DESIGN OF A BIOTRANSFORMATION PROCESS
FOR DEHALOGENATION OF
CHLORINATED ALIPHATIC COMPOUNDS

Natasha N. Rosen

A thesis submitted to the University of London
for the degree of Doctor of Philosophy

November 1994

Department of Chemical and Biochemical Engineering
University College London
Torrington Place
London WC1E 7JE
For Mum
ACKNOWLEDGEMENTS

I would like to thank a number of people who have all had a significant effect on my research and without whom the last three years would not have been as productive or as enjoyable:

- My supervisor at UCL, Professor Malcolm D. Lilly, for his vast knowledge and guidance and for helping me to take my hat off.
- My supervisor at the University of Kent, Professor Alan T. Bull, for all his help and advice in the first year of my project.
- My advisor, Dr John M. Woodley for his input and for asking the right questions.
- Dr David J. Hardman, for all his thoughts and helpful discussions.
- Dr Nigel J. Titchener - Hooker for planting the original idea.
- Dr. Paul J. Sallis for his valuable technical advice and for providing the bacterial strain.
- Dr. Jonathan Hunt for his help and advice on my project and my life!
- A very special thank you to Billy Doyle for all his ‘beyond the call of duty’ help in the pilot plant, the fermenter room and on the football pitch!
- Gordon and Stuart for the overnighters and for bringing me cakes in the morning.
- All the technical help that I have received from the workshop and all of the staff at UCL and the University of Kent, especially to Anna Alongi for all the long chats.
- To all my friends and colleagues at UCL and at the University of Kent for keeping me sane and making me laugh.
- To James for helping me to see the light.
- To Bradley for his love, food and diversions!
- And finally, to my Mum and Dad for their financial and emotional support and love always.

UCL is the Science and Engineering Research Council’s Interdisciplinary Research Centre for Biochemical Engineering and the Council’s support is gratefully acknowledged.
ABSTRACT

Biotransformations have traditionally been used for the synthesis of novel organic molecules but they can also be used for the degradation of toxic and environmentally harmful compounds, such as haloalkanes. Some haloaliphatic compounds used in industry are toxic, carcinogenic, and resistant to degradation and so tend to accumulate in the environment unless the halogen substituents are removed.

Large-scale use of these halogenated aliphatic compounds in industry has led to widespread contamination. *Rhodococcus erythropolis* Y2, which utilizes many of these compounds as carbon and energy sources, possesses a dehalogenase. The biotransformation involves the conversion of the haloalkane to the corresponding alcohol by cleavage of the carbon-halogen bond; further reactions allow complete mineralization of the haloalkane. The enzyme shows great industrial potential as it is a stable biocatalyst and does not require cofactors or oxygen to function.

The main objectives of this project were to demonstrate the production of the haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2 on a large scale and to examine the use of this halidohydrolase in an immobilized form for the removal of halogenated aliphatic compounds from aqueous process streams.

A small scale enzyme production process has been developed which has aimed to optimize the fermentation, enzyme induction and isolation procedures. Changes in the fermentation medium and conditions have resulted in an increase in the final cell concentration from 0.7 to 5.9 g<sub>dw</sub>/L. A less volatile more effective inducer, 4-chlorobutanol, has been found. When added to the culture at a cell concentration of 0.8 g<sub>dw</sub>/L and a substrate concentration of 0.74 mM, the specific enzyme activity increased from the previous value of 0.05 to 0.16 U/mg. The fermentation and enzyme isolation have been scaled-up to 1000 litres, resulting in an increase in the final cell concentration to 9.28 g<sub>dw</sub>/L and a three-fold increase in the specific growth rate to 0.31 h<sup>-1</sup>.

By understanding the kinetics of the halidohydrolase under various conditions, the reactor design has been evaluated. The enzyme has been immobilized from clarified cell extracts onto Eupergit C beads. Under optimal conditions, this gave an activity of 0.0185 U/g dry beads and 0.5 U/g dry beads on 1-chlorobutane and 4-chlorobutanol respectively. A packed bed reactor was able to dehalogenate completely saturated solutions of 1-chlorobutane. Lower percentage conversions were obtained with 4-chlorobutanol.
CONTENTS LIST

TITLE PAGE .............................................................................................................1
ACKNOWLEDGEMENTS .........................................................................................3
ABSTRACT ..................................................................................................................4
CONTENTS LIST .......................................................................................................5
LIST OF ABBREVIATIONS .......................................................................................11
LIST OF FIGURES ....................................................................................................12
LIST OF TABLES ......................................................................................................18
1.0 INTRODUCTION ..............................................................................................23
1.1 MICROBIAL BIOTRANSFORMATIONS ..........................................................27
   1.1.1 Introduction to Biotransformations .........................................................27
   1.1.2 Biocatalysts ...............................................................................................27
   1.1.3 Characteristics of the Biotransformation for Process Design ..................28
   1.1.4 Choice of Reactor System ........................................................................29
1.2 INTRODUCTION TO THE GENUS Rhodococcus AND RELATED ORGANISMS .................................................................................................32
   1.2.1 Introduction ..............................................................................................32
   1.2.2 Kinetics of Bacterial Growth on Chlorinated Aliphatic Compounds .......33
1.3 HALOALKANE DEHALOGENASES ...................................................................36
   1.3.1 Anaerobic Transformations .....................................................................36
   1.3.2 Aerobic Dehalogenation of Haloalkanes ..................................................39
      1.3.2.1 Glutathione-Dependent Dehalogenases ........................................41
      1.3.2.2 Oxygenase-type Dehalogenases ....................................................42
      1.3.2.3 Haloalkane Halidohydrolases ........................................................46
1.4 HALOALKANE HALIDOHYDROLASE PRODUCED BY Rhodococcus erythropolis Y2 ..................................................................................50
   1.4.1 Effect of Glycine ......................................................................................52
2.1.3 Medium Preparation .............................................................76

2.2 GROWTH CONDITIONS ..........................................................76
  2.2.1 Growth in Shake Flasks ......................................................77
  2.2.2 Growth in Batch Fermenters ................................................77
  2.2.3 Criteria for Scale-up ..............................................................78
  2.2.4 Data Determination During Fermentation ...........................78
  2.2.5 Determination of Bacterial Concentration ..........................79

2.3 WHOLE CELL STUDIES .............................................................79
  2.3.1 Assay for Dehalogenation in Whole Cell Suspensions............ 79
  2.3.2 Determination of Optimum pH for Dehalogenase Activity in Whole Cells ..............................................................80

2.4 PREPARATION OF CRUDE CELL FREE EXTRACTS... 80
  2.4.1 Disruption using the French Pressure Cell .........................80
  2.4.2 Disruption using the High Pressure Homogenizer ...............80
    2.4.2.1 Small Scale ...................................................80
    2.4.2.2 Large Scale ...................................................81

2.5 DEHALOGENATION DETERMINATION USING THE DEHALOGENASE IN THE CELL FREE EXTRACTS .................................................................82

2.6 INDUCTION OF DEHALOGENASE ACTIVITY .............82

2.7 ANALYTICAL PROCEDURES .........................................................82
  2.7.1 Determination of Halide Ion Concentration .......................82
    2.7.1.1 In Growing Cultures ..........................................82
    2.7.1.2 In Cell Free Extracts using Bergmann-Sanik Method ..........83
    2.7.1.3 In Cell Free Extracts using Florence and Farrar Method .......84
    2.7.1.4 Chloride Analyzer Method .....................................87
### 2.7.2 Gas Chromatographic Analysis

- **2.7.2.1 Determination of Standard Curves**
- **2.7.2.2 Determination of Extraction Efficiency**
- **2.7.2.3 Determination of Saturation**

### 2.7.3 Determination of Protein Concentration in Cell Free Extracts

### 2.7.4 Chemicals and Reagents

### 2.8 IMMOBILIZATION

- **2.8.1 Procedure for Immobilization on a Small Scale**
- **2.8.2 Determination of Immobilization Conditions**
  - **2.8.2.1 pH Value of the Binding Buffer**
  - **2.8.2.2 Ionic Strength of the Binding Buffer**
  - **2.8.2.3 Amount of the Enzyme**
  - **2.8.2.4 Immobilization Time**
  - **2.8.2.5 Determination of Binding Yield/Activity Yield and of Operational Stability**

### 2.9 DEVELOPMENT OF A SMALL SCALE REACTOR SYSTEM

- **2.9.1 Setting up the Plug Flow Reactor**
- **2.9.2 Measurement of Column Parameters**
  - **2.9.2.1 Determination of Bed Volume**
  - **2.9.2.2 Determination of Bed Flow Characteristics**
  - **2.9.2.3 Determination of Percentage Conversion vs Flowrate**
- **2.9.3 Use of a Mini Batch Reactor**

### 3.0 RESULTS

### 3.1 FERMENTATION DEVELOPMENT

- **3.1.1 Medium Development**
3.1.2 Effect of pH on Specific Growth Rate ........................................ 113
3.1.3 20 L Fermentation Data .................................................. 113

