillatory dynamics, as seen in Fig. 6.3. This instability is thought to be due to the inhibitory effects of catechol and its oxidation products. Understanding the effects of catechol oxidation on the cells will help in modeling this system better.

Figs. 6.1 and 6.2 show that continuous catechol production can be achieved at concentrations up to 0.08 mM using 0.24 mM phenol in the feed. Thus, steady catechol production in a continuous fermentor has been demonstrated experimentally. Continuous production of catechol would be advantageous for large scale industrial production.

CHAPTER 7
CONCLUSIONS AND RECOMMENDATIONS

Kinetic studies on two strains of *Bacillus stearothermophilus* i.e. BR219 and BR321 were carried out. *Bacillus stearothermophilus* BR219 can grow on high phenol concentrations (10 mM) at 55°C at high growth rates (1.3 hr^{-1}). The results of continuous culture experiments show that because of its high specific growth rate, extreme phenol tolerance, and potential unimportance of pH control and sterile conditions, this strain may be well suited for use in bioreactors to degrade phenol. *Bacillus stearothermophilus* BR321, a mutant of BR219, can convert phenol to the specialty chemical catechol while growing on a dilute medium like DP. Thus, it is well suited for continuous catechol production. A summary of important results obtained during the course of this research is given below.

Summary of Results

In continuous culture, *Bacillus stearothermophilus* BR219 achieved high specific growth rates (up to 1.3 hr^{-1}) on a dilute medium (DP) supplemented with 10 mM phenol. Steady states were achieved at dilution rates from 0.25 hr^{-1} to 1.3 hr^{-1}. Automatic pH control was not required, and microbial contamination did not become a problem, even after loss of aseptic conditions. The specific phenol degradation rate was relatively unaffected by the growth rate, varying only forty

five percent over a five fold increase in the growth rate.

In batch culture, *Bacillus stearothermophilus* BR321 grew rapidly (0.3 hr^{-1}) on a dilute medium (DP) while converting phenol to catechol. Catechol concentrations as high as 1 mM were achieved using 2.4 mM phenol feed. Cell death began immediately following the growth phase, presumably due to both a lack of substrate and catechol toxicity. Catechol autoxidation products may also affect the growth kinetics of this organism. A mathematical model was developed to explain the batch kinetics of this organism, and it included cell death due to lack of substrate and catechol toxicity. The model fit the growth and phenol-uptake data well.

In continuous culture experiments at a dilution rate of 0.12 hr^{-1}, BR321 produced catechol for phenol concentrations in the feed up to 0.24 mM. Above this concentration oscillatory dynamics were observed.

Further Studies

This research demonstrates the ability to biologically produce catechol from phenol. However, because catechol is more toxic than phenol, research is needed on the separation of catechol as it is formed in batch and continuous processes. The economic feasibility of this process also needs to be evaluated in detail. Scale-up studies would help in developing this process further. Kinetic studies on the autoxidation of catechol and its effect on growth are needed to improve the existing model and possibly increase reactor performance.

This research has given important insights in the potential use of *Bacillus stearothermophilus* for catechol production on a large scale. Future work may lead to a full scale commercial process.

