Continuous Degradation of Phenol at Low Levels using *Pseudomonas putida* Immobilised in Calcium Alginate

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PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
Continuous Degradation of Phenol at Low Levels Using *Pseudomonas putida* Immobilized in Calcium Alginate

MSc(Hons) Thesis
UWS Macarthur

Amendments to the thesis

The following amendments attend to the comments raised by the examiners. In the cases where I have agreed with the recommendation, I have outlined the change that needs to be made. Where I have not agreed with recommendations, I have provided my argument as to why I think that changes are not necessary.

Examiner 1

Changes agreed to

Page 4; line 6  Change “process” to “processes”

Page 6; line 2  Change “......the problem of phenol degradation at low levels.” to “......the problem of degradation of low concentrations of phenol.”

Page 7; line 1 & Page 57; line 17  Change “......*Pseudomonad...” to “......*pseudomonad(s)...”

line 16  Change “......sufficient energy source available.” to “......a source of sufficient energy available.”

line 17  Change “......of phenol can only sustain a low cell population.” to “......of phenol can sustain only a low cell population.”

Page 11; line 16  Change “(Alvarez et al., 1991; Berwick, 1984; Beunink et al., 1988; Janke et al., 1988 and Yu et al., 1994).” to “(Alvarez and Vogels, 1991; Berwick, 1984; Beunink and Rehm, 1988; Yu and Welander, 1994).”


Page 17; line 7  Change “If the addition of medium is repeated continuously, by drawing out an equal volume of liquid from the culture vessel as is replaced, then there is continuous production of cells.” to “...By drawing out an equal volume of liquid as is replaced, a continuous recycling of medium takes place, causing cell production to occur continuously.”

line 19  Change “......high throughput of wastewaters....” to “......a high throughput of wastewater....”

Page 23; last line  Change “Aeration and agitation are an important factor...” to “Aeration and agitation are important factors...”
Page 23; last line Change “Aeration and agitation are an important factor...” to “Aeration and agitation are important factors...”

Page 25; line 1 Change “…Lallai et al. (1989)…” to “…Lallai and Mura (1989)”

Page 26; line 13 Change “…difficult as depth of the bead increases…” to “…difficult as the diameter of the bead…”

Page 27; line 15 Change “…during phenol degradation < 100 mg L⁻¹ by a…” to “…during degradation of < 100 mg L⁻¹ phenol by a…”

Page 32; line 10 Change “These units were necessary to show how the system was being optimised, as either one or both units…” to “These values were necessary to show how the system was being optimised, as either one or both values…”

line 17 Change “…to describe how the systems ability…” to “…to describe how the system’s ability…”

Page 38 The average bead diameter of bead produced using 25G needles were ……..

Page 49; Fig 5 & Page 62 Fig 9 Add footnote: “Timer relay (TR) which is not shown on the diagram is part of component pH C.”

Page 67; line 19 Change “…to determine what affect D has…” to “…to determine what effect D has…”

Page 78, line 5 and 17 Change “…Lallai et al. (1989)…” to “…Lallai and Mura (1989)”

Page 80; last line Change “…however the density of cells was less…” to “However, Sₜ was lowest at…”

Page 90; line 5 Change “…batches of immobilised Ps.putida were prepared under identical conditions were used.” to “…batches of immobilised Ps.putida were prepared under identical conditions.”

Page 95; line 6 Change “The only difference will be the distribution of the cell population in the reactor, except that they were differently distributed.” to “The only difference will be the distribution of the cell population in the reactor.”

Page 96; line 8 Change “…with a diameter of 2 mm.” to “…with a bead diameter of 2 mm.”

Page 107 Table 5 Insert legend: “Below is an explanation as to how the Corrected r₅ was derived.” Change “To adjust the degradation rate, the latter value is divided by 3 to make it comparable to the Sₜ of 100 mg L⁻¹ used in this study.” to “To adjust the degradation rate, Sₜ is divided by 10 to make it comparable to the Sₜ of 100 mg L⁻¹ used in this study.”


of the condensor, making up the sterile venting assembly.

Section VIII  References in this section are correct. It is the in-text referencing that is incorrect in places.
Examiner 2

Changes agreed to (header numbers are from the examiner's report)

3; Page 16 Section 4.2.1
Change “substrate inhibition.”
to “substrate limitation.”

5 d) Add w/v to all % values e.g. Page 38 Change “2%” to “2% w/v”

5 e) Page 36; Section 7.5
Change “Inoculum was produced following the same procedure in all experiments (Figure 1).”
to “Inoculum was produced following the same procedure in all experiments (Figure 1). The orbital shaker used was a New Brunswick Innova 4330 with a 25.4 mm diameter orbit.”

6 b) Page 68, Section 12.2 line 8
Change “The amount of phenol present was Se for the run....”
to “The amount of phenol present was $S_0$ for the run...”

6 c) Page 70; Fig 13
Change “...the effect of dilution rate on the degradation rate of immobilised Ps. putida.”
to “...the effect of dilution rate on the degradation rate of phenol by immobilised Ps. putida.”

6 d) Page 81
Change “Figure 15: The effect of pH on the effluent phenol concentration using immobilised Ps. putida.”
to “Figure 15: The effect of reaction pH on the effluent phenol concentration using immobilised Ps. putida.”

8 a) Insert “Leahy and Colwell” (1990) in References: Done as per first examiners request.

8 b) Page 52
Change “Palleroni (1952)”
to “Palleroni (1984)”
Done as per first examiners request.

Changes not agreed to

4 This section was meant as an overall explanation of the experimental section of the thesis, and how it all ties together; not as a strict guideline to each experiment. Therefore, full names to experiments were not given.

5 a) A description of the weighing procedure is not generally outlined in a thesis, unless it is the object of the investigation, or an unusual procedure is adopted. Otherwise it is assumed to be a standard procedure. As my method for weighing materials was neither unusual, nor the object of this study, it was not included in the thesis. pH was mentioned in the thesis in an experimental section. Makes and models of general equipment are not added in a thesis, unless they are unusual or specifically designed pieces of equipment.

5b) Company names for media are sufficient unless the media is made by separately bought components. Media made this way was listed.

5c) A description of sterilisation techniques was given. This information should be sufficient as I did not practice any methods that were not considered routine.
Colorimetric methods are still highly used in research today, even though there are HPLC and GC techniques. The colorimetric method was used for this research as it was fast and easy, and many samples were tested at one time point. Also over a period of 4 days, sampling was performed at least every 4-6 hours, making it difficult to have access to equipment such as that suggested for such long durations. Colorimetric methods are most commonly used in the literature, making it a more acceptable method to use for comparison of results.

This could have been placed in the appendix, but it was chosen as part of the main thesis, as it was a series of experiments performed to develop an assay able to determine low concentrations of phenol. It is the basis for all the results in the thesis. The materials and methods listed describe any changes made and information that was not given in the method referenced from the American Public Health Association (1992). *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, American Water Works Association and Water Environment Federation, Washington DC.

A separate results and discussion was not given as the basis of this experiment was only to conclude if the method was accurate and able to be used as an assay technique.

Cell data could have been useful, but isn't performed for reasons of ease and speed. Only one reactor was available, so removal of beads would have altered the cell population during an experiment, thus affecting the results. Also removing beads was not able to be done without contaminating the system. Indirect methods would not be able to be performed for such reasons as described above.

A dissolved oxygen probe was not used as this would have required extra funding which we did not have (having purchased a reactor). This sort of data would have been helpful but it was not imperative.

Stanbury and Whitaker (1984) is located on Page 8.

Angela Mordocco
I dedicate this thesis and my work to my much loved sister Charmaine Mordocco and to my grandfather John Vassallo. I miss you both deeply, and wish you were here to see this with me.

The most important discoveries of the laws, methods, and progress in Nature have nearly always sprung from the examination of the smallest objects that she contains.

J.B. Lamarck
Statement of Authentication

The work as presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material either in whole or in part, for a degree at this or any other institution.

Angela Mordocco
Acknowledgments

I wish to thank my principal supervisor, Dr. Clem Kuek, for his advice, guidance and encouragement throughout the years, and primarily for his friendship. I also wish to thank Mr. Roger Jenkins for his help and support during the project.

My heartfelt thanks go to my Parents and Sisters for their support and endless faith in me.

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Mr. Richard Burgess for his expertise and help under dire circumstances;

The Lab Staff for their help; and

The Security Staff for their smiling faces and company at all times of the morning and night.
Abstract

Biodegradation is the breakdown of a compound by a biological organism. Over the past few decades, the biodegradation of compounds such as phenol has been researched extensively. Phenol research has shown that certain organisms are capable of utilising it as an energy source, and a variety of methods are available for its removal. Unfortunately, there is a lack of research on phenol degradation at low concentrations. The majority of research performed on phenol degradation has used concentrations above 500 mg L$^{-1}$, while phenol is highly toxic at levels below 25 mg L$^{-1}$ (Kirk-Othmar, 1982). The aim of this research was to pursue the problem of phenol degradation at below 100 mg L$^{-1}$ and develop a system able to degrade phenol at such levels. The system consisted of a bioreactor developed to run in continuous mode, using *Ps. putida* immobilised in calcium alginate. A standard method was modified to quantitatively analyze effluent phenol levels, and a medium designed to increase the longevity of calcium alginate beads in continuous culture. A continuous flow bioreactor was also designed using an overflow weir for use with immobilised cells.

Studies were performed on selected physicochemical parameters and optimisation carried out to improve phenol degradation. It was found that at 0.6 h$^{-1}$, the rate of phenol degradation was optimised to 51 mg L$^{-1}$ h$^{-1}$ and an $S_c$ of 16 mg L$^{-1}$ was achieved. Increasing the dilution rate past this point did not increase the phenol degradation rate and it was thought that this could be due to a low diffusion environment.
The system designed could be scaled up by increasing the bead volume and the dilution rate proportionally to increase the throughput of effluent. The effluent phenol level was not adversely affected and the rate of removal was increased proportionally. A maximum degradation rate of 124 mg L\(^{-1}\) h\(^{-1}\) was achieved through scaling up the reaction system.

A series of experiments were performed to optimise pH, temperature and bead diameter. From these experiments it was found that:

1. *Ps. putida* could grow effectively in the low acidic ranges and degrade phenol (pH range of 5.5 - 6.5),

2. phenol degradation was optimal between 20 - 30°C, and

3. the bead diameter should not be above 1 mm.

At dilution rates above 0.5 h\(^{-1}\), the immobilised cell system was found to be more efficient at degrading phenol than a free cell system. Based on the results obtained, immobilisation offers increased stability and increased protection for cells under extreme conditions and is able to use higher dilution rates than free cells under continuous culture.

Thus optimized, the immobilised system developed can take phenol levels from 100 mg L\(^{-1}\) down to 2.5 mg L\(^{-1}\) at a dilution rate of 0.6 h\(^{-1}\), giving a degradation rate of 58.5 mg L\(^{-1}\) h\(^{-1}\).
Abbreviations

D - Dilution rate (h⁻¹)

F - Flow rate (mL min⁻¹)

V - Reactor volume (L)

NB - Nutrient Broth

NA - Nutrient Agar

IPM - Inoculum Production Medium

IPA - Inoculum Production Agar

RM - Reaction Medium

MRM - Modified Reaction Medium

Sᵢ - Initial Phenol Concentration of the influent (always 100 mg L⁻¹)

Sₑ - Effluent Phenol Concentration (mg L⁻¹) - The concentration of phenol remaining at steady state using a given dilution rate

rₛ - Phenol Degradation Rate (mg L⁻¹ h⁻¹) - The amount of phenol utilised within one hour given a specific dilution rate and influent phenol concentration

\[ rₛ = (Sᵢ - Sₑ) \times D \]
# Table of Contents

Statement of Authentication  
Acknowledgments  
Abstract  
Abbreviations  
Table of Contents  
List of Figures  
List of Tables  
Publications

I: INTRODUCTION  
1. Phenol  
   1.1 Problems with Phenol  
   1.2 Phenol in Effluent  
2. Degradation of Phenol  
   2.1 Chemical Treatment  
   2.2 Biological Degradation  
3. The Problem - Degrading Phenol at Below 100 mg L\(^{-1}\)  
   3.1 Reasons for Researching Low Level Phenol Degradation  
   3.2 Problems to be Overcome in order to Degrade Phenol at Low Levels  
   3.3 The Broad Research Objective

II: LITERATURE REVIEW  
4. Essential Characteristics of the System Chosen for this Study on Phenol Degradation  
   4.1 Characteristics of the Microorganism  
      4.1.1 Aerobes or Anaerobes?  
      4.1.2 Pure Culture or Mixed Culture?  
      4.1.3 Bacteria, Fungi or Yeasts?  
   4.2 Options for Culture Mode  
      4.2.1 Batch Culture Mode
4.2.2 Continuous Culture Mode 17

4.3 The Choice of Immobilised Cells or Free Cells 18
  4.3.1 Advantages of Immobilised Cell Systems 19
  4.3.2 Problems with Cells Immobilised in Calcium Alginate 20
    4.3.2.1 Stability of Calcium Alginate Beads 21
    4.3.2.2 Low Mass and Gas Transfer in Calcium Alginate Beads 21

4.4 The Influence of Environmental Parameters on Continuous Degradation of Phenol at Low Levels 23
  4.4.1 Aeration and Agitation in an Immobilised Cell System 23
  4.4.2 pH of the Reaction System 24
  4.4.3 Temperature of the Reaction System 25
  4.4.4 Bead Diameter and the Effect on Phenol Degradation 26

5. Conclusion and Evaluation 27

III: EXPERIMENTAL PROGRAM 29

6. Introduction 30
  6.1 Preliminary Work and System Design 30
  6.2 Optimisation Studies 30
  6.3 Comparison Study - Free Cell Degradation Compared to Immobilised Cell Degradation of Phenol 31
  6.4 Characterisation of Bioreactor Performance 32

IV: EXPERIMENTAL METHODS 33
  7.1 Culture Maintenance and Preservation 34
    7.1.1 Culture Supply and Storage 34
    7.1.2 Short-term Culture Preservation 34
  7.2 Materials 34
  7.3 Media 35
  7.4 Sterilization 36
  7.5 Preparation of Inoculum 36
  7.6 Immobilisation of Ps. putida in Calcium Alginate 38
V: EXPERIMENTAL

8. Experiment 1: A Modified Colorimetric Method for the Determination of Phenol
   8.1 Introduction
   8.2 Materials and Methods
   8.3 Results and Discussion

9. Experiment 2: Determination of the Baseline Performance of the Continuous Immobilised Cell System used in this Study
   9.1 Introduction
   9.2 Materials and Methods
      9.2.1 Inoculum Production and Immobilisation
      9.2.2 Reaction Medium
      9.2.3 Phenol Degradation
   9.3 Results
   9.4 Discussion