3.2 INDUCTION ................................................................. 120
3.2.1 Choice of Inducer ..................................................... 120
3.2.2 Amount of Inducer ..................................................... 121
3.2.3 Induction Time .......................................................... 121

3.3 CELL DISRUPTION ..................................................... 126
3.3.1 Effect of Addition of Glycine to the Fermentation .............. 126
3.3.2 Effect of Number of Passes on Protein Concentration .... 127
3.3.3 Effect of Harvest Time on Protein Concentration in Cell Free Extracts .................................................. 128

3.4 EFFECT OF SCALE-UP ON THE FERMENTATION ...... 134
3.4.1 Data from 20 L and 1500 L Fermentations ................. 134

3.5 ENZYME KINETICS ..................................................... 139
3.5.1 Experiments with 1-Chlorobutane ............................. 139
3.5.1.1 Effect of Substrate Concentration on Activity .............. 139
3.5.1.2 Lineweaver-Burk Plots ........................................ 139
3.5.2 Experiments with 4-Chlorobutanol ............................. 145
3.5.2.1 Effect of Substrate Concentration on Activity .............. 145
3.5.2.2 Lineweaver-Burk Plots ........................................ 145
3.5.3 Effect of the Amount of Cell Free Extract on Halidohydrolase Activity .................................................. 146
3.5.4 Effect of Temperature on Halidohydrolase Activity ...... 153
3.5.5 Effect of pH on Halidohydrolase Activity .................... 157
3.5.6 Substrate Inhibition .................................................. 160
3.5.7 Product Inhibition .................................................. 160
3.5.8 Stability of Halidohydrolase Activity in Cell Free Extracts... 160

3.6 RESULTS OF ENZYME IMMOBILIZATION ............162
3.6.1 Immobilization Conditions .................................162
3.6.2 Kinetics of the Immobilized Enzyme ..................163

3.7 BIOREACTOR DESIGN ........................................172
3.7.1 Characteristics of the Biotransformation ............172
3.7.2 Enzyme Production Target ...............................174
3.7.3 Design of Mini-reactors .................................175
   3.7.3.1 Design of Mini-reactor with 1-Chlorobutane
   as Substrate..................................................175
   3.7.3.2 Design of Mini-reactor with 4-Chlorobutanol
   as Substrate..................................................176

3.8 RESULTS FROM SMALL SCALE TRIALS OF TWO
MODEL SYSTEMS ..............................................177
3.8.1 Packed Bed Plug Flow Reactor .........................178
   3.8.1.1 Percentage Conversion vs Flow rate Profiles
   with Saturated 1-Chlorobutane .......................178
   3.8.1.2 Percentage Conversion vs Flow rate Profiles
   with Saturated 4-Chlorobutanol .....................179
3.8.2 Mini Batch Reactor ......................................179

4.0 DISCUSSION ................................................186

4.1 LARGE-SCALE PRODUCTION OF THE
HALIDOHYDROLASE FROM RHODOCOCCUS
ERYTHROPOLIS Y2 .............................................186

4.2 ENZYME INDUCTION .......................................191

4.3 ENZYME ISOLATION .......................................192

4.4 STATE OF THE BIOCATALYST ..............................195
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>b.p</td>
<td>Boiling point</td>
</tr>
<tr>
<td>BSTR</td>
<td>Batch stirred tank reactor</td>
</tr>
<tr>
<td>CER</td>
<td>Carbon dioxide evolution rate</td>
</tr>
<tr>
<td>CPFR</td>
<td>Continuous plug flow reactor</td>
</tr>
<tr>
<td>CSTR</td>
<td>Continuous stirred tank reactor</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DOT</td>
<td>Dissolved oxygen tension</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>E_p_F</td>
<td>Amount of enzyme in a plug flow reactor</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme-Substrate complex</td>
</tr>
<tr>
<td>EDTA</td>
<td>Di-aminoethane tetraacetic acid (disodium salt)</td>
</tr>
<tr>
<td>EEC</td>
<td>European Economic Community</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione transferase</td>
</tr>
<tr>
<td>m.p</td>
<td>Melting point</td>
</tr>
<tr>
<td>NCIMB</td>
<td>National Collections of Industrial and Marine Bacteria Limited</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen uptake rate</td>
</tr>
<tr>
<td>P</td>
<td>Power</td>
</tr>
<tr>
<td>P</td>
<td>Product</td>
</tr>
<tr>
<td>PCP</td>
<td>Pentachlorophenol</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribo-nucleic acid</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiratory quotient</td>
</tr>
<tr>
<td>S</td>
<td>Substrate</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
</tbody>
</table>
NOMENCLATURE

A  Arrhenius constant (s⁻¹)
∂  Proportion of time for batch operation (hrs)
Dₑ  Effective diffusivity (cm² min⁻¹)
Dᵢ  Impeller diameter (m)
Eₐ  Activation energy for catalytic reaction (kJ mol⁻¹)
F₁  Flow rate through impeller region (L min⁻¹)
K  Temperature and pressure dependent rate constant (kgf cm⁻²)
k  First order rate constant (μmol min⁻¹ mg⁻¹)
ka  Reaction rate constant for activation (M⁻¹ s⁻¹)
kₐa  Constant volumetric oxygen transfer rate (cm s⁻¹)
Kₘ  Michaelis Constant (mM)
Kₘ(app)  Apparent Michaelis constant (mM)
Kₛ  Monod half-saturation constant (mM)
Nᵢ  Impeller tip speed (rpm)
pI  Isoelectric point
P/V  Constant volumetric power input (W m⁻²)
q  Flow rate (L min⁻¹)
R  Amount of protein released after N passes (g L⁻¹)
R  Gas law constant (J K⁻¹ mol⁻¹)
R  Particle radius (m)
Reₜ  Reynolds number in the turbulent region
Rₘ  Maximum amount of protein available for release (g L⁻¹)
S₀  Initial substrate concentration (mM)
T  Absolute temperature (K)

13
\( t_d \) Generation time (hrs)

\( U \) Moles/min substrate converted (mol min\(^{-1}\))

\( \mu_{\text{max}} \) Maximum specific growth rate (h\(^{-1}\))

\( v \) Rate of reaction (\( \mu \)mol mL\(^{-1}\))

\( V_{\text{max}} \) Maximum reaction rate (U mgprotein\(^{-1}\))

\( X \) Percentage conversion = \( (S_0 - S/S_0) \)
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Relationship between rhodococci and closely related bacteria,</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>based on partial sequencing of 16S ribosomal ribo-nuclei acids</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>Separation of rhodococci from other genera using the</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>electrophoretic heterogeneity of ribosomal protein AT-L30</td>
<td></td>
</tr>
<tr>
<td>1.2</td>
<td>Possible pathway for the reductive dehalogenation of</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.4</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>Proposed pathway for the reductive dehalogenation of</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>tetrachloroethylene</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>Standard curve for the Bergmann-Sanik enzyme assay to</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>determine chloride release</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Standard curve for the Bergmann-Sanik enzyme assay to</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>determine chloride release</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Standard curve for the spectrophotometric determination of chloride</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>using the mercury (II) thiocyanate method</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Standard curve for the spectrophotometric determination of chloride</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>using the mercury (II) thiocyanate method</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>Standard curve for the spectrophotometric determination of chloride</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>using the mercury (II) thiocyanate method</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Standard for 1-chlorobutane in heptane with column temperature at 45 °C and substrate concentrations of 0-5 mM ....................... 90
2.4 Standard for 4-chlorobutanol in ethyl acetate with column temperature at 60 °C and substrate concentrations of 0-60 mM ....................... 91
2.5 Extraction efficiency curve for 1-chlorobutane in heptane ........ 93
2.6 Extraction efficiency curve for 4-chlorobutanol in ethyl acetate..... 94
2.7 Gas chromatograph trace of saturated solutions of 1-chlorobutane in water ................................................................. 96
2.8 Gas chromatograph traces of a diluted saturated solution of 4-chlorobutanol in water ............................................................. 97
2.9 Standard curve for protein determination in cell free extracts using the Bradford protein assay ............................................. 99
2.10 Diagram to show the model plug flow reactor system .............. 104
2.11 Bed volume profile showing % conversion of a saturated solution of 1-chlorobutane at pH 7 vs volume through packed column........ 107
2.12 Plug flow profile showing the outlet absorbance of a 1 % acetone solution through a packed bed ........................................... 108
3.0 Comparison in cell concentration against fermentation time for a culture of Y2 grown on a 2 L scale under the same conditions with two different media .............................................. 112
3.1 Results of shake flask experiments to show the effect of pH on the specific growth rate of Rhodococcus erythropolis on medium B.115
3.2 OUR, DOT and CER profiles for a 20 L fermentation of Rhodococcus erythropolis Y2 .................................................. 116
3.3 OUR and CER profiles for two successive 20 L fermentations of Rhodococcus erythropolis Y2 ........................................... 117
3.4 Plot of cell concentration and absorbance at 600 nm against fermentation time for a typical 20L fermentation of *Rhodococcus erythropolis* Y2 .......................................................... 118