APPENDIX

```
C------ Ramkumar Subramanian ------------------------------------------------
C------ ChE 899 Masters Thesis Research ---------------------------------------
C------ This program solves the two differential equations using IMSL ----------
C------ subroutine IVPRK (by the 5th order Runge - Kutta Method) and  ----------
C------ evaluates the values of various parameters ----------------------------
C
        INTEGER XPARM,NEQ
        PARAMETER (MXPARM=50,NEQ=4)
        COMMON KP,YSX,S
        INTEGER IDO,ISTEP
        REAL FCN,PARAM(MXPARM),T,TEND,TOL,Y(NEQ),KP,YSX,S
        EXTERNAL FCN,IVPRK,SSET
C-------------------------------------------------------------------------------
        CHARACTER RFILE*80
        TYPE'(X,A,$)','ENTER THE FILE NAME FOR RESULTS :'
        ACCEPT'(A)',RFILE
        OPEN(UNIT=2,NAME=RFILE,TYPE='NEW')

C------ Nomenclature ----------------------------------------------------------

C       Y(1) = Cell Concentration (cells/100 micro L)
C       Y(2) = substrate Concentration (g/L)
C       Y(3) = Phenol Concentration (mM)
C       Y(4) = Catechol Concentration (mM)
C       KP = 3e-7 = Specific Phenol Degradation Rate
C       YSX = 2.4e-7 = Substrate Yield Coefficient
C       S = 0.05*YSX = Maintenance Term
C       Note : Only in the program I use S as the maintenance term
C       MU = 0.3 = Specific Growth Rate
C       KD = 0.3 = Specific Death Rate
C       N and KC are the Catechol Inhibition constants = 2.3 and 0.64 resp.

C------ Input initial conditions ----------------------------------------------
        Y(2)=0.3
        Write (*,*) ' T,X0,PHE,CAT,KP,YSX,S'
        READ (*,*) T,Y(1),Y(3),Y(4),KP,YSX,S
C-------Input data -----------------------------------------------------------
        TOL=0.1

C------ Setting param to default ----------------------------------------------
        CALL SSET(MXPARM,0,PARAM,1)
C------ Selecting values for PARAM(1,10) --------------------------------------
        PARAM(1) =1.E-04
        PARAM(4)=1.E4
        PARAM(10)=1.
C-------------------------------------------------------------------------------
        IDO=1
        WRITE(2,*)'     Time         [X]          [S]          [P]          [C]'
        WRITE(2,*)   T,Y
        DO 50 ISTEP=1,32
        TEND=FLOAT(ISTEP)

        CALL IVPRK(IDO,NEQ,FCN,T,TEND,TOL,PARAM,Y)
        WRITE(2,*) T,Y
50      CONTINUE


C-------------------------------------------------------------------------------
        IDO=3
        CALL IVPRK(IDO,NEQ,FCN,T,TEND,TOL,PARAM,Y)
        END
C------ Subroutine FCN begins now ---------------------------------------------
        SUBROUTINE FCN(NEQ,T,Y,YPRIME)
        COMMON KP,YSX,S
        INTEGER NEQ
        REAL    Y(NEQ),YPRIME(NEQ),MU,KC,YSX,KP,N,KD,S
```

```
C-------------------------------------------------------------------------------
      MU=0.3
      KC=0.63
      N=2.3
      KD=0.3

      IF(Y(2).GT.0.05) THEN
      IF(Y(4).LE.0.0) Y(4)=0
      YPRIME(1)=MU*Y(1)-(KC)*(Y(4)**N)*Y(1)
      ELSE
      YPRIME(1)=(-KC)*(Y(4)**N)*Y(1)-KD*Y(1)
      END IF

      IF(T.LE.2.5) YPRIME(1)=0.0

      YPRIME(2)=(-YSX)*(MU*Y(1)+S*Y(1))
      IF(T.LE.2.5 .or. Y(2).LT.0.01) YPRIME(2)=0.0

      YPRIME(3)=(-KP)*Y(1)
      YPRIME(4)=KP*Y(1)

      IF(T.LE.2.5.OR.Y(3).LE.0.02) YPRIME(3)=0.0
      IF(T.LE.2.5.OR.Y(3).LE.0.02) YPRIME(4)=0

      IF(Y(4).LE.0.0) Y(4)=0.0

      RETURN
      END