10. Experiment 3: The Development of a Medium Compatible with Extended Use of Calcium-Alginate Beads in a Continuous System
    10.1 Introduction
    10.2 Materials and Methods
       10.2.1 Inoculum Production and Immobilisation
       10.2.2 Modified Reaction Medium (MRM)
       10.2.3 Phenol Degradation
    10.3 Results
    10.4 Discussion

11. Experiment 4: Baseline Performance of a Bioreactor using an Overflow Mechanism for Continuous Phenol Degradation
    11.1 Introduction
    11.2 Materials and Methods
    11.3 Results
    11.4 Discussion
12. Experiment 5: The Effect of Dilution Rate on Phenol Degradation using Immobilised *Ps. putida*  67
   12.1 Introduction  67
   12.2 Materials and Methods  68
   12.3 Results  68
   12.4 Discussion  71
13. Experiment 6: The Effect on *r_s* and *S_s* as a Result of Scaling up the Immobilised Phenol Degrading System  72
   13.1 Introduction  72
   13.2 Materials and Methods  73
   13.3 Results  74
   13.4 Discussion  76
14. Experiment 7: The Effect of pH on Phenol Degradation using Immobilised *Ps. putida*  78
   14.1 Introduction  78
   14.2 Materials and Methods  79
      14.2.1 Inoculum Production and Immobilisation  79
      14.2.2 Modified Reaction Medium  79
      14.2.3 Phenol Degradation  79
   14.3 Results  80
   14.4 Discussion  83
15. Experiment 8: The Effect of Temperature on Phenol Degradation using Immobilised *Ps. putida*  84
   15.1 Introduction  84
   15.2 Materials and Methods  84
   15.3 Results  85
   15.4 Discussion  88
16. Experiment 9: The Effect of Bead Diameter of Immobilised *Ps. putida* on Phenol Degradation  89
   16.1 Introduction  89
   16.2 Materials and Methods  90
   16.3 Results  93
16.4 Discussion

17. Experiment 10: Comparison of the Degradation Rate of Two Continuous Systems: Free Cell *Ps. putida* versus Immobilised *Ps. putida*

17.1 Introduction

17.2 Materials and Methods

17.2.1 Free Cell Continuous Culture

17.2.1.1 Inoculum Production

17.2.1.2 Modified Reaction Medium

17.2.1.3 Phenol Degradation

17.2.2 Immobilised Cell Continuous Culture

17.2.2.1 Phenol Degradation

17.3 Results

17.4 Discussion

VI: GENERAL DISCUSSION AND FURTHER WORK

18.1 Degradation of Phenol at Levels Below 100 mg L\(^{-1}\) by an Immobilised Cell System

18.2 Comparison of \(r_S\) and \(S_e\) obtained for the Immobilised System Developed to Published Results

VII: CONCLUSION

VIII: REFERENCES
List of Tables

Table 1: Inoculum Production Medium (IPM) (Hill and Robinson, 1975) 35

Table 2: Volumes and Concentrations of assay reagents for the Standard Method for phenol detection (APHA, 1992) and the Modified Assay Method for phenol detection 42

Table 3: Mean absorbance values and respective standard deviations for the Standard Method and the Modified Method 44

Table 4: The Scale up Factor and Corresponding Bead to Medium Volume ratio and Dilution rates tested 73

Table 5: Comparison of the Phenol Degrading Capability of Ps. putida Bioreactors 107
List of Figures

Figure 1: Procedure for the production of inoculum of *Ps. putida* 37

Figure 2: Procedure for the immobilisation of *Ps. putida* in calcium alginate 39

Figure 3: Comparison of the mean absorbance values for the Standard Method and the Modified Method 44

Figure 4: Bubble Column Bioreactor 1 48

Figure 5: Schematic representation of the instrumentation for bioreactor 1 49

Figure 6: The continuous degradation of phenol using immobilised *Ps. putida* in RM at D = 0.3 h\(^{-1}\) 50

Figure 7: The effect of MRM at pH 6.5 on the degradation of phenol using *Ps. putida* at D = 0.3 h\(^{-1}\) 56

Figure 8: Bubble column bioreactor designed for continuous mode using an overflow weir 61

Figure 9: Schematic representation of the instrumentation for bioreactor 2 62

Figure 10: Photograph of the redesigned bioreactor containing fully grown beads 63

Figure 11: The effect on phenol degradation using the newly designed bioreactor at D = 1.2 h\(^{-1}\) 64

Figure 12: The effect of dilution rate on the effluent phenol concentration 69

Figure 13: The effect of dilution rate on the degradation rate of immobilised *Ps. putida* 70
Figure 14: The effect on $r_S$ and $S_o$ caused by scaling up the bead volume and dilution rate

Figure 15: The effect of pH on the effluent phenol concentration using immobilised *Ps. putida*

Figure 16: The effect of pH on the degradation rate of phenol by immobilised *Ps. putida*

Figure 17: The effect of temperature on the effluent phenol concentration using immobilised *Ps. putida*

Figure 18: The effect of temperature on the degradation rate of phenol by immobilised *Ps. putida*

Figure 19: The effect of bead diameter on the effluent phenol concentration

Figure 20: The effect of bead diameter on the phenol degradation rate

Figure 21: Comparison of the effluent phenol level achieved by a free cell system to an immobilised cell system

Figure 22: Comparison of the degradation rates achieved by a free cell system compared to an immobilised cell system
Publications

I: INTRODUCTION
1. Phenol

Phenol is an aromatic compound consisting of a benzene ring (a six-membered aromatic carbon) with a hydroxy group (-OH) attached. It is toxic at low concentrations and unlike single chain carbon molecules, phenol is highly stable and difficult to degrade (Autenrieth et al., 1991). However, there is a significant amount of work on the degradation of phenol.

1.1 Problems with Phenol

The toxicity of phenol has been widely documented and there is great concern over the disastrous effects phenol has upon both humans and the environment. The effects on aquatic life are destructive at low concentrations, and for humans the danger is just as great as 1 g of phenol is lethal (Bond and Straub, 1974). Continued exposure to phenol for humans is also quite damaging. The literature to date strongly points out the high toxicity of phenol, not only to humans but to aquatic life. For fish, 5 - 25 mg L\(^{-1}\) is lethal, and as low as 0.1 mg L\(^{-1}\) leaves an odour and an aftertaste (Kirk-Othmar, 1982). For humans, the allowed exposure is 20 mg day\(^{-1}\) for people working with phenol (Bond and Straub, 1974). It was also stated that small doses to humans are damaging causing dangerous and painful burns and adsorption through the skin may occur (Windholtz, 1983). Ingestion of phenol can cause vomiting, paralysis, lung failure and cardiac arrest (Windholtz, 1983). Although effluent can be diluted and then discharged into the waterways, this approach is not feasible for a chemical toxic at 5 - 25 mg L\(^{-1}\).
1.2 Phenol in Effluent

Phenol is commonly found in effluent and in waste particularly from petroleum sites. It is also produced in coking plants, oil refineries, and chemical industries involved with the production of pesticide resins, dyes and pharmaceuticals (Autenrieth et al., 1991). Effluent waters can contain as little as 10 mg L\(^{-1}\) to greater than 5000 mg L\(^{-1}\) of phenol.

The toxicity of phenol has been found to cause problems during treatment. Treatment plants consist of treating tanks such as settlement or activated sludge tanks and these consist of a wide range of bacteria and protozoa (Forster and Johnson, 1987). Many substrates are essential for the growth of effluent-treating-organisms, although as the concentration of certain chemicals increases above an optimal level, activity will decrease and if levels are too high activity may cease completely.

Phenol is a major concern in effluent treatment as there are few organisms that grow in treatment tanks able to degrade the substance. Even at low concentrations the cell population within the tanks is unable to degrade phenol due to inhibition. Cell death can occur if conditions are limiting and this can lead to irreversible changes in the cell populations within treating tanks (Barnes and Fitzgerald, 1987). To overcome the problem of toxicity, effluent containing phenol is commonly treated through separate methods.

2. Degradation of Phenol

There are a variety of methods available for phenol removal from effluent. The efficiency of these methods is dependent on the time taken for the reaction and
the initial concentration of phenol to be degraded. Some processes can also be very costly and this is a large determinant when choosing a system.

2.1 Chemical Treatment

Chemical processes available for the removal of phenol include chlorination, ozonation and benzo extraction, all giving > 97% removal efficiency (Bond and Straub, 1974). However, these process can produce toxic by-products and lead to secondary effluent problems. For example, during chlorination chlorine and phenol can produce compounds which taint the taste of fish (Autenrieth et al., 1991). Toxic emissions from such processes can also be produced and these can be more damaging than the reactant.

2.2 Biological Degradation

Under the correct environmental conditions, naturally occurring organic compounds are eventually degraded, hence they do not pose as great a threat to the environment as synthetic compounds do. The reason behind this is that microorganisms have had billions of years to adapt and utilise these compounds in their metabolic pathways (Atlas and Bartha, 1987). However, the development of the chemical industry has been sudden in comparison and with it many new synthetic compounds which were not previously known to microbial metabolism; or at the higher concentrations associated with intensive industrial activity.

The biodegradation of a compound can occur when the molecular structure and bonding sequence of the compound is recognizable by the metabolizing enzymes of a microorganism. For phenol, the basic structure is the benzene molecule (a six-
membered aromatic ring). This structure requires large amounts of energy to react as it is highly stable (Autenrieth et al., 1991). For chemical breakdown of this product high energy inputs are required, and for the biological breakdown the reaction may be slow as the cells require high amounts of energy to utilise the substrate. However, there are biological systems available to degrade phenol.

Over the last two decades, there has been a great deal of research on the degradation of phenol at concentrations between 500 - 4000 mg L\(^{-1}\) (Bettman and Rehm, 1985 and Lakhwala et al., 1992 and Yang and Humphrey, 1975). The systems used were diverse in nature and evolved as research progressed in this area in order to maximise the efficacy of the phenol degrading system. However, the aim of the majority of the work performed was to test the limits of the biological system in order to see what concentrations the cell population are able to utilise without being inhibited. Systems able to degrade up to 4 g L\(^{-1}\) were found, and it was observed that at higher levels inhibition occurred and productivity decreased (Bettman and Rehm, 1984).

Low degradation rates have been observed at low levels of phenol. In batch systems complete degradation occurs, but as the phenol concentration lowers in the reactor so does the biomass population, and so degradation rates decrease significantly (Bettman and Rehm, 1984). To overcome this, continuous reactors were used (Erhardt and Rehm, 1989; Khourey et al., 1992 and Yang and Humphrey, 1975). This allows a viable population of cells to be present for a longer duration. However, there has been less study on the problem of degradation at low levels, in either batch
or continuous systems. Therefore, the objective of this study was to perform research into the problem of phenol degradation at low levels.

3. The Problem - Degrading Phenol at Below 100 mg L\(^{-1}\)

3.1 Reasons for Researching Low Level Phenol Degradation

The majority of work performed to date on phenol degradation has been at high concentrations (Yang and Humphrey, 1975). Since phenol is a problem at concentrations as low as 0.1 mg L\(^{-1}\) (Section 1.1) the need for a system able to degrade phenol at low concentrations still remains. There is a lack of data available on systems able to remove phenol completely or even to safe levels. A thorough literature search was performed using Biological Abstracts on CD for records over the last 25 years. The records show that there are a large number of papers relating to phenol degradation at high levels. Keywords searched include: biodegradation, degradation, phenol and phenol removal. However, only three papers were found on phenol degradation at low levels, one of which used an immobilised cell system (Lakhwala et al., 1992).

There is a need for research on phenol degradation at below 100 mg L\(^{-1}\) if freshwater ponds and lakes, and the water used for drinking by communities is to be safe. Systems for phenol degradation could be designed to remove phenol to dischargeable levels or remove phenol completely ensuring that phenol would no longer pose a problem in effluent. Not only would this solve effluent problems, but systems such as this could then be used as a basis to design systems that are able to remove other hazardous chemicals such as the pesticide DDT using a mixed culture
system (Beunink and Rehm, 1988), or oil removal using a *Pseudomonad* and *Bacillus* isolate (Berwick, 1984).

### 3.2 Problems to be Overcome in order to Degrade Phenol at Low Levels

Few authors have presented work on phenol degradation at levels below 250 mg L\(^{-1}\) (Autenrieth et al., 1991; Hill and Robinson, 1975 and Lakhwala et al., 1992). In an immobilised cell system, the physical structure of the immobilising material has been found to inhibit degradation, due to restriction of oxygen and nutrient flow. Lakhwala et al. (1992) found that a lack of diffusion caused a decrease in reaction rates of one to two orders of magnitude. In designing a system to degrade phenol to low levels it would seem that optimising the bead environment and the reaction environment will be of prime importance if degradation is to be enhanced.

Another problem to be overcome in order to develop a system to degrade phenol at levels below 100 mg L\(^{-1}\) is that associated with cell density. The main problem is maintaining high biomass populations in the reactor on a low substrate concentration. To maintain a high cell population there must be a sufficient energy source available. This is where the problem lies. Low levels of phenol can only sustain a low cell population. This then gives low degradation rates and may cause effluent levels to rise (Hill and Robinson, 1975).

A common solution to this problem in a continuous reactor is to increase the dilution rate (D). When D is increased the amount of phenol flowing through the reactor in one hour is increased. Greater levels of phenol mean more cells are produced. However, D cannot be increased by large amounts otherwise the flow will be too great and cells will be washed out of the reactor at a rate greater than the
amount of cells being produced, causing cell loss. This is termed cell washout and causes the effluent concentration (\(S_e\)) to increase until it equals the concentration of the inflow (\(S_i\)), resulting in a complete decline of the degradation rate (Hill and Robinson, 1975 and Stanbury and Whitaker, 1984).

Therefore, the main problem is controlling biomass populations using \(S_e\). A population of cells controlled by the substrate concentration in the effluent is difficult to control, because it is the aim of degradation studies to bring the substrate effluent concentration to zero (or almost). If \(S_e\) is zero, then so is the substrate concentration in the reactor, and cell growth ceases. If cell numbers can be maintained at high levels to allow phenol to be consumed efficiently, then degradation rates and effluent levels can be improved.

3.3 The Broad Research Objective

The broad objective of this research was to develop and characterise a biological system able to degrade phenol at low levels.

The specific aims of this study were:

1. To develop a continuous system to degrade phenol below to 100 mg L\(^{-1}\) where the chosen microorganism will be \(Ps.\ putida\) which will be immobilised in calcium alginate.