3.5 Relationship between absorbance at 600 nm and bacterial concentration during a 20 L fermentation of *Rhodococcus erythropolis* Y2 .......................................................... 119

3.6 Plot of Chloride Release against time for a 20 L fermentation of *Rhodococcus erythropolis* Y2 .......................................................... 123

3.7 Chloride concentration against fermentation time for various amounts of inducer .......................................................... 124

3.8 Results using a Chlor-o-counter showing chloride concentration against time for cultures of *Rhodococcus erythropolis* Y2 induced at different times with 4-chlorobutanol .............................................. 125

3.9 Results of shake flask experiments to determine the final enzyme activity in cell free extracts for the amount of glycine added after 21 hours to a fermentation of *Rhodococcus erythropolis* Y2 .............. 129

3.10 Effect of addition time of glycine on chloride release .......... 130

3.11 Relative protein concentration against number of passes in a French pressure cell at 1.38 x 10^8 Pa .......................................................... 131

3.12 Relative protein concentration and enzyme activity against number of passes in the high pressure homogenizer ......................... 132

3.13 Protein concentration profile of 5th pass cell free extracts against fermentation time .......................................................... 133

3.14 Bacterial concentration against fermentation time for two fermentations of *Rhodococcus erythropolis* Y2 ......................... 136

3.15 Fermentation profile showing OUR, CER and DOT for a 20 L fermentation of *Rhodococcus erythropolis* Y2 ......................... 137
3.16 Fermentation profile showing OUR, CER and DOT for a 1500 L fermentation of *Rhodococcus erythropolis* Y2 .......................... 138

3.17 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 7 ......................................................... 141

3.18 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9 ......................................................... 142

3.19 Lineweaver-Burk plot showing 1/rate vs 1/substrate concentration for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 7 ....................... 143

3.20 Lineweaver-Burk plot showing 1/rate vs 1/substrate concentration for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9 ....................... 144

3.21 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 7 ......................................................... 147

3.22 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9 ......................................................... 148

3.23 Lineweaver-Burk plot showing 1/rate vs 1/substrate concentration for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 7 ....................... 149

3.24 Lineweaver-Burk plot showing 1/rate vs 1/substrate concentration for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9 ....................... 150

3.25 Activity vs amount of cell free extract for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane
solution at pH 7 ................................................................. 151

3.26 Activity vs amount of cell free extract for the halidohydrolase
from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane
solution at pH 9 ................................................................. 152

3.27 Activity vs temperature profile for the halidohydrolase
from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane
solution at pH 9 ................................................................. 154

3.28 Chloride concentration profile during enzyme assay performed at
different temperatures, for the halidohydrolase from *Rhodococcus
erthyropolis* Y2 towards 1-chlorobutane at pH 9 ................. 155

3.29 Activity vs temperature profile for the halidohydrolase
from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol
solution at pH 9 ................................................................. 156

3.30 pH profile of dehalogenase activity towards 1-chlorobutane in
clarified cell free extracts of *Rhodococcus erythropolis* Y2 grown
on 1-chlorobutane .............................................................. 158

3.31 pH profile of dehalogenase activity towards 4-chlorobutanol in
clarified cell free extracts of *Rhodococcus erythropolis* Y2 grown
on a rich yeast extract/glycerol medium (B) ......................... 159

3.32 Stability of Halidohydrolase activity in clarified cell free extracts
of *Rhodococcus erythropolis* Y2 grown on a rich yeast extract/
glycerol medium (B) towards 1-chlorobutane ......................... 161

3.33 Activity of immobilized enzyme against the pH of the binding
buffer ................................................................. 165

3.34 Immobilization of proteins onto Eupergit C via free amino
groups ........................................................................... 166

3.35 Activity against immobilization time profile ..................... 167
3.36 Activity of immobilized enzyme against the amount of cell free extract per gram of beads ............................................................. 168

3.37 Protein concentration absorbed and percentage of total protein absorbed against the amount of cell free extract per gram of beads... 169

3.38 Activity vs substrate concentration profile for the immobilized halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9 ...................................................... 170

3.39 Activity vs substrate concentration profile for the immobilized halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9 ...................................................... 171

3.40 Percentage conversion against flow rate of a saturated solution of 1-chlorobutane at pH 7 through a plug flow reactor packed with immobilized enzyme ...................................................... 180

3.41 Percentage conversion against flow rate of a saturated solution of 1-chlorobutane at pH 7 through a plug flow reactor packed with 100, 50 and 25 % percent of immobilized enzyme .......... 181

3.42 Percentage conversion against (flow rate of a saturated solution of 1-chlorobutane at pH 7)/(total amount of enzyme in the column).. 182

3.43 % conversion against flow rate of a saturated solution of 4-chlorobutanol at pH 7 through a plug flow reactor packed with immobilized enzyme ...................................................... 183

3.44 % conversion against reaction time in a batch stirred tank reactor with 5 mL of free enzyme in 45 mL of a saturated solution of 1-chlorobutane at pH 7 ...................................................... 184

3.45 % conversion against reaction time in a batch stirred tank reactor with 1 gdw of immobilized enzyme in 45 mL of a saturated solution of 1-chlorobutane at pH 7 ...................................................... 185
4.1 The relationship between first order rate constants and the operating pressure of a Manton-Gaulin homogenizer ....................... 194

4.2 Flow rate vs operation time for one continuous flow reactor compared to three continuous flow reactors in series ....................... 201

4.3 Percentage conversion of 1-chlorobutane vs the amount of haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2..... 205
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Industrial and agricultural uses of various haloalkanes</td>
<td>26</td>
</tr>
<tr>
<td>1.2</td>
<td>General characteristics of the main types of reactors</td>
<td>31</td>
</tr>
<tr>
<td>1.3</td>
<td>Comparison of the relative activities of the haloalkane dehalogenase towards common substrates</td>
<td>51</td>
</tr>
<tr>
<td>1.4</td>
<td>Relationship between properties for scale-up</td>
<td>56</td>
</tr>
<tr>
<td>1.5</td>
<td>Reasons for loss of immobilized biocatalyst productivity</td>
<td>60</td>
</tr>
<tr>
<td>3.0</td>
<td>Medium components for growth studies</td>
<td>110</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparison of bacterial concentration and specific growth rates</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>obtained on the media shown in Table 3.0</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Comparison of the properties of various inducers</td>
<td>122</td>
</tr>
<tr>
<td>3.3</td>
<td>Fermentation Conditions and Results</td>
<td>135</td>
</tr>
<tr>
<td>3.4</td>
<td>Characteristics of the Eupergit C matrix</td>
<td>166</td>
</tr>
<tr>
<td>3.5</td>
<td>Activity of immobilized enzyme against the molarity of the binding buffer</td>
<td>166</td>
</tr>
</tbody>
</table>
1.0 INTRODUCTION

Increased environmental awareness and stricter legislation (Table 1.0) has led to a realization that more needs to be done to remediate and prevent pollution. This means a clean up of existing hazardous waste sites and landfills, but more importantly the need to reduce deliberate discharge by end-of pipe or in process treatment of toxic substances in waste streams.

Moves are being made to change from traditional remediation methods to developing and employing a selection of environmental clean-up techniques. Initially, physicochemical technologies, especially physical containment, were used, as early biotechnological methods proved to be over-optimistic and unreliable. However, the enormity of the problem has meant that a more pragmatic approach has been taken in the usage of microbial metabolism in biodegradation. As a result, it is becoming an increasingly favoured technology which could prove to be a viable, cost-effective alternative for the remediation of many hazardous waste sites.

The use of preventative end-of pipe or in process treatment could remove the need for costly remediation practices. New treatment processes must be simple and cost-effective to deal with the large volumes and low unit values of waste. Thus new and improved biocatalysts will have widespread applications in industrial waste management. However, different classes of pollutants will require different approaches. Specific biocatalysts will need to be developed to treat particular environmental substances or mixtures of substances as is usual in most waste streams. In addition, mixed microbial populations might offer increased stability. Laboratory data suggest that microbes can be isolated which are capable of degrading the majority of pollutants found in toxic waste sites. However, such laboratory based biotransformations for the pollution control industry face
The text describes interesting problems of scale-up to cope with the very large volumes that need to be processed.

Table 1.0 EEC Council Directive for emission limits into rivers

(Compiled from EEC Directives Nos: 86/280, 88/347 and 90/415)

<table>
<thead>
<tr>
<th>Type of industrial plant</th>
<th>Period</th>
<th>Limit value (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon tetrachloride production by perchlorination</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Daily</td>
<td>3.0</td>
</tr>
<tr>
<td>Hexachlorobenzene production and processing</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Daily</td>
<td>2.0</td>
</tr>
<tr>
<td>Production of chloromethanes by methane chlorination</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Daily</td>
<td>3.0</td>
</tr>
<tr>
<td>Production of 1,2-dichloroethane</td>
<td></td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Daily</td>
<td>2.5</td>
</tr>
<tr>
<td>Production of perchloroethylene by perchlorination</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Daily</td>
<td>3.0</td>
</tr>
</tbody>
</table>

N.B In drinking water the emission limits for similar compounds are 3 µg/L.
In industry haloaliphatic compounds are widely used as solvents, lubricants, plasticizers, chemical intermediates and flame retardants (Hardman, 1991) (see Table 1.1). They are also used in agriculture as biocides. The total world production of chlorinated aliphatics has been estimated at $12 \times 10^9$ kg/yr (Kent, 1983).