C-------------------------------------------------------------------------------
```

```
C-------Ramkumar Subramanian-----------------------------------------------------
C-------ChE 899 Masters thesis-----------------------------------------------------
C-------Solution to Continuous model-----------------------------------------------

C------ This program solves the two differential equations using IMSL ----------
C------ subroutine IVPRK (by the 5th order Runge - Kutta Method) and  ----------
C------ evaluates the values of various parameters ----------------------------
C
        INTEGER XPARM,NEQ
        PARAMETER (MXPARM=50,NEQ=4)
        COMMON P,KP,YSX,S
        INTEGER IDO,ISTEP
        REAL FCN,PARAM(MXPARM),T,TEND,TOL,Y(NEQ),P,YSX,KP,S
        EXTERNAL FCN,IVPRK,SSET
C-------------------------------------------------------------------------------
        CHARACTER RFILE*80
        TYPE'(X,A,$)','ENTER THE FILE NAME FOR RESULTS :'
        ACCEPT'(A)',RFILE
        OPEN(UNIT=2,NAME=RFILE,TYPE='NEW')

C------ Nomenclature ----------------------------------------------------------

C       D = 0.12 = Dilution Rate (1/hr)
C       All other parameters are defined in the batch model

C------ Input initial conditions ----------------------------------------------
        Y(2)=0.3
        Write (*,*) ' T,X0,PHE,CAT,TIME LIMIT,STEP,KP,YSX,S'
        READ (*,*) T,Y(1),P,Y(4),LIMIT,LIMITS,KP,YSX,S
        Y(3)=P
C-------Input data -----------------------------------------------------------
        TOL=0.1

C------ Setting param to default ----------------------------------------------
        CALL SSET(MXPARM,0,PARAM,1)
C------ Selecting values for PARAM(1,10) --------------------------------------
        PARAM(1) =1.E-04
        PARAM(4)=1.E4
        PARAM(10)=1.
C-------------------------------------------------------------------------------
        IDO=1
        WRITE(2,*)'     Time        [X]          [S]          [P]          [C]'
        WRITE(2,*)   T,Y
        DO 50 ISTEP=1,LIMIT,LIMITS
        TEND=FLOAT(ISTEP)

        CALL IVPRK(IDO,NEQ,FCN,T,TEND,TOL,PARAM,Y)
        WRITE(2,*) T,Y
50      CONTINUE


C-------------------------------------------------------------------------------
        IDO=3
        CALL IVPRK(IDO,NEQ,FCN,T,TEND,TOL,PARAM,Y)
        END
C------ Subroutine FCN begins now ---------------------------------------------
        SUBROUTINE FCN(NEQ,T,Y,YPRIME)
        COMMON P,KP,YSX,S
        INTEGER NEQ
        REAL    Y(NEQ),YPRIME(NEQ),MU,KC,KP,KD,D,P,YSX,S
C-------------------------------------------------------------------------------
        MU=0.3
        KC=0.63
        N=2.3

        D=0.12
```

```
      KD=0.3

      IF(Y(2).GT.0.05) THEN
      YPRIME(1)=MU*Y(1)-D*Y(1)-(KC)*(Y(4)**N)*Y(1)
      ELSE
      YPRIME(1)=(-KC)*(Y(4)**N)*Y(1)-KD*Y(1)-D*Y(1)
      END IF

      YPRIME(2)=(-YSX)*(MU*Y(1)+S*Y(1)-D*(Y1))-D*Y(2)+D*0.3

      IF(Y(2).LE.0.0001) YPRIME(2)=0.3*D-D*Y(2)
      IF(Y(2).GT.0.05.AND.Y(3).GT.0.02) THEN
      YPRIME(3)=(-KP)*Y(1)-D*Y(3)+P*D
      YPRIME(4)=KP*Y(1)-D*Y(4)
      ELSE
      YPRIME(3)=P*D-D*Y(3)
      YPRIME(4)=D*Y(4)*(-1)
      END IF


      IF(Y(3).LT.0.0) Y(3)=0.0
      IF(Y(4).LT.0.0) Y(4)=0.0

      RETURN
      END
C-------------------------------------------------------------------------------
```

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BIBLIOGRAPHY

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