2. To optimise the continuous immobilised cell system for phenol degradation at levels below 100 mg L\(^{-1}\).
The literature review which follows discusses the considerations which led to the choice of the systems design used for this experimental work. Options that were considered have been mentioned and the reasons for choosing one over another have been discussed.
II: LITERATURE REVIEW
4. Essential Characteristics of the System Chosen for this Study on Phenol Degradation

Biodegradation is the utilisation (removal or alteration to a less harmful form) of a compound or group of compounds by a biological organism. In the literature, there are many compounds that have successfully been degraded by various organisms (Alvarez et al., 1991; Berwick, 1984; Beunink et al., 1988; Janke et al., 1988 and Yu et al., 1994). For phenol the biological systems designed for its treatment are diverse, and the characteristics of each system determines its appropriateness and efficacy at phenol degradation.

There are three basic elements of any fermentation. These are the substrate, the microorganism and the bioreactor system. In this study the substrate was phenol, the bioreactor was a modified bubble column bioreactor and the organism chosen was *Ps. putida*. These three elements can be further specified. Firstly, there was the culture mode and secondly the form in which the cells were used i.e. free cell, immobilised cells or enzymes. The research performed to date has shown the use of microorganisms in free cell culture, enzyme and immobilised cell systems. Enzyme systems are not feasible for large scale industrial use and research in this area is typically on small scale cultures. Thus, enzyme systems will not be considered here.

In the following literature review the reasons for choosing the main features of the system used for phenol degradation are explained further.
4.1 Characteristics of the Microorganism

4.1.1 Aerobes or Anaerobes?

In choosing a system for degradation of phenol a decision was made to use an aerobic system over an anaerobic system as it offers several advantages. Phenol can be degraded biologically via either aerobic or anaerobic pathways. Aerobic processes require molecular oxygen for complete degradation of phenol and the initial stages in the catabolism of phenol involves the use of oxygenases to oxidise the substrate. The pathway is known as the meta cleavage pathway (Feist and Hegeman, 1969). In batch and continuous culture systems the use of aerobic cultures has been widely researched (Bettman and Rehm, 1984; Feist and Hegeman, 1969; Hill and Robinson, 1975; Hutchinson and Robinson, 1988; Molin and Nilsson, 1985 and Tschech and Fuchs, 1987)

An anaerobic system does not utilise nor require oxygen. For anaerobic degradation of phenol a different pathway must be utilised to breakdown phenol as oxygenases will not be induced under anaerobic conditions. According to Tschech and Fuchs (1987), the carboxylation of phenol to 4-hydroxybenzoate by a denitrifying Pseudomonad can occur, and is the first step in the breakdown of phenol under anaerobic conditions. However, research relating to phenol degradation in anaerobic conditions pertains mainly to mixed consortia of organisms (Gallert and Winter, 1993; Jianmin et al., 1993; Khoury et al, 1992; Knoll and Winter 1987 and Satsangee and Ghosh, 1990).

A major disadvantage with anaerobic systems is the length of time required to completely degrade phenol. In an anaerobic system phenol degradation takes longer.
Wang et al. (1989) showed that for 0.5 g L\(^{-1}\) of phenol 75 hours was required for degradation, and this increased significantly for higher concentrations. Tolerance to phenol by the organisms is also limited in anaerobic cultures (Craik et al., 1992), with a limit of only 1 g L\(^{-1}\) shown, whereas in an aerobic system up to 4 g L\(^{-1}\) (Bettman and Rehm, 1984) have been degraded.

Another disadvantage for anaerobic systems is the difficulty in maintaining a system with complete lack of oxygen. Equipment costs are increased, maintenance of a culture collection is harder and control of the process is difficult. The disadvantages of anaerobic systems made the use of an aerobic process the choice for this work.

### 4.1.2 Pure Culture or Mixed Culture?

Systems designed for the degradation of phenol can use bacteria, yeast and fungi. Another factor of importance is whether the system uses a mixed or pure culture. If a mixed culture of microorganisms is chosen, then the organisms used should be highly suited to the substrate being degraded and the conditions required should be optimal for those organisms. Wang et al. (1989) and Satsangee and Ghosh (1990) performed studies in batch systems and found that there are basically 3 groups of interacting bacteria responsible for phenol degradation. These included phenol metabolisers, hydrogen-utilising methanogens and acetotrophic methanogens. However, in treatment tanks it has been found that bacteria are the predominant species present, and synergistic relationships may occur (Barnes and Fitzgerald, 1987). This relationship requires a balance between cell numbers and the degrading substrate and must be maintained if successful degradation of a compound is to happen (Barnes and Fitzgerald, 1987).
Defined mixed-culture systems studied by Morsen and Rehm (1990) and Zache and Rehm (1989) showed complete degradation of phenol at a concentration up to 17 g L\(^{-1}\) in a batch system using *Cryptococcus elinovii* H1 and *Pseudomonas putida*. It was found that compared to a free cell system, the mixed culture could degrade 1 g L\(^{-1}\) of phenol faster, and was likely to be due to higher cell numbers and the action of two ring cleavage systems (both meta and ortho cleavage systems were used, meta by the bacteria and the ortho by the yeast).

For mixed culture systems, the different populations of organisms makes optimisation studies difficult as conditions that are optimal for one organism may not be for the other. Zache and Rehm (1989) found that control of pH did not enhance the capabilities of the system, and different optima were necessary to optimise conditions for each microorganism. The changing conditions observed in the mixed culture caused problems in replicating the system as slight environmental changes would enhance a different organisms growth to that of previous investigations. Therefore, the basis for choosing was not entirely productivity, but the ease of operation, stability and ease of replication of the system, and based on this a pure culture was the choice for this study.

### 4.1.3 Bacteria, Fungi or Yeasts?

In a review of literature by Leahy and Colwell (1990) the most prominent hydrocarbon degrading bacteria were found to be *Archromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia* and *Pseudomonas* spp. Fungal species used for hydrocarbon degradation included the *Fusarium* (Anselmo and Novais, 1992) and *Aureobasidium spp*. (Cheetham and Bucke, 1984),
though less work has been performed using fungi. For yeasts *Rhodococcus* spp. (Straube et al., 1990) seems to be the best studied for phenol degradation and *Cryptococcus elinovii* (Morsen and Rehm, 1987; Morsen and Rehm, 1990 and Zache and Rehm, 1989).

In choosing an organism for degradation of phenol certain criteria must be met. Firstly, for this research it must be an aerobic organism and have a high growth rate in order to speed up reaction rates. It must be able to utilise phenol as its sole carbon source at varying concentrations, from low to extreme concentrations. The organism should be able to tolerate a wide range of conditions and be easy to maintain in sterile culture. If the organism has been studied in the area of phenol or hydrocarbon degradation then this is an advantage, though not necessary.

Erhardt and Rehm (1989) have performed various studies on the phenol degrading capabilities of the *Pseudomonas* spp. Work involving this species shows that it is highly suited to phenol degradation and utilises phenol readily at varying concentrations with efficient rates (Bettman and Rehm, 1984; Erhardt and Rehm, 1989; Hill and Robinson, 1975; Hutchinson and Robinson, 1988 and Molin and Nilsson, 1985). Hill and Robinson (1975) found that *Pseudomonas putida* ATCC 17484 gave the most efficient degradation rates for phenol compared to other systems studied, in batch culture and utilising substrate levels up to approximately 0.7 g L\(^{-1}\). This is within the lower range of phenol degradation, as some systems have shown degradation of up 4 g L\(^{-1}\) of phenol using the same organism (Morsen and Rehm, 1990). However, the work to be attempted here is in the lower phenol ranges, and Hill and Robinson (1975) have produced a highly effective batch culture system using
*Ps. putida* for phenol degradation at low levels. Hence, this was the organism chosen for this research.

### 4.2 Options for Culture Mode

#### 4.2.1 Batch Culture Mode

Fermentation processes can be run in various modes, and a choice of mode is usually dependent on the type of end product to be produced. Of the systems available there are batch, fed-batch, repeated-batch and continuous which are the most common. At the two extremes we have batch and continuous systems and these are the most commonly used systems for phenol degradation (Bettman and Rehm, 1984; Zache and Rehm, 1989; Morsen and Rehm, 1987 and Yang and Humphrey, 1975), and these will be the systems concentrated on.

In batch culture, many studies have shown degradation using high levels of phenol. Straube et al. (1990) used the yeast *Rhodococcus* to degrade phenol and found that it would grow on concentrations up to 2.8 g L\(^{-1}\), but substrate inhibition set in at levels above 0.25 g L\(^{-1}\). This is a very low level compared to other workers who have shown growth without inhibition at levels above 0.5 g L\(^{-1}\). When inhibition set in, the lag phase was prolonged and the growth rates were reduced. This sort of effect was also observed with a bacterial system utilising *Ps. putida* growing on phenol as a substrate and a lag phase of 10 hours was observed (Hill and Robinson, 1975). This means that reactor time is inefficient and to prolong the stage of maximum growth would be ideal. Systems are available that do this, in particular the continuous culture system.
4.2.2 Continuous Culture Mode

A possible solution to extending the length of maximum growth is to maintain conditions at an optimal level giving increased biomass growth, and therefore high phenol utilisation rates i.e. to prolong the exponential phase of growth. This is possible by the addition of fresh medium to the culture vessel (medium that is not growth limiting nor toxin producing). The new addition of medium to the culture will prolong the growth phase until the additional substrate is utilised. If the addition of medium is repeated continuously, by drawing out an equal volume of liquid from the culture vessel as is replaced, then there is continuous production of cells. This is continuous culture.

During steady state, the cell population and the concentration of the media components reach a state of equilibrium. The spent medium or effluent contains the same concentration of components as that in the reactor and the concentration of substrate can never fall to zero as the cell population would be destroyed. Therefore, an optimal system would be one in which D is high enough to sustain a high population of cells, but low enough to allow efficient utilisation of the medium components or substrate. Using this sort of system offers several advantages over a batch system for wastewater treatment including the ability to process a high throughput of wastewater's, a constant environment, and prolonging of the exponential growth phase of the organism (Autenrieth et al, 1991; Bettman and Rehm 1985; Lakhwala et al, 1992). However, problems do occur.

In pure culture, growth can be sustained on greater than 500 mg L\(^{-1}\) of phenol and investigations tended to work in this range and above with low dilution rates
(never going above 0.4 h\(^{-1}\)) (Hill and Robinson, 1975 and Yang and Humphrey, 1975). This was to avoid problems such as bacterial growth on reactor walls which is a major cause of shortening reactor usage time, due to blockage. Biomass adherence and retention also changes the dynamics of the system and causes non-Newtonian behavior and as a result steady state instability occurs (Erhardt and Rehm, 1989; Hill and Robinson, 1975 and Yang and Humphrey, 1975).

To avoid these problems continuous studies using pure cultures in continuous mode tended to use:

- phenol concentrations greater than 500 mg L\(^{-1}\) so providing a high enough carbon substrate level to enable a cell population to grow and be sustained, and avoid major fluctuations in steady state due to a dynamic population.

- very low dilution rates to avoid the critical D rate at which cell washout occurs so that complete degradation of phenol can occur, as high dilution rates did not achieve complete degradation (Erhardt and Rehm, 1989 and Yang and Humphrey, 1975).

However, the critical D rate is often very low and cell washout occurs early on, so the efficiency of the system is low. Running this sort of system is not feasible and a solution was required to overcome the problem of cell washout. This solution took the form of immobilisation.

4.3 The Choice of Immobilised Cells or Free Cells

In designing a system for phenol degradation there are several forms in which the cells can be used. For this research an immobilised cell system was chosen for the
continuous degradation of phenol. Immobilised whole cells are cells that are physically confined within a defined area, while retaining most if not all their metabolic activities (Chibata, 1978). Immobilised cells were chosen because of the advantages they offer for repeated or continuous use, due to their attachment to an inert surface (Chibata, 1978).

Cell immobilisation has been used for many years now and has wide use in phenol degradation (Bettman and Rehm, 1985; Hannaford, 1992 and Lakhwala et al., 1992). There are a variety of methods available to immobilise cells including cross-linking, entrapment and encapsulation. Detailed reviews and methods of such processes are widely found (Cheetham and Bucke, 1984; Chibata et al., 1986 and Webb, 1987), therefore a detailed review will not be undertaken here. For this research the immobilisation technique of entrapment within calcium alginate was used. It is a simple technique whereby cells are combined with an alginate solution, then extruded dropwise into a stirring solution of calcium chloride where it gels to form solid beads. These are immobilised cells or beads.

4.3.1 Advantages of Immobilised Cell Systems

Immobilised cells offer several advantages compared to free cell systems. A major feature of immobilisation is the high density of cells that can be contained, and the ease with which cells can be separated from the culture liquor. There are also many other advantages including:

- Continuous or repeated-batch use of cells. With immobilisation a high throughput of liquid can pass through the reactor while the cells are retained within, and no cell washout occurs. Also the culture liquor can be removed at the end of a batch
and the vessel refilled with fresh medium, and reuse of the same population of cells occurs. This removes the need for reculturing of cells for each new batch (Webb, 1987).

- High productivity, as cells are confined and density is high in the reactor.

- Tolerance to increased levels of toxic substances, owing to a barrier being present between the cells and the liquid phase, and

- Immobilised cells can withstand large fluctuations in liquid loading and no significant change in the cell population occurs. This is advantageous at high dilution rates.

However, the primary reason for using an immobilised system for this research was not in terms of productivity, but the advantages gained in operation. Immobilised cell systems offer several advantages during continuous operation over a free cell system. For instance, immobilised cells do not undergo biomass adherence to reactor walls and blockage is not a problem (Anselmo and Novais, 1992). Dilution rates can be higher than that of free cell systems as washout does not occur; and large dynamic changes in liquid loading do not cause significant disruption of the cell population. This enables stable steady states to be achieved (Anselmo and Novais., 1992 and Bettman and Rehm, 1985).

4.3.2 Problems with Cells Immobilised in Calcium Alginate

The advantages offered by an immobilised cell system make it an ideal choice for phenol degradation. However, there are disadvantages and problems that must be overcome if a productive immobilised cell system for phenol degradation at low levels
is to be developed. The efficacy of an immobilised cell system is dependent on the stability of the beads in the reaction environment and the ease of mass and gas transfer through the bead. These areas can be problematic and are discussed further below.

### 4.3.2.1 Stability of Calcium Alginate Beads

Alginate beads are formed when calcium binds with alginate to form a gelatinous structure. This structure is relatively stable in the continuous culture environment but problems are known to occur. The chemical bonding may not be sound if other ions or chelating agents such as phosphate are present. This causes destabilisation of the alginate gel due to preferential binding ions which may substitute for calcium (monovalent ions) or chelating agents (McLoughlin, 1994).