Naturally occurring halogenated compounds are widely produced by bacteria, fungi, marine macroalgae, sponges, protozoa, marine hemichordates, plants and mammals. At least 5 million t/yr of chloromethane are released into the environment from natural sources worldwide. Some haloaliphatic compounds are toxic, carcinogenic, and resistant to degradation so tend to accumulate in the environment unless the halogen substituents are removed (Armfield, 1990).

Microorganisms have been isolated which are capable of metabolising a wide range of haloaromatics and haloaliphatics. Dehalogenation of such halosubstituted compounds can occur as the result of chemical or biological activities (Vogel et al., 1987). The haloalkane dehalogenase produced by *Rhodococcus erythropolis* Y2 specifically catalyzes the cleavage of the carbon-halogen bond thus enabling the haloalkane to be mineralized. Rhodococci are aerobic, Gram-positive actinomycetes. They are pleomorphic but often form a primary mycelium that soon fragments into bacillary and coccoid elements. Most strains grow well on standard laboratory media and are able to utilize a wide range of carbon sources for energy and growth (Goodfellow, 1986).
Table 1.1  Industrial and agricultural uses of various haloalkanes (Belkin, 1992)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloromethane</td>
<td>Intermediate, refrigerant</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Solvent</td>
</tr>
<tr>
<td>Trichloromethane</td>
<td>Intermediate, solvent</td>
</tr>
<tr>
<td>Chlorobromomethane</td>
<td>Intermediate, fire extinguisher, solvent</td>
</tr>
<tr>
<td>Bromomethane</td>
<td>Soil and space fumigant, intermediate</td>
</tr>
<tr>
<td>Dibromomethane</td>
<td>Intermediate (agrochemicals and biocides)</td>
</tr>
<tr>
<td>Chloroethane</td>
<td>Intermediate, solvent, refrigerant</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>Solvent, intermediate (dyes, perfumes)</td>
</tr>
<tr>
<td>1,1,1-Trichloroethane</td>
<td>Solvent</td>
</tr>
<tr>
<td>1,1,2-Trichloroethane</td>
<td>Solvent</td>
</tr>
<tr>
<td>1,1,2,2-Tetrachloroethane</td>
<td>Solvent</td>
</tr>
<tr>
<td>Hexachloroethane</td>
<td>Solvent, explosives, rubber vulcanization</td>
</tr>
<tr>
<td>Bromoethane</td>
<td>Ethylating agent, refrigerant</td>
</tr>
<tr>
<td>Dibromoethane</td>
<td>Anti-knock additive, soil fumigant, solvent</td>
</tr>
<tr>
<td>1-Bromopropane</td>
<td>Intermediate (Pharmaceuticals, insecticides)</td>
</tr>
<tr>
<td>1,2-Dichloropropane</td>
<td>Soil fumigant, solvent</td>
</tr>
<tr>
<td>1,2 Dibromo-3-chloropropane</td>
<td>Soil fumigant</td>
</tr>
<tr>
<td>1-Bromobutane</td>
<td>Intermediate (Pharmaceuticals)</td>
</tr>
<tr>
<td>1-Bromoocytane</td>
<td>Intermediate (UV absorbers, dyes)</td>
</tr>
</tbody>
</table>
1.1 MICROBIAL BIOTRANSFORMATIONS

1.1.1 Introduction to Biotransformations

Biotransformations have traditionally dealt with the synthesis of novel organic molecules but they can also be used for degradation of toxic and environmentally harmful compounds, such as haloalkanes. The use of biotransformation technology in a degradative mode affects the selection and design of the reactor, partly dependent on the kinetics of the process at very low substrate concentrations.

Biotransformations occur when a product is obtained from a substrate through one or more reactions catalyzed by enzymes in the cells. In this project, the enzymes are used to degrade the substrate, the haloalkane and thus enable it to be mineralized to a safer product. Biotransformations may be carried out using the enzyme in a number of forms, as whole cells or isolated enzymes or as immobilized cells and enzymes. When developing a biotransformation process, biocatalyst selection and preparation, biotransformation, product isolation and recovery all need to be considered.

1.1.2 Biocatalysts

Biocatalysis involves the catalytic conversion of one compound to another using a microorganism, plant or animal cell whether growing, resting or as a cell extract in a crude or purified form.

Biocatalysts are theoretically of great industrial potential but there seem to be only a small number of commercial uses. The disadvantages of biocatalysts lie in their lack of commercial availability, operational stability and the economic comparison with competing chemical technology. However, some of the major advantages of enzymes are their high selectivity, high efficiency and activity under mild reaction conditions and their ability to function in non-aqueous media. In addition, the
introduction of a biocatalytic step into a reaction pathway may allow for a reduction in the total number of steps required. The biocatalysts with the most short term potential are the non-cofactor requiring enzymes, the most versatile group of biocatalysts being the hydrolases.

The greatest obstacle for the increased use of biocatalysts are the inherent disadvantages of biocatalysts, the existence of efficient chemical technology and regulations about changing current pharmaceutical production processes. As a result, few biocatalytic processes have been commercialized and those that have involve a single reactant of low molecular weight and high solubility in water. Biocatalysts should not therefore be used as replacements but as a new type of catalysts for which new uses and processes must be developed.

1.1.3 Characteristics of the Biotransformation for Process Design

In the design of a biotransformation process, several factors need to be considered. The properties of the substrate and product, the characteristics of the reaction and biocatalyst, and the interactions between the substrate and product and the biocatalyst must all be taken into account (Woodley and Lilly, 1994).

It is first necessary to identify the state of the substrate and product under reaction conditions, be they water-soluble or poorly water-soluble gases, liquids or solids. An understanding of their melting and boiling points is therefore essential (Freeman et al., 1993). The water-solubility of the substrate and product must also be determined as biocatalysts have been shown to be active in non-aqueous media. Some catalysts require water to operate and this must be assessed if the medium composition is to be correct. In addition, the operational pH and temperature can be determined by the stability of one or more of the reaction components.
An understanding of the reaction characteristics is also an important factor in the design of a biotransformation process. The equilibrium of the reaction is pH and temperature dependent and these may be adjusted to obtain the desired fractional conversion. Many biotransformations are accompanied by a change in pH and this may affect the selection and operation of the bioreactor. If the substrate or product is a gaseous substance, its consumption or production may need to be controlled. To reduce the amount of gas phase in the reactor, either an enriched gas phase may be used or the pressure in the reactor could be increased to dissolve some of the gas in solution.

The characteristics of the biocatalyst, especially its intracellular or extracellular nature, will determine the method of catalyst production and whether the enzyme should be used in the whole cell, as an isolated enzyme or in an immobilized form. Knowledge of the site of reaction is important for reactor selection and scale-up, in particular where a reaction occurs at the interface and a balance must be struck between catalyst activity and stability.

Finally, the interactions between biocatalyst, substrate and product must be considered. An understanding of substrate and product inhibition and substrate activity dependent on substrate concentration, pH and temperature must be gained. In addition, substrate and product toxicity and catalyst activity and stability as a function of substrate and product concentration, pH and temperature should be determined.

1.1.4 Choice of Reactor System

The choice of a reactor system for a biotransformation depends on many factors. The criteria for selection depend on the characteristics of the reaction, the properties of the substrate and product, the physical properties of the biocatalyst and finally the
characteristics of the reactor. These criteria can be illustrated by comparing three basic types of reactor: batch stirred tank; plug-flow (usually a packed bed); and a continuous stirred tank reactor (CSTR) as in Table 1.2. The choice will be influenced by the reactor output requirement. If this is small then a CSTR will not be economical. A batch system is probably the most flexible and cost effective.

The operational requirements of the reaction may also restrict the choice of reactor. Temperature and pH can be more easily controlled in a well-mixed stirred tank reactor. As the reaction proceeds, it may be necessary to feed substrate and this can be achieved most easily in a batch or tubular reactor. In addition, if the feed contains undissolved solids, a packed bed reactor cannot be used. Where the enzyme is reused, the reactor choice will be limited to those where retention systems can be fitted.

The most commonly used reactors for biotransformations are the well-mixed stirred tank batch reactor and the plug-flow or packed bed reactor. CSTRs have rarely been used except in biotransformations where the reactor is designed to give a series of mixed regions. This is mainly due to the fact that for a first order reaction, CSTRs require nearly twenty times as much biocatalyst as the batch or plug-flow reactor to carry out the same reaction to 99% conversion.
<table>
<thead>
<tr>
<th>Table 1.2  General Characteristics of the main types of reactors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Woodley and Lilly, 1994)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
1.2 INTRODUCTION TO THE GENUS *Rhodococcus* AND RELATED ORGANISMS

1.2.1 Introduction

The identification and classification of the genus *Rhodococcus* has been largely based on their distinctive cell-wall composition, often containing long-chain mycolic acids and tuberculostearic acid (Finnerty, 1992). The partial sequencing of 16S RNA has enabled the supragenic relationships of actinomycetes to be established, (Figure 1.0), placing *Rhodococcus, Nocardia and Mycobacterium* among the nocardioform actinomycetes, (Goodfellow, 1989). *Corynebacterium, Gordona* and *Tsukamurella* are closely related organisms, and several species have been moved among these genera. For example, Ochi (1992) has separated *Gordona* and *Tsukamurella* from *Rhodococcus* by measuring the electrophoretic heterogeneity of ribosomal protein AT-L30 (Figure 1.1).