Instability may also occur through mechanical or physical methods. The beads are mechanically sound but contact with other beads and against the reactor wall may cause abrasion of the beads causing bead breakage or cell leakage, and hence cell growth in the culture liquor. Kuek (1991) overcame this problem through the addition of calcium ions as a medium component. With excess calcium ions present, bead breakage was not a problem. However, this was only proven in batch or repeated-batch cultures and problems were still encountered in continuous culture (Bettman and Rehm, 1984).

### 4.3.2.2 Low Mass and Gas Transfer in Calcium Alginate Beads

Entrainment of cells involves the building of a matrix around the population of cells and the structure that develops will determine the reaction capabilities of the cell
population. The strength of binding is represented by the pore size. Pore size can effect the diffusion of substrates or products through the bead and inhibit the production or breakdown of products. If the pore size is too small then only low molecular weight substrates and products diffuse through the membrane, and if the pore size is too large then problems may occur with cell leakage.

In aerobic systems, diffusion of oxygen through the bead is necessary to support growth of cells within the beads. This is difficult as oxygen must diffuse through gelatinous layers to reach the center of the bead. It is known that cell growth occurs primarily on the outer most layers of the bead, with decreasing density towards the bead core (Bettman and Rehm, 1984). If cell growth becomes too dense at the outer layers, the diffusion of oxygen and nutrients becomes almost non-existent to the core, and cell growth does not occur. This produces beads that are sparsely populated and so degradation efficiency is decreased.

Diffusion may also be a hindrance in terms of transport of substrates at low concentrations throughout the bead. It was shown by Lakhwala et al. (1992) that compared with a membrane attached microbial reactor the degradation rates achieved by a gel entrapped system were lower when using phenol concentrations below 250 mg L\(^{-1}\). The decrease in efficiency was attributed to diffusional resistance of the gel matrix. As biomass is entrapped in an external matrix, the cells gain protection from high levels of toxic or inhibitory compounds. However, this can be a disadvantage when low concentrations of substances need to diffuse through this barrier in order to be utilised by the cells.
4.4 The Influence of Environmental Parameters on Continuous Degradation of Phenol at Low Levels

Once a fermentation system has been chosen and is performing at a satisfactory level, fermentation parameters which may affect the reproducibility of the system and phenol degradation should be optimised. Parameters such as aeration and agitation, pH, temperature and bead diameter are of prime importance. In order to run the system at its optimum it would be best if the organism chosen could withstand a wide range of fermentation conditions, as it would therefore require less control. The influence of various parameters on phenol degradation at low levels has not been greatly studied, and so it is difficult to determine whether the same responses seen at high phenol concentrations, will also occur at low phenol concentrations. The effect of various parameters on phenol degradation by an immobilised cell system at low levels has been discussed below.

4.4.1 Aeration and Agitation in an Immobilised Cell System

Keeping aeration and agitation at an optimum is essential for maximum productivity for any aerobic system. The level required for each system is not always known though, and will differ for each system studied. Economically, a system that can tolerate low aeration and agitation, and not show any difference in phenol degradation would be ideal for an effluent treatment system as it requires less power input (Forster and Johnson, 1987).

Aeration and agitation are an important factor in determining the distribution of cells in a liquid fermentation; and for an immobilised cell system, are of extreme
importance as they not only determine the distribution of immobilised cells throughout the reactor, but also the density and distribution of cells within the beads (Hannaford, 1992 and McLoughlin, 1994). A fermentation system should be homogenous in distribution and cell density to allow the best utilisation of solutes possible (McLoughlin, 1994). If cell distribution is not even throughout the reactor then solute demand will be low or sporadic, steady state levels may increase and phenol degradation will decrease (Lakhwala et al., 1992).

Aeration and agitation will also determine the maximum volume of beads that can be placed in a reactor. There will be a packing volume where beads move freely and aeration and agitation are not restricted. However, as the volume of beads increases in the reactor, mixing becomes restricted and phenol degradation slows down (Hannaford, 1992).

By optimising aeration and agitation the distribution of beads, cells within beads and bead volume can be optimised to increase degradation.

4.4.2 pH of the Reaction System

Cell growth and productivity have long been known to be dependent upon the pH of a reaction system. For an immobilised cell system the importance of pH is no less. However, for an industrial treatment plant, a system that can operate under a wide range of pH would be more efficient and require less control.

During phenol degradation, pH approaches the acidic region as phenol is being utilised, and as the pH lowers, the degradation rate decreases (Bettman and Rehm, 1985). Temporary lags in cell growth have also been observed (Hannaford, 1992 and
Lallai et al., 1989) and a low pH of 4.5 has been reported by Lallai et al. (1989), and with this a cessation in growth and degradation.

Immobilisation has been said to infer increased protection for cells against environmental conditions, therefore the optimal pH observed for free cell systems may not be the same for an immobilised cell system. Also, according to McLoughlin (1994) alginate gel matrices have a negative charge upon them, and so the pH of the surrounding environment will influence the diffusion of charged substrates, and the excretion of products throughout the bead. Therefore, alteration of pH may affect the amount of phenol utilised.

4.4.3 Temperature of the Reaction System

Temperature is an important parameter and influences cell growth rates (Brock, 1970) and phenol degradation. There are generally two ways temperature can effect an organism: 1) increases in temperature can cause chemical and enzymatic reactions in the cell to occur more rapidly and an increase in cell growth, and 2) cellular components such as proteins and DNA are sensitive to high temperatures, and can be irreversibly damaged (Brock, 1970).

It is expected that there is a minimum temperature where growth does not occur, an optimum temperature at which growth is best, and a maximum temperature where growth will cease. As the temperature of a reaction increases past the maximum temperature for growth, lethal effects become apparent. As immobilisation is said to infer increased protection of cells, it may be found that phenol degradation is not inhibited at temperatures above 25°C, which is the optimum temperature for free cells of *Ps. putida* during phenol degradation (Bettman and Rehm, 1985 and
Hannaford, 1992). A high temperature of 30°C was the reported optimum for Yang and Humphrey (1975), however this has not been supported elsewhere.

Determining the optimum temperature range is essential for phenol degradation using immobilised cells, as temperature can affect not only the cell growth rates, but the dissolved oxygen ratio in the reactor, which in turn affects phenol degradation (McLoughlin, 1994).

4.4.4 Bead Diameter and the Effect on Phenol Degradation

A significant problem with immobilised cells is that of diffusion; the diffusion of oxygen and nutrients through the gel matrix (Doherty et al., 1995; Lakhwala et al., 1992 and McLoughlin, 1994). Cell conditions at the surface, and surrounding the bead will be optimal as there is no limitation to the diffusion of oxygen and solutes to the cells. This is due to the liquid layer surrounding the bead allowing easy transport of nutrients to the cells. However, diffusion becomes increasingly difficult as depth of the bead increases, inhibiting the transfer of oxygen and nutrients through the gel matrix (Doherty et al., 1995). When diffusion becomes limited, cell growth ceases or is limited and can cause several significant consequences:

- zonation of the microenvironment within the bead which may alter intraparticle growth, metabolism and product formation (Doherty et al., 1995 and McLoughlin, 1994).

- Low cell numbers at the center will generate unproductive regions and influence particle density (McLoughlin, 1994).
• accumulation of cells on the bead surface which may rupture the gel surface, resulting in outgrowth and leakage (McLoughlin, 1994).

• Non-homogenous distribution of cells can cause insufficient diffusivity of solutes (Doherty et al., 1995)

• at low substrate concentrations, a concentration gradient can form and sufficient substrate may not be available for cellular processes (McLoughlin, 1994).

The obvious answer to the problem is to decrease the diameter of the bead to overcome problems with diffusion. Dalili and Chau (1987) suggest using a bead diameter of 1 mm to obtain uniform distribution of cells within the beads. Others have agreed with this suggestion as it is said that only the outer 0.3 - 0.5 mm of the bead contain cell growth (McLoughlin, 1994). Therefore, the smaller the bead the less volume that is wasted.

5. Conclusion and Evaluation

The literature review has explained the development of the system used in this research and the problems expected to occur during phenol degradation < 100 mg L⁻¹ by a continuous immobilised cell system. Degradation rates have been shown to be affected by the organism used, culture mode, immobilisation and physical factors, such as pH, temperature, bead diameter, and aeration and agitation.

Problems that may occur have been outlined, and it has been suggested that the greatest limit to the efficacy of this system will be $S_e$, as it will control biomass levels throughout the reactor.
As outlined in the introduction (Part I), the aim was to develop a system able to overcome these problems and efficiently degrade phenol at < 100 mg L\(^{-1}\). The effect of other physicochemical parameters such as pH, temperature and bead diameter has also been investigated and conditions optimised.
III: EXPERIMENTAL PROGRAM
6. Introduction

The experiments performed were divided into three research stages. The first stage involved the preliminary work and the design of a fermentation system. The second phase was the optimisation of the system designed, and the third was a final comparison between the immobilised cell system developed and a free cell system.

6.1 Preliminary Work and System Design

The first experiment was the modification of a colorimetric assay for the determination of phenol using 5 mL sample volumes. A standard procedure was known, however a volume size in excess of 100 mL was required to test between the concentration range of 0.1 mg L\(^{-1}\) to 10.0 mg L\(^{-1}\). Thus, a modified assay method to detect phenol between this concentration range and using a 5 mL sample size was developed.

Experiments 2 to 4 were baseline studies of the system. In these experiments first observations were made of the system and improvements on system design or conditions were carried out. Included in this was the redesign of the bubble column bioreactor for continuous use. This then produced a stable continuous immobilised cell system that could be used to study phenol degradation below 100 mg L\(^{-1}\).

6.2 Optimisation Studies

Over Experiments 5 to 9 the limits of the system designed were tested. In Experiment 5 investigations on dilution rate and the effect it has upon \(S_e\) and \(r_S\) were performed. By studying dilution rates the behaviour of the system at low and high
dilution rates could be observed and based on the results achieved, one dilution rate was chosen for use as a base D in further studies.

The aim of Experiment 6 was to determine if scaling the system up would cause $S_e$ and $r_s$ to change. By increasing the throughput of the system, and increasing dilution rates and bead volume, it was expected that no difference would be seen in the effluent phenol concentration as there would be the same proportion of beads available to the volume of liquid flowing through the system. However, the degradation rate was expected to increase proportionally as the bead volume increased, and hence cell numbers were increased.

Experiments 7 to 9 were optimisation studies where investigation of the optimum pH required to grow cells within the bead and degrade phenol was performed, the optimum temperature was determined, and the relationship between bead diameter and phenol degradation was investigated. It was expected that there would be an optimum bead diameter favorable for diffusion, where $S_e$ and $r_s$ are best.

### 6.3 Comparison Study - Free Cell Degradation Compared to Immobilised Cell Degradation of Phenol

The final experiment performed was to compare the phenol degrading ability of a free cell system to an immobilised cell system. In a continuous system the biomass population is determined by the dilution rate at steady state. No matter what history the cells have had, the dilution rate placed upon the population would determine the effect on phenol degradation. From the results, it could be determined
whether the free cell system is as effective or more effective than the immobilised cell system at degrading phenol at low levels.

6.4 Characterisation of Bioreactor Performance

Two units were used to measure the efficacy of the system developed. $S_e$, is the steady state effluent phenol concentration produced after a minimum of six residence times. This unit represented the concentration of phenol available in the reactor and in the effluent. It is used to derive the quantity of phenol remaining from the initial concentration of 100 mg L$^{-1}$. This information was then used to calculate the second value, $r_S$, the rate at which phenol was being removed from the reactor by the cell population present, and at a particular dilution rate. These units were necessary to show how the system was being optimised, as either one or both units could be changed after varying conditions in the system. For example two systems (A and B) could have an $S_e$ of 25 mg L$^{-1}$. However, system A was run at 0.3 h$^{-1}$ while system B was run at 0.9 h$^{-1}$. $S_e$ gives the impression that the two systems are equal, but in fact they are not. When $r_S$ is calculated it can be seen that system B removed a greater amount of phenol than system A ($r_S :$ system A = 22.5 mg L$^{-1}$ h$^{-1}$ and system B = 90 mg l$^{-1}$ h$^{-1}$). Hence, the use of both units to describe how the systems ability to degrade phenol had changed.
IV: EXPERIMENTAL METHODS
7.1 Culture Maintenance and Preservation

7.1.1 Culture Supply and Storage

*Pseudomonas putida* ATCC 11172 was from the American Type Culture Collection (Maryland, USA). The culture was revived and maintained on Nutrient Agar (NA) slants.

7.1.2 Short-term Culture Preservation

Four NA slants were stored at 4°C until required and/or sub-cultured every two months (48 h at 25°C).

From this collection one slant was used to produce approximately three working cultures of which one would be chosen for the production of inoculum for experiments performed.

7.2 Materials

All chemicals required for media production and performance of the phenol assay were of analytical grade from BDH (Kilsyth, Victoria). The 4-aminoantipyrine required for the phenol assay was supplied by the Sigma Chemical Company (St. Louis, Missouri). The sodium alginate used for immobilisation was Manugel GMB (Batch 556921) supplied by Kelco AIL, Sydney.

Terumo Neolus 25 G (0.50 x 25 mm) hypodermic needles were used for immobilising and sampling. All samples were filtered using sterile 0.22 μm Millipore GS membrane filters.
7.3 Media

The medium used to produce inoculum for experiments was Inoculum Production Medium (IPM) a Mineral Salts Medium by Hill and Robinson (1975) (Table 1).

Table 1: Inoculum Production Medium (IPM) (Hill and Robinson, 1975)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>0.500</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.840</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.750</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.488</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.060</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.060</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.060</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.060</td>
</tr>
</tbody>
</table>

In agar (IPA) form this media contained an additional 11.0 g L⁻¹ of agar and was used to prepare inoculum (Section 7.5)

For cell culture within beads, all medium constituents were increased to compensate for the volume of water in the beads. The concentration change was calculated by multiplying the concentration of the medium components in Table 1 by the total volume of beads and media in the column, and then dividing this value by the
volume of media used. Other media used is described within the experimental sections.

7.4 Sterilization

All media and apparatus were autoclaved at 121°C for 15 minutes.

7.5 Preparation of Inoculum

Inoculum was produced following the same procedure in all experiments (Figure 1).
Working culture of *Ps. putida* as described in Section 7.1.2

- Transfer colonies onto 3 x IPA plates

In incubate plates at 25°C for 3 days

- Transfer 3 colonies into each of 5 Macartneys with 5 mL IPM (0.5 gL⁻¹)

In incubate with lids loose and static, at 25°C for 48 h

- Transfer 5 mL of inoculum into a 250 mL flask containing 45 mL IPM (0.5 gL⁻¹)

In incubate flasks with lid loose at 25°C for 24 h and 150 rpm in an orbital shaker

Inoculum is now able to be used directly for the production of *Ps. putida* immobilised in calcium-alginate (Section 7.6)

Figure 1: Procedure for the production of inoculum of *Ps. putida*. 
7.6 Immobilisation of *Ps. putida* in Calcium Alginate

All procedures for the immobilisation of *Ps. putida* were performed aseptically and all materials and equipment were sterilized prior to use.

The inoculum produced following the procedure outlined in Section 7.5 was added to a 3% sodium alginate solution (except for Experiment 2 in which a 2% sodium alginate solution was used). The ratio of inoculum to alginate was 1:9. Enough alginate-inoculum solution was prepared to give a 1:11 ratio of beads to media in the column. An immobilisation procedure adapted from Kuek (1991) was used (Figure 2). Beads were produced by pumping the suspension through a 25 gauge hypodermic needle, 5 cm above a stirring solution of 0.1 M calcium chloride. The beads were then allowed to set for 10 - 15 minutes in the calcium chloride solution, after which they were washed twice in distilled water, drained and placed directly into the bioreactor.
Ps. putida
Inoculum from Section 7.5

3% sodium alginate soln.

inoculum-alginate soln. in a
1:9 ratio

alginate-inoculum solution extruded through a hypodermic needle into
a stirring solution of calcium chloride via a pump

Beads allowed to set for approx. 15 minutes in the calcium chloride solution

Beads washed twice in distilled water and drained

Beads placed directly into bioreactor. Experiment started.

Figure 2: Procedure for the immobilisation of *Ps. putida* in calcium alginate
V: EXPERIMENTAL
8. Experiment 1: A Modified Colorimetric Method for the Determination of Phenol

8.1 Introduction

The method most commonly used for the detection and quantification of phenol in water and wastewater is the Direct Photometric Method as outlined in the Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1992) (APHA). This method relies on phenol reacting with 4-aminoantipyrine with potassium ferricyanide present, thus forming a coloured antipyrine dye. The absorbance of aminoantipyrine is then measured at a wavelength of 500 nm in a 40 mm cuvette. The volume of sample required by this method to detect the minimum detectable concentration of phenol (0.1 mg L\(^{-1}\)) is 100 mL (Standard Method in Table 2). A 100 mL sample size would be too large to collect (about 40 min.) from a 300 mL to 400 mL bioreactor during a fermentation, and therefore would not be representative of that time. To overcome this problem a small sample size of 4 - 5 mL would be used, as this would be a more reasonable sample volume to collect from the bioreactor, and would give an adequate sample volume for use with a 40 mm cuvette (16 mL capacity). However, scaling the assay reagent volumes down by this ratio (1:19) would cause errors in absorbencies, as the volumes of sample and reagents required would be too small to be dispensed precisely and repeatedly. Thus, a dilution in reagent concentration was to be used leaving all reagent volumes the same, but allowing a smaller sample size to be used.

In the modified method a 40 mm cuvette was used with a sample size of 5 mL. It was anticipated that this would give increased absorbencies over a wider range,
without affecting the linearity and accuracy of the results. The linearity and sensitivity of the modified assay method were tested

Table 2: Volumes and Concentrations of Assay Reagents for the Standard Method for Phenol Detection (APHA, 1992) and the Modified Assay Method for Phenol Detection.

<table>
<thead>
<tr>
<th>Reagent/Sample Type</th>
<th>Standard Method (APHA, 1992)</th>
<th>Modified Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original Values</td>
<td>Sample size 4 mL</td>
</tr>
<tr>
<td>Conc.</td>
<td>Volume*</td>
<td>Conc.</td>
</tr>
<tr>
<td>Sample</td>
<td>--</td>
<td>100 mL</td>
</tr>
<tr>
<td>Ammonium Hydroxide</td>
<td>0.5 N</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Phosphate Buffer pH 6.8</td>
<td>0.56M</td>
<td>~2.5 mL</td>
</tr>
<tr>
<td>4-amino antipyrine</td>
<td>2%</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td>8%</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>107 mL</td>
<td>4.28 mL</td>
</tr>
</tbody>
</table>

~ = approximate volume (reagent is added until a pH of 6.5 is reached)

Volume* = Reactant Volumes
8.2 Materials and Methods

A blank of distilled water and phenol standard concentrations of 0.1, 2.0, 4.0, 6.0, 8.0 and 10.0 mg L\(^{-1}\) were prepared. The Modified Method and the standard colorimetric method (APHA, 1992) was performed on these samples. Three replicates were performed on each sample. The absorbance 500 nm was then determined for all samples using both methods in a 40 mm cell. The results for the triplicate samples of each method were averaged and the standard deviation calculated (Table 3), to determine the distribution of data.
Figure 3: Comparison of the mean Absorbance values for the Standard Method and the Modified Method

Table 3: Mean absorbance values and respective standard deviations for the Standard Method and the Modified Method

<table>
<thead>
<tr>
<th>Phenol Concentration (mg L⁻¹)</th>
<th>Standard Method</th>
<th>Modified Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Abs.</td>
<td>Std Dev.</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0160</td>
<td>0.0030</td>
</tr>
<tr>
<td>2.0</td>
<td>0.2620</td>
<td>0.0117</td>
</tr>
<tr>
<td>4.0</td>
<td>0.5600</td>
<td>0.0100</td>
</tr>
<tr>
<td>6.0</td>
<td>0.8020</td>
<td>0.0067</td>
</tr>
<tr>
<td>8.0</td>
<td>1.1430</td>
<td>0.0420</td>
</tr>
<tr>
<td>10.0</td>
<td>1.4570</td>
<td>0.0547</td>
</tr>
</tbody>
</table>

Mean Abs. = Mean Absorbance₅₀₀  Std Dev. = Standard Deviation
8.3 Results and Discussion

The standard method for phenol determination is linear when measured at 500 nm in a 40 mm cell. The range of linearity is from 0.1 mg L\(^{-1}\) to 10.0 mg L\(^{-1}\) (Figure 3). It was shown that the modified method also gave a linear standard curve within the 0.1 mg L\(^{-1}\) to 10.0 mg L\(^{-1}\) range, although the absorbance values obtained were higher (Figure 3).

The standard deviation of the mean absorbance values was calculated for both the Standard Method and the Modified Method (Table 3). Error bars could not be placed on the Figure 3 as the amount of deviation was small, so it would not have been accurate. As the dispersal of data from the mean was small, accuracy was high.

The Modified Method is sensitive to phenol in the concentration range of 0.1 mg L\(^{-1}\) to 10.0 mg L\(^{-1}\) and could be used for measuring phenol with a 5 mL sample size without the accuracy of the assay being affected.
9. Experiment 2: Determination of the Baseline Performance of the Continuous Immobilised Cell System used in this Study

9.1 Introduction

Bioreactor studies commenced with initial observations on the capacity of the system designed, to choose an appropriate dilution rate and to determine a feasible design of the column and apparatus for use in a continuous capacity.

9.2 Materials and Methods

9.2.1 Inoculum Production and Immobilisation

The inoculum was prepared as outlined in Section 7.5 of which 2.5 mL was then added to 22.5 mL of a 2% sodium alginate solution. The immobilised cells were then prepared following the method outlined in Section 7.6 (Figure 2).

9.2.2 Reaction Medium

The components of the Reaction Medium (RM) (Hill and Robinson, 1975) were (g L\(^{-1}\)): Phenol, 0.100; KH\(_2\)PO\(_4\), 0.420; K\(_2\)HPO\(_4\), 0.375; (NH\(_4\))\(_2\)SO\(_4\), 0.244; NaCl, 0.030; CaCl\(_2\), 0.030; MgSO\(_4\), 0.030 and FeCl\(_3\), 0.030.

9.2.3 Phenol Degradation

Bioreactor

Twenty-five milliliters of immobilised \textit{Ps. putida} were placed in the bubble column bioreactor (Figure 4) with 275 mL of RM (1:11 bead: medium ratio). The pH was regulated by a pH controller (Figure 5). The dilution rate used was 0.3 h\(^{-1}\) and was chosen from similar experiments described in the literature. Air was supplied to
the column at 1.0 L min\(^{-1}\). Temperature was maintained at 25°C. Samples of a minimum of 5 mL were collected via the outlet tube and assayed for phenol. The sample pH was also measured. Fresh medium and spent medium was added and removed using the same pump (with dual pumping heads) (Figure 5).

**Treatment of Air Supply**

Compressed air was filtered through a sterile 0.2 μm cellulose acetate filter (Millipore Corp., Massachusetts, USA) to exclude microorganisms. The air was humidified by sparging through a vessel containing sterile distilled water. Loss of liquid in the column was minimised by manipulating the temperature of the humidifier.
Figure 4: Bubble Column Bioreactor 1
1 = exhaust condensor socket; 2 = addition ports (2 shown only) for acid, base and medium; 3 = water jacket for temperature control; 4 = outlet port; 5 = air sparger (orifice diameter of 0.5 mm); 6 = pH electrode port; 7 = assembly for sterile venting; 8 = exhaust condensor; 9 = assembly for sterile venting; 10 = exhaust outlet.

Arrows indicate flow of air.
Internal diameter =3.0 cm; height = 69.5 cm;
height of sparger orifice from base = 1.3 cm
Figure 5: Schematic representation of the instrumentation for bioreactor 1
MR - Medium reservoir; WR - Waste reservoir; DP - Dual pump head; WB - Water bath; BC - Bubble Column; P - Peristaltic pump; A - Acid; B - Base; TR - Timer relay; pH C - pH controller; pH M - pH meter; H - Humidifier; SF - Sterile Filtration; F - Flowmeter; PR - Pressure Regulator; C - Compressor
Figure 6: The continuous degradation of phenol using immobilised *Ps. putida* in RM at $D = 0.3$ h$^{-1}$
9.3 Results

During growth the beads changed in colour from a translucent white (just after immobilisation), to a light cream colour. After one day in the bubble column in continuous mode the beads changed to a dark-brown colour.

The level of attenuation within the first four hours (Figure 6) was high giving 98% removal of phenol. At a dilution rate of 0.3 h⁻¹ phenol remained below 10 mg L⁻¹ for the entire period, giving a phenol degradation rate of 29.4 mg L⁻¹ h⁻¹. The ability of the beads to degrade phenol is 1.18 mg L⁻¹ h⁻¹ mL⁻¹ of beads. At 22 h the fermentation system became very turbid, and it was found that the beads had dissolved in the system.

9.4 Discussion

The phenol concentration in the reactor decreased significantly within the first 5 h suggesting that cell growth occurred within the beads and the cell population present was high enough to give almost complete degradation of phenol within a few hours. After this point the phenol concentration steadied at below 10 mg L⁻¹. This represented the steady state level for the dilution rate of 0.3 h⁻¹. During steady state, the cell population present is at a dynamic equilibrium within the calcium alginate bead. The cells utilised the phenol concentration at a level at which the cell populations could grow and be maintained.

The dissolution of beads observed at 22 h was most likely in response to chelating agents. The beads are made from a sodium-alginate solution which gels when in contact with calcium chloride to form a solid-like structure. However, when
in contact with solutions containing chelating agents with a greater affinity for calcium ions than the alginate, the beads dissolve (Cheetham and Bucke, 1984). The media used in this experiment contained phosphates which act as the chelating agent and so the beads dissolved. The Reaction Medium used also contained a low concentration of calcium ions and this may have contributed to the dissolution of the beads, as the presence of calcium ions is required throughout operation to replace the ions that have been removed by chelating agents, such as phosphate, and through the natural course of running the bioreactor. It was necessary to then modify the media used to increase bead longevity, so that the beads would not be destroyed during an experiment.
10. Experiment 3: The Development of a Medium Compatible with Extended Use of Calcium-Alginate Beads in a Continuous System

10.1 Introduction

Based on results from Experiment 2 it was decided that a medium which is more compatible with bead longevity i.e. one which results in less bead dissolution, was required. It was suspected that the large amount of phosphates and the low concentration of calcium chloride present in the RM used in Experiment 2 were detrimental to the stability of the beads. In a continuous system, the calcium chloride must be continually added to give a constant supply of calcium ions (Cheetham and Bucke, 1984) and the phosphate content reduced. However, there is evidence to suggest that phosphate may be required by a microorganism in order to effectively degrade phenol (Robertson and Alexander, 1992). Thus, the reduction of the total phosphate in the medium has to be made without affecting phenol degradation.

The concentration of alginate can also be altered to increase the strength of the beads. As the alginate concentration is increased, the pore size decreases producing a closely linked matrix in which the cells are entrapped between a calcium alginate structure of greater strength (Cheetham and Bucke, 1984 and Chibata et al., 1986). Therefore, the aim of this experiment was to give increased longevity of the beads, without compromising phenol degradation by altering the Reaction Medium
and the system in the following three ways:

1. decreased total phosphate concentration
2. increased calcium chloride concentration
3. increased sodium alginate concentration for immobilisation from 2 - 3%

10.2 Materials and Methods

10.2.1 Inoculum Production and Immobilisation

The inoculum was prepared as outlined previously in Section 7.5 (Figure 1), A 3% sodium alginate solution was used for immobilisation instead of a 2% solution. The immobilisation procedure was as outlined in Section 7.6 (Figure 2).

10.2.2 Modified Reaction Medium (MRM)

RM was modified in phosphate and calcium concentration. The phosphate was reduced to a quarter of the content used previously. The calcium was increased to 0.25 M, a concentration found to give increased stability of calcium alginate beads (Kuek, 1986). From hereafter the medium was known as the Modified Reaction Medium (MRM). The amounts added were (g L⁻¹): KH₂PO₄, 0.1055; K₂HPO₄, 0.095 and CaCl₂, 2.775.

10.2.3 Phenol Degradation

This study was conducted using the bubble column bioreactor and the methodology described previously (Section 9.2.3, Experiment 2). The culture pH was maintained at pH 6.5 ± 0.2 by the automatic addition of 0.5 M NaOH or 0.5 M HCl, regulated by a pH controller. The electrode was sterilized by immersion in 70%
ethanol (pH 4) for 10 minutes before use. Electrode drift was corrected daily by comparisons of pH controller readings against an external pH meter.

10.3 Results

The beads did not dissolve during phenol degradation for over 70 hours (Figure 7). This was 50 hours more bead stability than in Experiment 2. The increase in calcium content, and decrease in phosphate content in the MRM coupled with the increase in sodium-alginate concentration from 2% to 3%, showed a definite increase in the longevity of bead life without affecting the degradation of phenol.