Due to their persistence in the environment, their apparent lack of catabolite repression and their tolerance to starvation, rhodococci have been found to be useful in bioremediation tests. They display a diverse range of metabolic capabilities and are able to degrade short-chain, long-chain, and halogenated hydrocarbons, and numerous aromatic compounds, including halogenated and other substituted aromatics, heteroaromatics, hydroaromatics and polycyclic aromatic hydrocarbons (Warhurst and Fewson, 1994). They possess a variety of pathways for degradation and modification of aromatic compounds, including monooxygenase and dioxygenase ring attack, and cleavage of catechol by both ortho- and meta- routes.

Rhodococci are able to produce emulsifiers and flocculants (Finnerty, 1992) and these may be important as commercial products and as necessary components of rhodococcal biotransformations. *Rhodococcus erythropolis* S-1 produces a
bioflocculant, NOC-1 (Kurane et al, 1986), which is able to flocculate a wide range of materials including kaolin clay, yeast and livestock water.

1.2.2 Kinetics of Bacterial Growth on Chlorinated Aliphatic Compounds

Biological removal of halogenated aliphatic compounds is an option provided that efficient elimination of these potentially recalcitrant substrates can be achieved. The elimination efficiency depends on the process technology but more importantly on the degradation properties of the bacterial strains employed. Important aspects of bacterial growth are the Monod half-saturation constant (\(K_g\)) and the maximum growth rate (\(\mu_{\text{max}}\)). In a process where the concentration of the growth substrate and \(K_g\) values are of the same order of magnitude, the degree of elimination is influenced by the \(K_g\) value. The kinetic parameters also affect the rate of the removal in biofilms and thus influence reactor design. Wijngaard et al (1993) described the kinetics of growth of *Ancylobacter aquaticus* AD20 and AD25, *Xanthobacter autotrophicus* GJ10 and *Pseudomonas* sp. strain AD1. Monod kinetics were observed during growth in chemostat cultures on 1,2-dichloroethane, 2-chloroethanol and 1,3-dichloro-2-propanol. The \(K_g\) values of strains GJ10, AD20 and AD25 for 1,2-dichloroethane were 260, 222 and 24 \(\mu\)M respectively. The results indicated that the efficiency of chlorinated compound removal was influenced by the kinetic properties and the cellular content of the first catabolic enzyme.

This study aimed to determine the growth parameters and the kinetics of the halidohydrolase produced by *Rhodococcus erythropolis* under various conditions and with different substrate concentrations in order to determine the reactor design.
Figure 1.0 Relationship between rhodococci and closely related bacteria, based on partial sequencing of 16S ribosomal ribo-nucleic acids (Goodfellow, 1989).
Figure 1.1  Separation of rhodococci from other genera using the electrophoretic heterogeneity of ribosomal protein AT-L30 (Ochi, 1992).
1.3 HALOALKANE DEHALOGENASES

Halogenated alkanes can function as carbon and energy sources, cometabolic substrates and as electron acceptors in bacteria. In general, organic compounds act as electron donors and undergo oxidation. However, due to the electronegative character of the halogen substituents on aliphatic compounds, polyhalogenated alkane compounds often behave as electron acceptors and are therefore reduced.

Haloalkanes, especially the short-chain molecules, are easily transported through the soil by groundwater resulting in aquifer contamination. Usually, C_{10-18} straight chain haloalkanes are the most amenable to biotransformation, although the gaseous C_{1.4} and liquid C_{5.9} haloalkanes have been studied more frequently because of their environmental importance and their significance for public health.

Hydrolytic removal of halogen substituents is commonly the first step in the degradation of haloaliphatic compounds by aerobic bacteria. Hydrolytic dehalogenations are catalyzed by specific dehalogenases, a group of enzymes which has been extensively studied in bacteria and which does not seem to occur in mammals. The application of bacteria possessing hydrolytic dehalogenases comprises two major fields: treatment processes for industrial waste streams and biotransformations for the production of chiral building blocks (see Section 1.10.3).

1.3.1 Anaerobic Transformations

The role of anaerobic microorganisms has been demonstrated in the rapid removal of chloroforms and tribromomethanes from anaerobic aquifers. Methanogenic bacteria are anaerobes which use formate for growth. Pure cultures of methanogenic bacteria have been reported to metabolize halogenated hydrocarbons. Mixed cultures of acetate-utilizing methanogens, exposed to C_{1} and C_{2} halocarbons can cometabolize
haloalkanes, thus allowing them to be mineralized. Vogel and McCarty (1985) proposed a reductive dehalogenation of tetrachloroethylene (Figure 1.2).

Belay and Daniels (1987) first reported the metabolism of halogenated hydrocarbons by pure cultures of methanogenic bacteria. They suggested that degradation was due to both dehydrohalogenation and reductive dehalogenation reactions which could occur independently or together depending on the substrate. In the case of bromoethane, conversion could occur via a dehydrodehalogenation (Equation 1.0) to produce ethylene or by reductive mechanism (Equation 1.1) to produce ethane.

(Equation 1.0)
Dehydrodehalogenation

\[
\begin{align*}
\text{Dehydrodehalogenation} \\
X - C - C - X & \rightarrow X - C - C + XH \\
\end{align*}
\]

(Equation 1.1)
Reductive dehalogenation

\[
\begin{align*}
\text{Reductive dehalogenation} \\
X - C - C - X + H_2 & \rightarrow X - C - C + HX \\
\end{align*}
\]
Figure 1.2 The possible pathway for the reductive dehalogenation of tetrachloroethylene (Vogel and McCarty, 1985).
In addition to methanogens, reductive dehalogenation of highly chlorinated aliphatics by other strict anaerobes has been reported. The sulphate-reducing organism *Desulphobacterium autotrophicum* converts 1,1,1-trichloroethane to 1,1-dichloroethane (Egli *et al*., 1987) and a *Clostridium* sp., an anaerobic heterotroph transformed 1,1,1-trichloroethane and carbon tetrachloride by reductive dehalogenation. Egli *et al* (1988) also investigated the reductive dechlorination of carbon tetrachloride by *Acetobacterium woodii* and four other anaerobes and showed that the reduction of chlorinated methanes only occurred in organisms which possessed the acetyl-CoA pathway (Figure 1.3).

The use of methanogens in anaerobic and aqueous environments for biodegradation is therefore possible where halogenated compounds occur at low concentrations. Further biodegradation of the dechlorinated products may then occur under anaerobic conditions.

### 1.3.2 Aerobic Dehalogenation of Haloalkanes

Isolation of organisms able to use haloalkanes as carbon and energy sources has been shown to be extremely difficult (Omori and Alexander, 1978). They isolated aerobic bacteria that utilize halosubstituted C\textsubscript{1} and C\textsubscript{2} compounds but differ from methanogens in three ways; they utilize the haloalkane directly for growth; they can withstand high concentrations of substances; and they have lower degradation rates than methanogens. Janssen *et al* (1988) reported that out of seven chlorinated aliphatic hydrocarbons tested, only trans 1,2-dichloroethene was relatively non-toxic to a mixed methanogenic culture. Batch enrichment techniques have also isolated aerobic bacteria that utilize long-chain haloalkanes.
Figure 1.3 Proposed pathway of carbon tetrachloride degradation in bacteria that possess the acetyl-CoA pathway.

Key
1. Sequential Reductive Dehalogenation
2. Substitutive Transformation
3. CO$_2$ Assimilation by the Acetyl-CoA Pathway
Initial studies (Anders, 1982) showed that prokaryotes and eukaryotes possess two catalytic mechanisms for carbon-halogen bond cleavage of haloalkanes, either reductive or oxidative. These reactions are catalyzed by a glutathione-dependent nucleophilic replacement catalyzed by glutathione-S-transferase or by cytochrome P<sub>450</sub>. Recent studies (Janssen et al, 1987) have isolated enzymes known as haloalkane halidohydrolases that catalyze hydrolytic dehalogenation. The choice of enrichment substrate affects the selection of the dehalogenase types and hydrolytic dehalogenases are selected by substrates with chain lengths of C<sub>1</sub>-C<sub>4</sub>.

1.3.2.1 Glutathione-Dependent Dehalogenases

*Pseudomonas sp.* DM1 was shown to convert dichloromethane to formaldehyde by a mechanism that was strongly induced by dichloromethane but was not detectable in the cell-free extracts (Brunner et al, 1980). Stucki et al, (1981) described an inducible, glutathione-dependent enzyme that converted DCM to formaldehyde and inorganic chloride in cell-free extracts of *Hyphomicrobium sp.* DM2. (Figure 1.4). The dehalogenase activity was strongly inducible and was strictly dependent on GSH. A number of glutathione-S-transferases have been detected although none has yet been purified.