A rapid decrease in the effluent phenol concentration within 5 h was found (Figure 7). The effluent phenol concentration remained below 20 mg L\(^{-1}\) throughout the experiment with 80% to 98% degradation achieved. A degradation rate of 27 mg L\(^{-1}\) h\(^{-1}\) was obtained and the bead activity was 1.1 mg L\(^{-1}\) h\(^{-1}\) mL\(^{-1}\) of beads.

A fluorescent yellow pigment was observed in the reaction vessel at approximately 30 h. A sample was removed from the column at 30 h and observed with methylene blue staining under the microscope at 1000x. Free cells were not observed. Crystalline structures were found confirming the presence of a precipitate in the medium.

Rapid fluctuations in the level of liquid in the column were observed throughout the entire experiment. These changes amounted to as much as 40 mL in a total culture volume of 275 mL. It was found that continual wear upon the inlet and outlet pump tubing caused inconsistencies between the inflow and outflow pumped volumes, thus resulting in variations in culture volume.
Figure 7: The effect of MRM at pH 6.5 on the degradation of phenol using *Ps. putida* at $D = 0.3 \text{ h}^{-1}$
10.4 Discussion

The reduction in the phosphate concentration of the MRM combined with an increase in sodium-alginate concentration from 2% to 3%, and the increase of calcium chloride concentration, resulted in sustained phenol degradation (over 70 hours) in the bioreactor without dissolution of the beads. The problem of increasing bead longevity has been rectified by altering the calcium chloride concentration, the available phosphate levels and the sodium alginate concentration from 2 - 3% in the reaction medium.

Phenol degradation was not affected by the changes in the medium or alginate concentration, as the degradation rates and bead activity of this experiment are comparable to that of Experiment 2 over the period when the beads remained intact. The large initial drop in phenol concentration was comparable to that achieved in Experiment 2 and can be used in further experiments as an indication that cell growth has occurred within the beads.

The yellow colour in the medium was possibly a result of pigment production by the cells. According to Palleroni (1952), the production of a fluorescent pigment occurs with the Pseudomonads and in particular in media with a low iron content as was the cause with the RM used. However, production of a pigment can be erratic or cease even in the defined media (Palleroni, 1952).

Rapid fluctuations in the level of liquid in the column occurred. A possible reason for this volume change was thought to be the column and pump set up being used. The column used was originally designed for batch culture. It was adapted for continuous culture by using two pump heads to push liquid through the reactor.
However, wear upon the inlet and outlet pump tubing caused inconsistencies. To overcome this, a new column was to be designed in which liquid was only pumped in. The ability of the new reactor design to keep a stable liquid volume was investigated in the following experiment. If steady state is to be achieved so that optimisation of the system can be performed, then the column volume (V) and the flow of medium and effluent (F) must be constant, to give a consistent D. As a result, a bioreactor had to be designed in which a different method achieving a continuous flow is used to attain a consistent culture volume.
11. Experiment 4: Baseline Performance of a Bioreactor using an Overflow Mechanism for Continuous Phenol Degradation

11.1 Introduction

In Experiment 3, steady state was not achieved. It was observed in the previous experiment that there were large deviations in reactor volume. The system was running at a dilution rate of 0.3 h\(^{-1}\) and it was assumed that this was not high enough to cause the system to behave erratically, so another explanation was needed. It was observed that the flow of liquid in and out of the column was not equal and this led to major volume changes and it was decided that this was the probable cause of the system being unable to achieve steady state. So before steady state experiments could be performed the design of the continuous bioreactor needed to be modified. This required designing a new bioreactor. A reactor with an overflow weir was custom made. This new bubble column needed to be specially designed for immobilised cells, as in traditional continuous systems cells are lost in the efflux. To retain the immobilised cells a T-piece was added to the overflow weir (Figure 8, Part 4).

The aim of this experiment was to observe the effectiveness of the new column at maintaining an equal inflow and outflow, so that steady state can be achieved and maintained for continuous degradation of phenol.
11.2 Materials and Methods

The configuration of the bioreactor setup (Figure 8) is shown in Figure 9. The methodology used is as outlined in Section 10.2, Experiment 3. An increased dilution rate of 1.2 h\(^{-1}\) was used to test the bioreactor initially at high dilution rates (previously 0.3 h\(^{-1}\)).

11.3 Results

Continuous culture was found possible with consistent culture volumes using the modified bioreactor. Immobilised cells were not lost from the column, no blockage occurred at the overflow mechanism, and there was minimal fluctuation in reactor volume. Figure 10 shows the column containing beads which are fully grown.

An initial decrease in the effluent phenol concentration occurred in the first 10 hours (Figure 11). After a period of 10 hours had elapsed a general increase in \(S_e\) was observed until steady state was achieved (at approximately 30 h). The established steady state phenol concentration remained at approximately 45 mg L\(^{-1}\) for the duration of the experiment. At steady state, the degradation rate was 64.8 mg L\(^{-1}\) h\(^{-1}\) at a dilution rate of 1.2 h\(^{-1}\). Minor fluctuations were observed in \(S_e\), though this was within the range of ± 5 mg L\(^{-1}\) and did not influence the results.
Figure 8: Bubble column bioreactor designed for continuous mode using an overflow weir.  
1 = exhaust condensor socket; 2 = addition ports (1 shown only) for media; 3 = capillary addition ports (1 shown only) for acid and base; 4 = overflow weir for immobilised cell use; 5 = outlet port; 6 = water jacket for temperature control; 7 = bubble column; 8 = draining port; 9 = pH electrode port; 10 = air sparger (orifice diameter of 0.5 mm).  
Arrows indicate flow of air. 
Internal diameter of lower section = 2.7 cm; height of sparger orifice from base = 1.3 cm; height = 59 cm; Internal diameter of upper section = 5.7 cm.
Figure 9: Schematic representation of the instrumentation for bioreactor 2
MR - Medium reservoir; WR - Waste reservoir; DP - Dual pump head; WB - Water bath; BC - Bubble Column; P - Peristaltic pump; A - Acid; B - Base; TR - Timer relay; pH C - pH controller; pH M - pH meter; H - Humidifier; SF - Sterile Filtration; F - Flowmeter; PR - Pressure Regulator; C - Compressor
Figure 10: Photograph of the redesigned bioreactor containing fully grown beads
Figure 11: The effect on phenol degradation using the newly designed bioreactor at $D = 1.2 \text{ h}^{-1}$.
11.4 Discussion

The redesigned bubble column bioreactor held a consistent culture volume and no loss of beads occurred during the continual degradation of phenol, over a period greater than six residence times. This experiment was performed at a $D$ of 1.2 h$^{-1}$, four times greater the rate used in Experiment 3 in order to see if the new column could run at high dilution rates and still produce a stable system. This aim was achieved as shown in Figure 11 in which the $S_e$ levels were stable, unlike the scattering of $S_e$ values obtained in Experiment 3 (Figure 7).

The decrease in effluent phenol concentration in the first 8 h may be caused by a high initial cell population which is able to quickly utilise the phenol available in the bioreactor. By the eighth hour, the phenol concentration was probably low enough to cause a decrease in population size. Due to this decrease in cell numbers $S_e$ gradually increased. However, as phenol began to rise so did the cell numbers. This continued until an equilibrium had been reached between cell numbers and phenol present. This is when steady state began (between 10 - 110 h).

As this experiment was performed at $D = 1.2$ h$^{-1}$, the level of attenuation is much less than that produced in Experiment 3 (or 2). This is due to the dilution rate being four times greater in this experiment. However, in this experiment the degradation rate was higher. At such a high dilution rate the cell population should be greater than at 0.3 h$^{-1}$, and although complete attenuation cannot be achieved, the cell population is able to utilise more phenol over the same time period. Thus a higher degradation rate. However, in achieving a high $r_S$, $S_e$ was higher.
A stable system capable of degrading phenol had been designed and using this system it was then possible to perform studies to determine the working limits of the system in terms of the dilution rate, scale, pH, temperature and bead diameter. By testing these parameters optimal conditions were to be determined and used to improve phenol degradation.
12. Experiment 5: The Effect of Dilution Rate on Phenol Degradation using Immobilised *Ps. putida*

12.1 Introduction

The dilution rate used in Experiment 4 was arbitrarily chosen, so further work was required to determine a dilution rate that will produce a system able to achieve steady state in order to perform optimisation studies. Therefore, the aim of this experiment was to observe the effect of dilution rate upon the degradation of phenol by immobilised *Ps. putida*.

Free cell systems used for degrading phenol are commonly operated at low dilution rates. This produces a biomass population able to achieve good levels of attenuation. To improve attenuation levels the dilution rate was increased. However, in a free cell system increasing dilution rates causes cell washout to occur. With the use of immobilised cells this problem is avoided. Unlike free cell systems, immobilised cells do not undergo cell washout at low dilution rates, and it was expected that an immobilised cell system is able to run at higher dilution rates than a free cell system. Immobilised cell particles would need a much higher throughput in the reactor before they are washed out from the column. Investigating the effect D might have upon phenol degradation was therefore required in order to determine what affect D has upon an immobilised cell reactor. It was expected that as D is increased, attenuation levels would rise until the limit of the system had been reached, at which point $S_e$ would equal $S_i$, and phenol degradation would cease. It was also expected that the level of attenuation and degradation rates achieved would be more
efficient for an immobilised system than a free cell system, though this was further investigated in Experiment 10.

12.2 Materials and Methods

The following set of experiments were performed using the bioreactor and methodology described previously in Experiment 4 (Section 11). To investigate the effect of dilution rate, separate batches of immobilised *Ps. putida* were prepared under identical conditions and placed in the column. Separate batches of beads prepared under identical conditions were used as the cells would have no history which may influence cell behavior. When the effluent phenol concentration was stable for a minimum of six residence times, steady state was said to be achieved. The amount of phenol present was Se for the run, and each experiment was continued for a minimum of 70 hours. All experiments were performed at 25°C and pH 6.5. The only variable altered was the dilution rate for each set of beads. The dilution rates tested were 0.3 h⁻¹, 0.6 h⁻¹, 0.9 h⁻¹ and 1.2 h⁻¹.

12.3 Results

At all four dilution rates studies there was some level of degradation attained, though complete attenuation was never reached. The amount of degradation lessened as D increased, with 0.3 h⁻¹ giving the most attenuation (below 10 mg L⁻¹) (Figure 12).

The degradation rate did not continue to increase at the same rate as Se. Instead rs appeared to equilibrate between 0.6 and 0.9 h⁻¹ (Figure 13). From 0.6 h⁻¹ to 1.2 h⁻¹ the degradation rates reached a maximum between 51 - 56 mg L⁻¹ h⁻¹.
Figure 12: The effect of dilution rate on the effluent phenol concentration
Figure 13: The effect of dilution rate on the degradation rate of immobilised *P. putida*
12.4 Discussion

The degradation rate obtained for the immobilised cell system did not proportionally increase as in the case of $S_e$ (Figure 13). The activity of the bead population increased until approximately 0.6 h$^{-1}$, after which the rate of degradation reached a plateau until 1.2 h$^{-1}$ (Figure 13). This same effect was encountered by Lakhwala et al. (1992) and was attributed to an increase in dissolved oxygen demands by the cell population. At low dilution rates, diffusion of oxygen is not such a problem because cell numbers are lower (Gosmann and Rehm, 1986). However, as the dilution rate increases so does the available phenol, and as a result cell numbers increase. With increasing cell numbers in the bead, oxygen is consumed faster than it can diffuse into the beads, and so cells have to compete for oxygen (Gosmann and Rehm, 1986). As a result, the rate of removal of phenol was slowed, and when oxygen can no longer be transferred across the matrix, degradation ceased.

For further work, a dilution rate of 0.6 h$^{-1}$ was used. This dilution rate gave a degradation rate within the optimal range, and it was also the saturation point for $r_S$. However, the effluent phenol concentration achieved could still be optimised further. Therefore, by using this dilution rate the effect of selected parameters on $S_e$ and $r_S$ could be seen.
13. Experiment 6: The Effect on $r_s$ and $S_e$ as a Result of Scaling up the Immobilised Phenol Degrading System

13.1 Introduction

The limit of any system to deal with effluent can be measured by the amount of effluent that can be effectively degraded in an hour. This is the throughput capability. An optimal system is one that can degrade high volumes of liquid without the degradation capability of the system being diminished.

In this experiment, the aim was to increase the throughput of liquid without causing a significant increase in the level of attenuation. In general, scaling up is in terms of throughput. This was done by increasing the dilution rate. By proportionally increasing the bead to medium volume and dilution rate, the system should be able to be scaled up without having to increase bioreactor size. In performing scale up studies the limit of the system to deal with large volumes of liquid was determined, and the data gained from this study could be used in designing effective reactors capable of dealing with large volumes of effluent.

In this experiment, it was expected that when the bead to medium volume and dilution rate were increased in proportion, $S_e$ would remain relatively constant as the ratio of cells to the amount of phenol in the reactor per unit time would remain the same. Therefore, the aim of this experiment was to determine whether increasing the throughput capacity of the system would deleteriously effect phenol degradation.
13.2 Materials and Methods

The following set of experiments were performed using methodology described previously in Experiment 4, Section 11. All experiments were performed at 25°C and pH 6.5. In Experiment 5, 33 mL beads were produced and placed in the column with 267 mL of media. This was a 1:11 ratio. A dilution rate of 0.3 h⁻¹ was set. In this experiment, 5 levels of scaling up the dilution rate and bead to medium volume were tested (Table 4). Each experiment was begun when $S_e$ was constant for a minimum of six residence times. When steady state was reached, the phenol concentration in the reactor was determined repeatedly every 4 - 6 hours, for greater than 70 hours. This was $S_e$.

Table 4: The Scale up Factor and corresponding Bead to Medium Volume ratio and Dilution rates tested

<table>
<thead>
<tr>
<th>Scale up Factor</th>
<th>Bead Volume (mL)</th>
<th>Total Reactor Volume (mL)</th>
<th>Dilution Rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>16.5</td>
<td>400</td>
<td>0.15</td>
</tr>
<tr>
<td>1</td>
<td>33</td>
<td>400</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>400</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>400</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>132</td>
<td>400</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Note: '1' represents the base value of bead to medium volume and a dilution rate used previously.
13.3 Results

Scaling up the dilution rate and bead to medium volume resulted in an increase in the effluent phenol concentration (Figure 14). However, even at the highest dilution rate $S_e$ never increased above 10 mg L$^{-1}$ and could be said to be steady throughout the tested range. The agitation of the beads did slow as the volume of beads in the reactor was increased to the 99 mL volume and above.