A new strain of methylotrophic bacterium DM1 has been isolated that uses DCM or dibromomethane as sole sources of carbon and energy (Scholtz et al, 1988). This strain demonstrated clear structural and catalytic differences from the original DCM isolates. The characterization of a new enzyme activity led to the suggestion that there are two groups of glutathione-dependent dehalogenases; group A represented by isolates DM2 and DM4 and group B by DM1.
These enzymes differ from those bacterial glutathione transferases recently isolated from a number of bacterial strains. (Piccolomini et al, 1989). The bacterial GSTs were all dimeric proteins composed of 2 subunits with a molecular weight of 22.5 kd and formed between 0.002-0.06 % of the total cytosolic protein with at least two classes of GSTs having a role in defence against the effect of antibiotics.

1.3.2.2 Oxygenase-type Dehalogenases

These enzymes have a broad substrate range and catalyze the dehalogenation of haloaromatic, haloalkane and haloalkanoic acids. The methane monooxygenases usually function to oxidize methane to methanol via a NADH, oxygen dependent reaction. Alkanes up to pentane are oxidized at similar rates, although an increase in the size or number of halogen substituents does have an adverse effect on the rate of oxidation.

Methane monooxygenases exist in a soluble or particulate, membrane-bound form, and the distribution between both forms has been shown to be dependent on growth conditions. The methane-oxidizing bacterium H2, oxidized mono- and dichloromethanes to the corresponding acids and haloacids, which may then become substrates for 2-haloacid dehalogenases in the environment. Some monooxygenases act as terminal alkane hydroxylases while others produce a mixture of 1- and 2- alcohols from n-alkanes.

Higgins et al, (1979) reported that the methane monooxygenase of Methylosinus trichosporium OB3 catalyzed a wide range of reactions including condensations, oxidations and the dehalogenation of halogenated methanes and chlorophenols.
Figure 1.4  Glutathione-dependent dehalogenation of dichloromethane by *Hyphomicrobium* sp. DM2 (Stucki et al., 1981).

\[
R-\overset{\bigcirc}{C}-\overset{\bigcirc}{C}l + O_2 + NAD(P)H \rightarrow \text{ALKANE MONOOXYGENASE}
\]

\[
\left[ R-\overset{\bigcirc}{C}-\overset{\bigcirc}{C}l \right] + H_2O + NAD(P) \rightarrow \text{SPONTANEOUS}
\]

\[
R-\overset{\bigcirc}{C}=O + HCl \rightarrow \text{DEHYDROGENASE}
\]

\[
R-\overset{\bigcirc}{C}=O \rightarrow \text{DEHYDROGENASE}
\]
Methane monooxygenases could be isolated as particulate or soluble enzymes in methylotrophs depending on their growth conditions (Stanley et al., 1983). The particulate form differed in its substrate specificities, in that it could not oxidize aromatic or higher alkanes whereas the soluble enzyme could.

Yokota et al. (1986) reported the failure of resting cell suspensions of the hydrocarbon-utilizing bacterium *Pseudomonas butanovara* to dehalogenate haloalkanes under anaerobic conditions and so concluded that an oxygenase was involved in the dehalogenation of haloalkanes. The alkane monooxygenase, induced by the n-alkane moiety catalyzed n-terminal dehalogenation of haloalkanes to aldehydes which were subsequently converted to the corresponding acids by dehydrogenases (Figure 1.5).

An oxygenase is produced by *Rhodococcus erythropolis* Y2 using 1,9-dichlorononane or 1-chlorohexadecane as enrichment substrate (Omori and Alexander, 1978). This enzyme is induced by C$_7$ to C$_{16}$ 1-haloalkanes and n-alkanes. The oxygenase activity dehalogenated C$_4$ to C$_{19}$ 1-chloroalkanes with an optimum activity towards 1-chlorotetradecane. (Armfield et al., 1990) By catalyzing the initial dehalogenation, the enzyme provides metabolites that can feed directly into the beta-oxidation cycle.
Figure 1.5  Proposed scheme for the dechlorination of 1-chloroalkanes by the n-alkane monooxygenase (Yokota et al., 1986).
1.3.2.3 Haloalkane Halidohydrolases

Haloalkane halidohydrolases are enzymes that are capable of hydrolytic cleavage of carbon-halogen bonds by catalyzing a nucleophilic substitution with water. Recently it has been found that several chlorinated hydrocarbons of environmental and toxicological relevance may be converted by hydrolytic dehalogenation in bacteria. A hydrolytic dehalogenase with activity towards 1,2-dichloroethane, 1,2-dibromoethane, 3-chloropropane and methylchloride was described in a strain of Xanthobacter autotrophicus that utilizes chlorinated aliphatics for growth (Janssen et al. 1987) (Figure 1.6). This dehalogenase was found to be a polypeptide with a molecular mass of 36 kDa showing activity toward various C₁ to C₄ 1-halogenated or n-alkanes, and in common with other halidohydrolase-type dehalogenases required no co-factors or oxygen for activity (Janssen et al., 1988).

Recently, Verschueren et al. (1993a) have performed crystallographic and fluorescence studies on the interaction of the haloalkane dehalogenase with halide ions. The crystal structure of the enzyme has been determined at 1.9 Å resolution at two different pH values, the pH of crystallization (pH 6.2) and the pH of optimal activity (pH 8.2). The putative active site is located between the two domains of the protein in an internal, predominantly hydrophobic cavity. Asp₁₂₄, His₂₈₉, Asp₂₆₀, which are located in this cavity, have been proposed to be the catalytic residues. The other residue, Asn₁₄₈, helps stabilize the conformations of two of these active site residues. Comparison of the models at pH 6.2 and pH 8.2 revealed one major structural difference. At pH 6.2, a salt-bridge is present between the N atom of His₂₈₉ and the O atom of Asp₁₂₄, while at pH 8.2, the salt-bridge is absent, indicating that the N atom of the histidine residue is deprotonated at the pH of optimum activity. Crystal structures of the dehalogenase enzyme complexed with iodoacetamide, chloroacetamide, iodide and chloride revealed a halide binding site.
between the ring NH's of two tryptophan residues, Trp\textsubscript{125} and Trp\textsubscript{175}, located in the active site. The halide ion lies on the intersection of the planes of rings of the tryptophans. Figure 1.7 shows the putative reaction mechanism in which the O atom of Asp\textsubscript{124} performs a nucleophilic attack on the substrate, resulting in an intermediate ester (Verschueren et al., 1993b). Subsequently, an hydrolytic water molecule, activated by His\textsubscript{289}, cleaves this intermediate, releasing the alcohol product and restoring the Asp\textsubscript{124} side-chain.

The isolation and characterization of haloalkane halidohydrolases have also been reported from Corynebacterium sp. strain m15-3 (Yokota et al., 1986); Arthrobacter sp. HA1 (Scholtz et al., 1987) and Rhodococcus erythropolis Y2 (Sallis et al., 1990). The biochemical characteristics of these organisms are all similar. The enzymes all have pH optima ranging from pH 9.2 to 9.5 and are monomeric proteins with a molecular weight of 34 to 36 kDa. They also have similar isoelectric points and activation energies.

H\textsuperscript{+} ions are produced in this reaction and thus a drop in pH is observed during fermentation. To control the pH, alkali is added to the fermentation and the choice of alkali has implications for the reactor choice.

Comparison of substrates common to all of the haloalkane halidohydrolases indicates that the enzyme from X. autotrophicus GJ10 is different to the others with respect to relative substrate activities. The type of haloalkane halidohydrolase isolated may depend on the choice of enrichment substrate. X. autotrophicus GJ10 was isolated on 1,2-dichloroethane whereas the others were isolated on 1-chlorobutane or 1-chlorohexane.
Figure 1.6  Suggested pathway for the metabolism of 1,2-dichloroethane by *Xanthobacter autotrophicus* GJ10 (Janssen et al, 1987). The reaction of interest is the one catalyzed by the haloalkane halidohydrolase. It should be noted that no cofactors or oxygen are required for this reaction.
Figure 1.7  Reaction mechanism at the optimal pH (pH 8.2) of haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 as deduced from the crystal structures of the native enzyme without substrate (Verschueren et al. 1993c).
The five haloalkane halidohydrolases already isolated and purified show substrate activity towards more than sixty haloalkanes and some haloalcohols.

The producer organisms are readily isolated from soil using batch-enrichment cultures. These isolated aerobic bacteria are more beneficial than methanogens in that they can withstand higher concentrations of substrates and they have lower degradation rates. In addition they are able to utilize the haloalkane directly for growth. All of these properties have fuelled a growing interest in research into haloalkane halidohydrolases and their producer organisms.

1.4 HALOALKANE HALIDOHYDROLASE PRODUCED BY

*Rhodococcus erythropolis* Y2

*Rhodococcus erythropolis* Y2, capable of utilizing 1-chlorobutane as its sole source of carbon and energy, was isolated by batch enrichment culture from a soil sample previously exposed to haloalkanes (Sallis et al, 1990). In addition to growth on 1-chlorobutane, *Rhodococcus erythropolis* Y2 can use the following as growth substrates; 1-chloropropane, 1-chloropentane, 1-chlorohexane, 1-chlorododecane, 1-chlorotetradecane, 1-chlorohexadecane and 1-chlorooctadecane. The dehalogenase was inducible and active towards a larger range of halogenated aliphatic compounds than would support growth of the organism. The enzyme also catalyzed the cleavage of a number of mono- and di-substituted chloro- and bromoalkanes with carbon chain lengths from C1-C16. The product of its activity towards 1-chlorobutane was n-butanol, and towards 4-chlorobutanol, the product was 1,4-butanediol (Equations 1.2 and 1.3). This indicates a hydrolytic dehalogenation catalyzed by the enzyme. Table 1.3 shows a comparison of the relative activities (standardized with respect to 1-chlorobutane degradation rates) of the haloalkane dehalogenase towards common substrates.
Table 1.3 Comparison of the relative activities (standardized with respect to 1-chlorobutane degradation rates) of the haloalkane dehalogenase towards common substrates. (Sallis et al, 1990).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloropropane</td>
<td>8</td>
</tr>
<tr>
<td>1-chlorobutane</td>
<td>100</td>
</tr>
<tr>
<td>1-bromobutane</td>
<td>100</td>
</tr>
<tr>
<td>1-chloropentane</td>
<td>132</td>
</tr>
<tr>
<td>1-chlorohexane</td>
<td>107</td>
</tr>
<tr>
<td>1-bromohexane</td>
<td>66</td>
</tr>
<tr>
<td>1-chloroheptane</td>
<td>104</td>
</tr>
<tr>
<td>1-chlorooctane</td>
<td>72</td>
</tr>
<tr>
<td>1-chlorononane</td>
<td>77</td>
</tr>
<tr>
<td>1-chlorodecane</td>
<td>85</td>
</tr>
<tr>
<td>1-chlorododecane</td>
<td>53</td>
</tr>
<tr>
<td>1-chlorotetradecane</td>
<td>10</td>
</tr>
<tr>
<td>1-chlorohexadecane</td>
<td>8</td>
</tr>
<tr>
<td>1-chlorooctadecane</td>
<td>0</td>
</tr>
<tr>
<td>1,2-dichloromethane</td>
<td>6</td>
</tr>
<tr>
<td>1,2-dibromoethane</td>
<td>802</td>
</tr>
<tr>
<td>1,3-dichloropropane</td>
<td>202</td>
</tr>
<tr>
<td>1,4-dichlorobutane</td>
<td>232</td>
</tr>
<tr>
<td>1,6-dichlorohexane</td>
<td>168</td>
</tr>
<tr>
<td>1,9-dichlorononane</td>
<td>61</td>
</tr>
<tr>
<td>2-chlorobutane</td>
<td>3</td>
</tr>
<tr>
<td>2-bromobutane</td>
<td>67</td>
</tr>
<tr>
<td>1,2-dichloropropane</td>
<td>4</td>
</tr>
<tr>
<td>1,2-dibromopropane</td>
<td>132</td>
</tr>
<tr>
<td>2-chloroethanol</td>
<td>15</td>
</tr>
<tr>
<td>3-chloropropan-1-ol</td>
<td>18</td>
</tr>
<tr>
<td>4-chlorobutan-1-ol</td>
<td>121</td>
</tr>
<tr>
<td>Bis(2-chlorobutan) ether</td>
<td>60</td>
</tr>
</tbody>
</table>
The dehalogenase showed an optimum activity at pH 9.2 and retained more than 50% of its maximum activity over a pH range of 7.5-11.0, but was very low at pH 5-6. Activity increased with assay temperature. Sallis et al. (1990) reported that the enzyme had an isoelectric point of 4.7, an activation energy of 43 kJ/mol, a $K_m$ of 0.26 mM and a $V_{max}$ of 0.38 for 1-chlorobutane. Its molecular mass is 36 kDa and it is a monomeric protein. The N-terminal sequence for the first twenty residues of the purified enzyme is as follows: Ser-Glu-Ile-Gly-Thr-Gly-Phe-Pro-Phe-Asp-Pro-His-Tyr-Val-Glu-Val-Leu-Gly-Glu-Arg. The presence of similar haloalkane dehalogenases in the environment suggests that they have not evolved recently. The possibility of parallel evolution from a common ancestral protein or the spread of dehalogenase genes or plasmids is a plausible explanation for this homology.

1.4.1 Effect of Glycine
Due to the resistant nature of the Gram-positive cell to disruption, it is necessary to look at ways of weakening the cell wall to facilitate enzyme extraction. Sallis et al. (1990) showed that *Rhodococcus erythropolis* Y2 was insensitive to lysozyme, but subsequent studies showed that growth in the presence of 2% (w/v) glycine and the use of freeze thaw cycles rendered the isolate slightly sensitive to the action of lysozyme.
Other work with glycine has indicated that it has the ability to alter cell wall structure and increase protein production. Miyashiro et al (1980) demonstrated that glycine and L-isoleucine were prominent in stimulating protein production in Bacillus brevis, although the mode of action of each was different. Glycine made cells more sensitive to lysozyme and caused a large decrease in the alanine content of the cell wall fraction. These findings supported the possibility that glycine alters cell wall structure in such a way as to facilitate protein excretion. Hara et al (1983) added 1.5 % glycine to the growth medium of Bacillus mesentericus niger. The results suggested that glycine might stimulate protein excretion as well as protect the excreted protein from protease.

1.5 SCALE-UP

Rhodococcus erythropolis has previously been grown on a small scale, up to 2 L, and therefore the effects of scaling-up the fermentation were not known. In addition, it was considered to be important to demonstrate the feasibility of the proposed biotransformation by developing a pilot plant scale fermentation. For these reasons and to produce sufficient amounts of enzyme for the study, a large scale process has been developed.

The scale-up of this system followed a similar pathway to that shown in Figure 1.8, where a 1% (v/v) inoculum was used whenever possible.

Scale-up means reproducing the optimized small scale environmental conditions on a larger scale. It was first necessary before scaling-up to ensure that the process was scaleable. This meant that data needed to be obtained from fermentations performed on a small scale in a stirred tank fermenter and not on a shake flask scale. In this project the optimized small scale process (20 litres) was scaled-up to 1000 litres. The conditions required for growth in a successful biochemical process have been
established in a laboratory fermenter. These include pH, temperature, nutrient concentration, oxygen concentration and shear rate. Scale-up involved maintaining these conditions independent of vessel volume. If conditions were the same, and if no mutations occurred which might have caused the growth kinetics or the metabolic products to change, the production rate per unit volume should have been the same in the large and small scale systems.

Figure 1.8 Typical pathway for the scale-up of a process to 1000 litres
The criteria for scale-up generally differs for chemical reactors and bioreactors. The following have been proposed for bioreactor design and were considered when calculating the fermentation conditions when scaling up from 20 litres to 1500 litres:

- Constant impeller tip speed = \( \pi \times \text{impeller speed} \times \text{impeller diameter} \) (Equation 1.4)
  Impeller tip speed determines the maximum shear rate. This may then influence both the size of the microbial floc or maximum stable bubble size and any damage to viable cells.

- The Reynolds number in the turbulent region, \( Re_t \), determines the root mean square velocity, \( u_{rms} \), and thus the bubble mass transfer coefficients.

- Constant volumetric power input (P/V). This value tends to decrease with increased scale. Using this value through the turbulent Reynolds region enables mass transfer coefficients and particulate sizes to be determined. It also allows the size of motor needed during fermentation to be calculated.

- Equal mixing times. In practice, mixing time increases with scale. If it is a well-mixed vessel, then there is a characteristic circulation time. If the liquid recirculation flow rate \( F_i \) through the impeller region varies as a cross-sectional area \( \pi D_i^2 \) and the average impeller velocity in the tank varies as \( N_i D_i \), then \( F_i / V \) is proportional to \( N_i D_i^3 / D_i^3 \) which equals \( N_i \).

- Constant volumetric oxygen transfer rate or \( k_L a \). This is a useful constant if oxygen transfer is critical but it may be difficult to achieve in large volumes. When the \( k_L a \) value is the same, other mixing and flow characteristics such as circulation time or maximum shear rate are not the same in vessels of different scale. Table 1.4
illustrates an example of scale-up from a small scale 80 L to a large scale 10,000 L agitated bioreactor (Oldshue, 1966).

Table 1.4  Relationship between properties for scale-up (Oldshue, 1966)

<table>
<thead>
<tr>
<th>Property</th>
<th>80 L</th>
<th>Large scale, 10⁴ L</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>1.0</td>
<td>125</td>
</tr>
<tr>
<td>P/V</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ni</td>
<td>1.0</td>
<td>0.34</td>
</tr>
<tr>
<td>Di</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Fi</td>
<td>1.0</td>
<td>42.5</td>
</tr>
<tr>
<td>Fi/V</td>
<td>1.0</td>
<td>0.34</td>
</tr>
<tr>
<td>NiDi</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Rei</td>
<td>1.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>

N.B The bold numbers are the parameters that were kept constant at that value and the other values are those that resulted from this action.