The phenol degradation rate was found to increase by double as the scaled up factor was increased by double. The degradation rate increased by the same magnitude as the scale-up factor for each change in scale implemented showing a linear relationship between the dilution rate, bead to medium volume and the degradation rate. This can be seen by the first ratio. At 0.15 h$^{-1}$ and with 16.5 mL of beads a degradation rate of 14.9 mg L$^{-1}$ h$^{-1}$ was given. After doubling both the dilution rate and the population of beads in the reactor (to give 0.3 h$^{-1}$ and a 33 mL of beads) the phenol degradation rate doubled to 28.8 mg L$^{-1}$ h$^{-1}$. This relationship continued for all dilution rates tested (Figure 14).
Figure 14: The effect on $r_S$ and $S_e$ caused by scaling up the bead volume and dilution rate
13.4 Discussion

The relationship between dilution rate and bead volume with respect to phenol degradation in an immobilised continuous culture system was proportional. In increasing the dilution rate up to 1.2 h\(^{-1}\) the performance of the reactor was not limited as long as proportional changes were made to the bead volume. It was found that the throughput of the reactor could be increased four fold without affecting the level of attenuation. Although a small increase in the effluent phenol level was seen as the system was scaled up (Figure 14), it was thought that this increase was due to volumetrically determining the increase in beads and not by actual bead counts, and this resulted in small deviations in the effluent concentration.

The relationship between bead volume and \(r_s\) was complex. It showed a doubling in \(r_s\) as the dilution rate and the bead volume was increased. This was because the number of beads (and hence the number of cells) present is doubled or increased by the magnitude represented by the scale up factor, and so a greater numbers of cells were available to degrade phenol, hence doubling \(r_s\).

A stable and steady system has been designed to achieve degradation over a wide range of dilution rates. The dilution rate of 0.6 h\(^{-1}\) was used with a bead:medium ratio of 1:11 in the previous experiment (Experiment 5) and will be used for further experiments. Under these conditions an \(S_c\) of 16 mg L\(^{-1}\) and an \(r_s\) of 51 mg L\(^{-1}\) h\(^{-1}\) were produced. The aim of the following experiments was to improve these values by altering pH, temperature and bead diameter.
Therefore, scaling up the bioreactor (by increasing the dilution rate and bead volume) did not adversely affect the degradation rate nor the effluent concentration.
14. Experiment 7: The Effect of pH on Phenol Degradation using Immobilised *Ps. putida*

14.1 Introduction

It was previously shown by Hannaford (1992) that the optimum pH for phenol degradation by immobilised *Ps. putida* in a batch reactor was 6.5. Lallai et al. (1989) observed that as phenol was degraded a decrease in pH occurred and a temporary lag in cell growth was seen (Section 4.4.2). A similar event was seen by Bettman and Rehm (1985), whereby the degradation of phenol was accompanied by a pH drop to as low as 4.5 in both batch and continuous culture.

Immobilising an organism may alter the optimum pH for a biotransformation performed by it. This may decrease the efficiency of the reaction due to the negative charge upon the gel matrix, altering the diffusion capacity of the bead (McLoughlin, 1994).

If pH is controlled within the optimum range, cell growth within the beads will not be affected and degradation will be kept at a maximum. It was expected that as pH was brought towards the acid region, phenol degradation would slow (Bettman and Rehm, 1985 and Lallai et al., 1989). The aim of this set of experiments was to determine the optimal pH range for phenol degradation by immobilised *Ps. putida*. 
14.2 Materials and Methods

14.2.1 Inoculum Production and Immobilisation

The inoculum was prepared as outlined previously in Section 7.5 (Figure 1). A 3% sodium alginate solution was used for immobilisation. The immobilisation procedure was as outlined in Section 7.6 (Figure 2).

14.2.2 Modified Reaction Medium

The modified reaction medium was used. The components were (g L$^{-1}$):

Phenol, 0.100; KH$_2$PO$_4$, 0.1055; K$_2$HPO$_4$, 0.095; (NH$_4$)$_2$SO$_4$, 0.488; NaCl, 0.060; CaCl$_2$, 2.775; MgSO$_4$, 0.060 and FeCl$_3$, 0.060.

14.2.3 Phenol Degradation

This study was conducted using bubble column bioreactor 2 (Figure 8 and 9) and the methodology described previously (Section 11.2, Experiment 4). The culture pH was maintained at the test pH by the automatic addition of 0.5 M NaOH or 0.5 M HCl, regulated by a pH controller. The electrode was sterilized by immersion in 70% ethanol (pH 4) for 10 minutes before use. Electrode drift was corrected daily by comparisons of pH controller readings against an external pH meter.

Five separate continuous cultures were run for this experiment where separate, identically produced batches of immobilised *Ps. putida* were prepared. All experiments were performed at 25°C and a dilution rate of 0.6 h$^{-1}$. The only variable altered was the pH of the reaction for each set of beads. When the effluent phenol concentration was stable for a minimum of six residence times, steady state was said
to be reached. The run was continued for a minimum of 70 hours. The pH values investigated were: 5.0, 5.5, 6.0, 6.5 and 7.0.

14.3 Results

The degradation of phenol was not adversely affected by pH between 5.5 and 6.5. The effluent phenol concentration was lowest in the range of 5.5 to 6.5 giving an $S_e$ below 10 mg L$^{-1}$ (Figure 15). At pH 5.0, $S_e$ approached the influent phenol concentration of 100 mg L$^{-1}$ and was also high at pH 7.0. The degradation rate was lowered at pH 7.0 and degradation was almost non-existent at pH 5.0 (Figure 16). In all experiments growth was observed in the beads, however the density of cells was less after growth at pH 7.0 compared to pH 5.5 to 6.5 (Figure 15).
Figure 15: The effect of pH on the effluent phenol concentration using immobilised *Ps. putida*
Figure 16: The effect of reaction pH on the degradation rate of phenol by immobilised *Ps. putida*
14.4 Discussion

The degradation of phenol is optimal between the pH ranges of 5.5 to 6.5, and is severely limited beyond the two limits. This suggests that regulation of pH is required to allow degradation of phenol by *Ps. putida* to remain at an optimal level. However, it seems that the beads are able to withstand a pH in the acidic region lower than observed for free cell systems. Lallai et al. (1989) reported a minimum of 5.5 for phenol degradation at 101 mg L$^{-1}$, while this research points out a pH low of 5.0. This effect may be attributed to the immobilisation of the cells within calcium alginate as the beads may produce an environment in which the cells are protected through a diffusion gradient.

In further experiments on optimisation, a pH of 6.5 was used. This pH was within the optimal range and was also found to be in the pH range commonly recorded for phenolic wastes. Autenrieth et al. (1991) found phenolic wastes to be at approximately 6.8, while Bond and Straub (1974) found the range to be 6.25 - 9.15.
15. Experiment 8: The Effect of Temperature on Phenol Degradation using Immobilised *Ps. putida*

15.1 Introduction

The range of reaction temperatures is typically determined by the microorganism in use and for *Ps. putida* it has been found that approximately 25°C is the optimum (Bettman and Rehm, 1985 and Hannaford, 1992) (Section 4.4.3). The aim of this experiment was to determine the optimum temperature range for immobilised *Ps. putida* to degrade phenol continuously.

15.2 Materials and Methods

The following set of experiments were performed using methodology described previously in Experiment 7 (Section 14). To investigate the effect of temperature, four separate continuous cultures of immobilised *Ps. putida* were run for this experiment where separate, identically produced batches of beads were used. When the effluent phenol concentration was stable for a minimum of six residence times, steady state was said to be achieved. The amount of phenol present was the \( S_0 \) for the run, and the run was continued for a minimum of 70 hours. All experiments were performed at pH 6.5 and a D of 0.6 h\(^{-1}\), and with 33 mL of beads. The only variable altered was the temperature of the reaction for each set of beads. The temperatures tested were: 20°C, 25°C, 30°C and 35°C.
15.3 Results

Phenol degradation was optimal between 25°C and 30°C, with 30°C producing the lowest effluent phenol concentration (Figure 17). At 30°C the phenol degradation rate had increased to 58 mg L⁻¹ h⁻¹ (Figure 18) and at 35°C degradation was almost non-existent (Figure 18). The effect of colder temperatures on phenol degradation was not as severe, with 20°C only showing a halving in the degradation rate.
Figure 17: The effect of temperature on the effluent phenol concentration using immobilised *Ps. putida*
Figure 18: The effect of temperature on the degradation rate of phenol by immobilised *Ps. putida*
15.4 Discussion

The optimum temperature range for degradation of phenol by immobilised *Ps. putida* was found to be 25°C to 30°C, indicating that the immobilised bacteria has a higher temperature optimum than that reported in the literature. Hannaford (1992) or Bettman and Rehm (1985) both reported 25°C as their optima with phenol degradation failing above or below this temperature.

The inability of *Ps. putida* to degrade phenol at 35°C (Figure 17) was likely due to the inhibition of metabolic pathways by enzyme denaturation. It seems that *Ps. putida* was able to behave as a psychrophile as degradation was halved at 20°C rather than completely inhibited.

For the following experiments a temperature of 30°C was used as this was the optimum temperature achieved.
16. Experiment 9: The Effect of Bead Diameter of Immobilised *Ps. putida* on Phenol Degradation

16.1 Introduction

In Experiment 6 it was found that the throughput of the reactor, and hence phenol degradation can be improved by increasing the volume of beads in the bioreactor. This showed that an increase in bead volume (which is an increase in cell numbers) is directly related to phenol consumption. Thus, by increasing cell load in the reactor, the rate of phenol degradation would be increased. Another factor known to affect phenol degraded is the bead diameter of the immobilised cells.

The spatial distribution of cells entrapped within a bead is suspected to be primarily on the surface of the beads, and decreasing in density towards the center (McLoughlin, 1994). The productivity of a bead is affected by solute transport and oxygen transport, which is highly affected by the distribution of cells throughout the bead. An ideal situation is one in which the cell loading is high and evenly distributed throughout the bead, and hence biomass would be at a maximum. However, according to McLoughlin (1994) cell growth occurs only in the outer 0.3 - 0.5 mm of the bead and the distribution of cells will affect diffusivity of oxygen and solutes (Section 4.4.4). The distribution of cells will also determine steady state levels as they will be dependent on the relationship between biological demand, and diffusion or supply of nutrients. To enhance diffusion the bead diameter of the immobilised cells needs to be investigated. Thus, the aim of this set of experiments was to determine the optimum bead diameter for phenol degradation.
16.2 Materials and Methods

The following set of experiments were performed using methodology described previously in Experiment 7 (Section 14). To investigate the effect of bead diameter, separate 33 mL batches of immobilised Ps. putida were prepared under identical conditions were used. All experiments were performed at 30°C, pH 6.5 and a D of 0.6 h⁻¹. When the effluent phenol concentration was stable for a minimum of six residence times, steady state was said to be achieved. The amount of phenol present was Sₑ for the run, and the run was continued for a minimum of 70 hours. The only variable altered was the diameter of the beads. The diameter of the beads investigated were: 1 mm, 2 mm and 3 mm. The bead diameter was altered using different sized hypodermic needles. The following needle sizes were used for the respective bead diameters: 18G for 3 mm; 25 G for 2 mm; and 30 G for 1mm beads. An average bead size for each needle was determined by measuring the diameter of 100 freshly made beads under a microscope at 100x and using a slide with a ruled grid.
Figure 19: The effect of bead diameter on the effluent phenol concentration
Figure 20: The effect of bead diameter on the phenol degradation rate
16.3 Results

As bead size decreased, the degradation rate increased and the effluent phenol concentration decreased. The use of various sized beads produced only a small increase in the degradation rate as bead size was reduced (an increase of 4.5 mg L\(^{-1}\) h\(^{-1}\) from 3 mm to 1 mm beads). The smallest beads produced the highest degradation rate (Figure 20).

A significant change was observed in the effluent phenol concentration (Figure 19). As the bead diameter increased (by 1 mm each time) the effluent phenol concentration approximately doubled (Table 4).

16.4 Discussion

This experiment showed that a smaller bead diameter enhanced phenol degradation as the degradation rate was increased, and the effluent concentration was lowered to 2.5 mg L\(^{-1}\). The findings in this experiment may be explained by the observation of McLoughlin (1994) that mass transport of solutes and oxygen is affected by the cell distribution within the beads and that beads are primarily populated in the outer 0.3 - 0.5 mm. Thus, it is probable that as the bead diameter was increased, cell distribution became more and more limited to the exterior of the beads and degradation rates diminished. Therefore, the results given confirm the observation made by McLoughlin (1994) and it was suggested that 1 mm diameter beads be used in order to give optimal mass transfer of oxygen and nutrients to the cell population within the beads.
17. Experiment 10: Comparison of the Degradation Rate of Two Continuous Systems: Free Cell *Ps. putida* versus Immobilised *Ps. putida*

17.1 Introduction

A problem found by many authors using immobilised cell systems is low mass transport of oxygen and solutes into the beads (McLoughlin, 1994 and Lakhwala et al., 1992). It has been suggested that increased bead diameter influences the transfer of solutes and oxygen to the cell population within a bead (Experiment 9, Section 16.4).

In terms of phenol degradation there has been little comparison performed to determine the efficacy of a free cell system to an immobilised cell system for phenol degradation. Although diffusion seems to be the greatest problem for immobilised cells, free cells are prone to the problem of cell washout at relatively low dilution rates.

The aim of this experiment was to compare a free cell continuous system to an immobilised cell system and determine which of the two was more efficient in degrading phenol at low concentrations.

17.2 Materials and Methods

This experiment used dilution rate for a comparison study, on two separate population samples. In order to compare the two systems, it was assumed that there was an equivalent population size in both systems at identical dilution rates. Dilution rate controls the population size in a reactor, regardless of what history the cells have.
If D is the same (and all environmental conditions are identical) over two separate runs, steady state conditions should be the same. This suggests that the resultant capacity for phenol degradation would be achieved by an equivalent number of cells in both of the systems. When an immobilised cell system and a free cell system are compared, the same philosophy stands. The only difference will be the distribution of the cell population in the reactor, except that they were differently distributed.

17.2.1 Free Cell Continuous Culture

17.2.1.1 Inoculum Production

Inoculum was prepared as outlined previously in Section 7.5 (Figure 1). Enough inoculum was made to fill the 400 mL bioreactor (10 x 50 mL aliquots).

17.2.1.2 Modified Reaction Medium

The modified reaction medium was used. The components were (g L⁻¹):

Phenol, 0.100; KH₂PO₄, 0.1055; K₂HPO₄, 0.095; (NH₄)₂SO₄, 0.488; NaCl, 0.060;
CaCl₂, 2.775; MgSO₄, 0.060 and FeCl₃, 0.060.

17.2.1.3 Phenol Degradation

Four hundred milliliters of inoculum was added to the bioreactor (Figure 8). The culture pH was maintained at 6.5 and the temperature at 25°C. A dilution rate of 0.3 h⁻¹ was used to begin the experiment. When steady state was reached and maintained for a minimum of 6 residence times, the dilution rate was increased to 0.6 h⁻¹. This procedure was then repeated for 0.9 h⁻¹, and then for 1.2 h⁻¹.
17.2.2 Immobilised Cell Continuous Culture

17.2.2.1 Phenol Degradation

Conditions were identical and comparison was made on the basis of dilution rate in this series of experiments. In this comparison, observations were made of $S_e$ and $r_s$ at steady state, across the various dilution rates for the free and immobilised cell systems. For this experiment the methodology previously described in Experiment 5 (Section 12.2) was used. All experiments were performed at 25°C and pH was set to 6.5 with a diameter of 2 mm. When $S_e$ was steady and maintained for a minimum of six residence times, the dilution rate was increased. The dilution rates tested were identical to those of Section 17.2.1.3 for free cell culture: 0.3 h$^{-1}$, 0.6 h$^{-1}$, 0.9 h$^{-1}$ and 1.2 h$^{-1}$.
17.3 Results

The immobilised cell system appeared to be better than the free cell system, at degrading phenol at $D > 0.6 \, \text{h}^{-1}$ (Figure 21 and 22). At $0.9 \, \text{h}^{-1}$ and $1.2 \, \text{h}^{-1}$ the immobilised cell system gave much better phenol degradation than the free cell system, with the degradation rate being almost twice the magnitude for the immobilised cells (Figure 21).

Problems were encountered in the free cell system at the highest dilution rate (noted by the non-filled point in Figures 21 and 22) as cells grew into large flocs causing the effluent level to become unstable, and cell growth was observed on reactor walls soon after the $1.2 \, \text{h}^{-1}$ run began. As flocs occurred $S_e$ began to increase, giving the appearance of an increase in efficiency. However, the experiment was stopped at this point. The system was running under conditions not dynamically equivalent for free cell systems, and therefore the results at $1.2 \, \text{h}^{-1}$ must be considered to be unreliable.
Figure 21: Comparison of the effluent phenol level achieved by a free cell system to an immobilised cell system
Figure 22: Comparison of the degradation rates achieved by a free cell system compared to an immobilised cell system.
17.4 Discussion

Free cells were able to degrade phenol as efficiently as immobilised cells at a dilution rate of 0.3 h\(^{-1}\). As increments smaller than 0.3 h\(^{-1}\) were not tested, it is not known whether the two systems remain comparable until 0.6 h\(^{-1}\). However, the slope of the graph suggest that the free cell and immobilised systems have comparable efficacies of degradation at \(D < 0.5\) h\(^{-1}\). As the dilution rate was increased further the capacity for phenol degradation decreased in the free cell system, and at \(> 0.9\) h\(^{-1}\) significantly decreased (Figure 22). It was thought that the dynamics of the system changed as \(D\) increased further, so that the cells could continue to degrade phenol efficiently. However, the cell population present could not effectively degrade such a high throughput of liquid, resulting in an increase in the effluent concentration as \(D\) increased (Figure 21).

The immobilised cell system showed a greater ability to degrade phenol at high dilution rates. Unlike the free cell system, significant cell loss did not occur as cells were bound to an inert material (calcium alginate), keeping cell density high in the reactor.

As the dilution rate increased, growth of flocculants and adherence of biomass to reactor walls occurred in the free cell system. This caused instability and fluctuating phenol levels in the effluent and led to a decrease in the rate of phenol removal. Hill and Robinson (1975) and Anselmo and Novais (1992), experienced this same problem, though no solution was suggested, except the use of a biomass supported structure such as immobilised cells (Anselmo and Novais, 1992). By using immobilised cells in continuous culture higher dilution rates could be used and
problems related to morphological growth characteristics of the organism could be overcome. This was found to occur, and the immobilised cell system was more efficient than a free cell system when $D > 0.5 \text{ h}^{-1}$.

At the lowest dilution rate, the free cell system was 6% more efficient than the immobilised system. This changed as $D$ increased, and the immobilised system was observed to have an increased degradation capability over the free cell system. As $D$ increased to 0.6 h$^{-1}$, the immobilised system was 18% more effective at degrading phenol than the free cell system, and at 0.9 h$^{-1}$, the efficacy of the immobilised system increased to 36%, twice the magnitude. This was expected to continue as $D$ increased, though the exact $D$ where the free cell system became dynamically different is not known.

In conclusion, the immobilised cell system was able to degrade a higher throughput of effluent containing phenol more effectively than a free cell system.
VI: GENERAL DISCUSSION
AND FURTHER WORK
18.1 Degradation of Phenol at Levels Below 100 mg L\(^{-1}\) by an Immobilised Cell System

The broad objective to develop a biological system able to degrade low levels of phenol has been achieved. Selected physicochemical parameters of the system were studied and optimised to improve phenol degradation. The system consisted of \textit{Ps. putida} immobilised in calcium alginate in a continuous flow bioreactor and attenuation of phenol achieved was degraded from 100 mg L\(^{-1}\) to below 2.5 mg L\(^{-1}\).

At the start of the project the system's capability was characterised by an \(S_e\) of 16 mg L\(^{-1}\) and \(r_s\) of 51.5 mg L\(^{-1}\) h\(^{-1}\). Through optimisation, the level of attenuation was improved 6 fold (\(S_e = 2.5\) mg L\(^{-1}\)), while the degradation rate was only marginally improved (\(r_s = 58.5\) mg L\(^{-1}\) h\(^{-1}\)). All results were obtained at a dilution rate of 0.6 h\(^{-1}\).

The direct conclusions that can be made from the research undertaken were:

a) The dilution rate affects phenol degradation. The level of attenuation was best if the flow of influent was low (i.e. \(D\)), but the rate of degradation was increased by the dilution rate. The increase in degradation rate ceased if the dilution rate was too high. This may have been due to increased cell growth in the beads causing an oxygen limited environment. These results were as expected as more phenol produces more cells and so the rate of utilisation would increase. Further work on conditions such as aeration and agitation could be used to enhance the flow of oxygen to the cells through the calcium alginate bead surface.

b) The system designed could handle increased flow of medium (or influent) provided that the volume of beads was proportionally increased with the dilution rate. Increased dilution rates did not affect the concentration of phenol in the
effluent, but the rate of degradation was increased. During scale up, there seemed to be no loss of degrading ability of the system. It was also seen that even with minimal movement of the beads through the reactor, degradation did not suffer, suggesting that the aeration rate could be lowered without affecting degradation, as a system that can perform as well with less aeration and agitation uses less power, and this is a major concern when designing reactors or treatment plants.

c) Phenol degradation was optimal between the pH ranges of 5.5 to 6.5, and was severely limited beyond the two limits. The ability of cells to grow and degrade under acidic conditions was possibly attributed to increased protection by immobilisation.

d) *Ps. putida* has an optimum temperature range from 25 - 30°C and was able to behave as a psychrophile as degradation was halved at 20°C, rather than completely inhibited.

e) The diameter of a bead should be no larger than 1 mm. Using smaller bead sizes is thought to enhance mass transport of solutes and oxygen. It is most probable that the larger the bead, the more the cells populate the outer regions of the bead, and so cells growing towards the center of the bead must compete for oxygen (Bettman and Rehm, 1984 and Gosmann and Rehm, 1986). Studies on the spatial distribution of cells by Scanning Electron Microscopy (SEM) would give detailed images of cell growth in a bead population, and so provide more evidence for their possible effect on mass and gas diffusion. Further studies on gel type and concentration would produce more data to explain how diffusion can be controlled so that competition for nutrients and oxygen is decreased, and
examining the longevity of the beads in continuous culture would also be
necessary. The beads used throughout this research could be used for over 70
hours of continuous culture. However, the limit of sustained degradation and
bead integrity should be studied.

f) The immobilised cell system could effectively degrade a higher throughput of
phenol compared to a free cell system. The immobilised system was better than a
free cell system at $D > 0.5 \text{ h}^{-1}$, using $S_e$ and $r_S$ as measures of efficacy. It is
expected that if the optimised conditions were used at dilution rates higher than
that studied, $S_e$ and $r_S$ would rise, but the extent to which $D$ can be raised without
degradation becoming inhibited should be tested. Cell washout was not observed
in the immobilised system, because of cell confinement within calcium alginate.
However, there is a dilution rate at which the beads would be removed, because
the flow of liquid through the reactor would cause increased turbulence due to
such a high velocity. This limit needs to be determined, to find the highest dilution
rate that the system can perform under. The results from the research performed
and others, suggest that immobilisation acts as a protective barrier over the cells,
giving increased stability and this may be the reason for a more efficient degrading
system being produced.
18.2 Comparison of $r_S$ and $S_e$ obtained for the Immobilised System Developed to Published Results

From the literature, the systems that have been designed to degrade phenol are not able to remove phenol to safe levels or below 10 mg L$^{-1}$. In order to compare systems published with the one reported here, the level of attenuation could be used. Table 5 shows $S_e$, $r_S$ and related values for the systems. The dilution rates used by these systems were lower than the 0.6 h$^{-1}$ used in this study. It was assumed that the dilution rates reported in these papers were the highest appropriate for these studies, and it was observed in some systems that washout occurred (Erhardt and Rehm, 1989 and Yang and Humphrey, 1975), or the system became unstable (Molin and Nilsson, 1985) at dilution rates not much higher that quoted here. In fact, only one system was able to completely degrade phenol at a dilution rate higher than 0.6 h$^{-1}$, and this was using a biofilm (Molin and Nilsson, 1985). All systems, apart from Molin and Nilsson (1985) used dilution rates lower than in the study reported here, and could only reduce phenol to between 100 mg L$^{-1}$ and 5 mg L$^{-1}$. In comparing these results, the $S_e$ of the system developed is much lower than achieved by other systems (except Erhardt and Rehm (1989), who reported complete degradation, though the level at which the phenol assay was accurate to was not mentioned). Overall, it can be said that the level of attenuation achieved in this research is comparable if not better than that produced by other systems.
Table 5: A Comparison of the Phenol Degrading Capability of *Ps. putida* Bioreactors

<table>
<thead>
<tr>
<th>System</th>
<th>D (h⁻¹)</th>
<th>Type of System</th>
<th>( S_i ) (mg L⁻¹)</th>
<th>( S_e ) (mg L⁻¹)</th>
<th>( r_s ) (mg L⁻¹ h⁻¹)</th>
<th>Corrected ( r_s ) (mg L⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Thesis</td>
<td>0.6</td>
<td>immobilised (free beads)</td>
<td>100</td>
<td>2.5</td>
<td>58.5</td>
<td>58.5</td>
</tr>
<tr>
<td>Scaled-up</td>
<td>1.2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>10.0</td>
<td>112.0</td>
<td>112.0</td>
</tr>
<tr>
<td>Molin and Nilsson, 1985</td>
<td>1.3</td>
<td>immobilised (biofilm)</td>
<td>500</td>
<td>12.0</td>
<td>720</td>
<td>124</td>
</tr>
<tr>
<td>Erhardt and Rehm, 1989</td>
<td>0.3</td>
<td>Free Cells</td>
<td>1000</td>
<td>100.0</td>
<td>270</td>
<td>27</td>
</tr>
<tr>
<td>as above</td>
<td>0.25</td>
<td>Activated Carbon</td>
<td>2500</td>
<td>0</td>
<td>360</td>
<td>12</td>
</tr>
<tr>
<td>Yang and Humphrey, 1975</td>
<td>0.4</td>
<td>Free Cells</td>
<td>200</td>
<td>5.0</td>
<td>78</td>
<td>39</td>
</tr>
</tbody>
</table>

Another method for comparison is the system’s ability to remove phenol. This is the degradation rate. In comparing \( r_s \), it must be noted that the other systems reported in Table 5 used much higher phenol levels in the influent \( S_i \). Increased phenol means increased cell numbers, and so the degradation rate would be greater when using a high \( S_i \). So to compare, the degradation rates of other systems have been corrected for an \( S_i \) of 100 mg L⁻¹. Assuming that biomass numbers increase proportionally with phenol levels, studies using an \( S_i \) higher than in this study can be compared by adjusting the \( r_s \) values reported. For example, a system with an \( S_i \) of 1 g L⁻¹ of phenol and a degradation rate of 300 mg L⁻¹ h⁻¹. To adjust the degradation rate, the latter value is divided by 3 to make it comparable to the \( S_i \) of 100 mg L⁻¹ used in this study. This is termed the corrected \( r_s \). Only one system using a biofilm (Molin and Nilsson, 1985), could achieve a corrected \( r_s \) better than that achieved in
this study of 124 mg L\(^{-1}\) h\(^{-1}\). If the optimised biofilm system developed by Molin and Nilsson (1985) is compared to the system in this study that had been scaled up to run at 1.2 h\(^{-1}\) (Table 5), then the two systems would appear to be comparable. Therefore, under scaled up conditions the rates of degradation appear to be equivalent. In comparing the corrected \(r_s\) with the other systems (Table 5), it would seem that the system developed in this project was more efficient at degrading phenol at low levels than those listed, when the initial amount of phenol is taken into account. The degradation rate of the system researched is equal if not better than those described above, after adjustment for \(S_i\).
VII: CONCLUSION
The research reported here has begun exploration of the problem of removing phenol at levels below 100 mg L\(^{-1}\). This problem is often seen in many free cell systems and is characterised by low dilution rates and/or cell washout. For immobilised cell systems, the problem is not washout at low dilution rates, but the effective transfer of solutes and gases to the cell population entrapped with the bead. It has been suggested that mass and gas transfer problems caused by immobilisation cause phenol consumption to be inhibited (Erhardt and Rehm, 1989; Molin and Nilsson, 1985; and Yang and Humphrey, 1975). However, in this research a system has been developed that does not undergo cell washout at high dilution rates, and has been optimised to enhance diffusion. The system developed is also comparable to systems published in the literature that deal with phenol degradation.

Further research is necessary if the system developed is to be adapted for treating effluent on industrial sites. The conditions found optimal for this system are similar to those by which a treatment plant or tank may use. For instance, the pH at which optimal degradation occurs is representative of the pH found for effluent requiring treatment. The temperature range that the system can use to degrade phenol is wide and not drastically outside the temperatures that would be experienced for a tank of effluent during a day and night. This points out the appropriateness of this system to an industrial situation or how the approach taken here can be used to design other systems able to tackle the effluent problem.
VIII: REFERENCES


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117