If geometrical similarity is maintained between scales, it is not usually possible to use more than one criteria for scale-up. It is therefore necessary to choose, as a scale-up basis, the transport property most critical to the performance of the particular bioprocess. This may prove difficult, given the sensitive and diverse responses of bacteria to each of the transport phenomena.

To achieve successful scale-up of the enzyme production system, it is also necessary for the design and type of reactor to be considered.
1.6 STATE OF BIOCATALYST

Having established a large scale process for its production, it is necessary to decide on the state of the catalyst, whether the whole cell or the isolated enzyme should be used, and whether that cell or enzyme should be in a free or immobilized form (Figure 1.9). If immobilization is chosen, an appropriate immobilization technique must be found for the system.

Biotransformations may be performed using whole cells or isolated enzymes as biocatalysts. Improvements in extraction and isolation technologies have meant that the use of intracellular enzymes is a viable option. The use of the isolated enzyme can be beneficial in many ways. Downstream processes may require a less turbid solution than would be obtained if whole cells were used, and as immobilized cells, growth of the cells during reaction may be a problem which may lead to diffusional limitations and release of cells into the broth.

The choice between the free and immobilized forms of an enzyme depends on the nature of the biotransformation and the relative operational stabilities of the two forms. In general, enzymes would appear to show little difference in their thermal stability whether in vivo, isolated in dilute solution, or immobilized. In practice, there is not much difference between the immobilized cell or immobilized purified enzyme as far as stability is concerned. One advantage in the use of the immobilized purified enzyme, as opposed to the immobilized cell, is that it is possible to obtain higher loadings of catalyst. The maximum activity per gram of solid can be ten times that of a preparation of immobilized cells from which the enzyme was obtained. However, immobilized catalysts of high activity are often subjected to a diffusion limitation which may result in inefficient use of the catalyst. As the purification process is a costly one, the resulting enzyme should ideally be used at 100% efficiency (Trevan, 1980). The use of immobilized cells is not a viable proposition.
Figure 1.9 Possible pathways leading to biotransformation
With the introduction of new separation technology, it is now possible to prepare intracellular microbial enzymes on the scale required for their use as industrial catalysts. Whether the effort involved in the purification is justified may depend on the enzymic activities demonstrated by the microbial cell.

The most important factor affecting the selection of an immobilized enzyme process is one of cost. If the enzyme is readily available at a low cost, the expense of immobilization may not be justified. Where the enzyme is expensive, the extra cost of immobilization, and therefore the ability to reuse the catalyst, will be less than continually adding more enzyme to the system. In fact, if the immobilization cost equals the production cost of the enzyme, reuse becomes economic after two uses. All immobilized enzymes are generated for the equivalent of at least fifty uses.

The stability of the enzyme is also an important consideration in relation to the average residence of substrate within the reactor. A balance must be achieved between flow rate, reactor volume, and enzymic activity. Ideally, the immobilized enzyme particle must have the maximum possible enzyme loading that will not induce significant diffusional limitations (Chibata and Tosa, 1976). Another consideration is that of the operational half-life of the system. The immobilized enzyme must be sufficiently stable under operational conditions or it will continually need replacing. It would also be beneficial if the polymer matrix could be recycled when its enzyme activity had been lost, especially if the half-life of the enzyme was short. This half-life may be influenced by many factors, as listed in Table 1.5.

Other aspects include the nature of the reaction which may impose certain constraints on the choice of process. The type of polymer material should ideally be inexpensive, retainable within the reactor, mechanically stable, incompressible, resistant to microbial attack, have a high surface area to volume ratio, be capable of
binding large quantities of enzyme and exist in the desired physical form (Messing, 1975).

This project will concentrate on a large scale system for the production of an intracellular enzyme, a haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2. The enzyme will be used in an immobilized form in a packed bed plug flow reactor for the removal of haloaliphatic compounds from aqueous process streams. The immobilized enzyme was chosen because of the high cost of producing the enzyme and the need to protect the enzyme from extremes of pH and temperature as well as the possibility of low solubility substrates. In addition, the practical training associated with both the production of an enzyme to carry out a biotransformation and the immobilization of such an enzyme system will be invaluable.

Table 1.5 Reasons for loss of immobilized biocatalyst productivity (Lilly, 1977).

- **Loss of enzyme activity**
  - denaturation
  - poisoning

- **Reduced enzyme substrate contact**
  - change in flow pattern
  - change in enzyme distribution
  - fouling

- **Loss of enzyme**
  - solubilization of enzyme and support
  - attrition of support
1.7 ENZYME KINETICS

In the design of a bioreactor, it is necessary to understand the kinetics of the enzyme and the corresponding biotransformation. The majority of enzyme catalyzed reactions are believed to involve a series of bimolecular or unimolecular steps. The simplest type of enzymatic reaction involves a single substrate. The substrate forms an unstable intermediate complex with the enzyme (ES), which subsequently undergoes decomposition to release the product or to regenerate the substrate.

1.7.1 Rate Equations

For a given temperature and pH, the conversion of a single substrate (S) to product (P) by an enzyme (E) may be written as:

\[
E + S \rightleftharpoons ES \rightleftharpoons E + P
\]

where:

\[k_1, k_2, k_3\] are individual rate constants.

If the substrate concentration is much greater than the concentration of the enzyme then the rate of reaction (\(v\)) can be described by the following Michaelis-Menten equation:

\[
v = -\frac{dS}{dt} = k_3[E]S/S + K_m
\]

where the Michaelis constant (\(K_m\)) is represented as:

\[K_m = \frac{(k_2 + k_3)}{k_1}\]
Equation 1.3 can be rearranged as follows:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \left(\frac{1}{S}\right) \tag{Equation 1.8}
\]

where:

\( V_{\text{max}} = \) the maximum reaction rate = \( k_3[E] \)

By plotting \( \frac{1}{v} \) against \( \frac{1}{S} \), a Lineweaver-Burk plot is obtained which enables \( V_{\text{max}} \) and \( K_m \) to be determined.

Although the Michaelis-Menten equation is applicable to a wide range of enzyme catalyzed reactions, it is not appropriate for reversible reactions or multi-substrate reactions.

### 1.7.2 Enzyme Inhibition

Enzyme catalyzed reactions are often inhibited by species that do not participate in the reaction as well as by products of the reaction. In some cases, the reactants themselves may act as inhibitors. Inhibition usually results from the formation of various enzyme-inhibitor complexes which decrease the amount of enzyme available for the normal reaction. Inhibitors may act reversibly or irreversibly. Irreversible inhibitors form a stable complex with the enzyme which cannot be removed. There are three types of reversible inhibition: competitive, non-competitive and substrate. The effect of competitive inhibition may be overcome by high substrate concentration as may non-competitive inhibition to a lesser extent. Substrate inhibition is not as common but in some cases the reaction rate may decrease at high substrate concentrations. By determining what compounds behave as inhibitors and what type of kinetic patterns are followed, it may be possible to assess the nature of the reaction, the active site of the enzyme and the overall effect this may have on reactor design.
1.7.3 Effect of pH and Temperature on Enzyme Kinetics

Most enzymes are efficient catalysts only within relatively narrow ranges of pH and temperature. The catalytic site and the conformation of the enzyme will be affected by changes in pH. Enzyme activity generally passes through a maximum at an optimum pH which may be due to a change in the stability of the enzyme, a change in the enzyme-substrate affinity or a reversible effect of pH on the maximum reaction rate ($V_{\text{max}}$).

When the rate of an enzyme catalyzed reaction is measured as a function of temperature, it is found that the rate passes through a maximum. In the low temperature range, there is little denaturation, and increasing the temperature increases the rate of the catalytic reaction (Equation 1.9).

**Arrhenius equation**

$$k_a = Ae^{-E_a/RT} \quad \text{(Equation 1.9)}$$

where:

$k_a$ = reaction rate constant for activation  
$A$ = Arrhenius constant  
$E_a$ = activation energy for catalytic reaction  
$R$ = gas law constant  
$T$ = absolute temperature

As the temperature rises, deactivation arising from protein denaturation becomes more and more important, so the observed overall rate will eventually begin to fall off. At temperatures in excess of 50 - 60 °C, most enzymes are completely denatured and the observed rates are essentially zero.
1.8 IMMOBILIZATION

1.8.1 Introduction

During the past twenty years, increasing emphasis has been placed on enzyme immobilization. A variety of immobilization techniques and carriers is available although there is no ideal solution. The method of attachment and carrier must be determined by considering the application, the enzyme and the proposed use.

There are many varied advantages to enzyme immobilization. These include the following: multiple use of a single batch of enzymes; the ability to process on a continuous basis; the ability to stop or control the extent of a reaction rapidly by removing or limiting the enzyme; the possibility of enzyme stabilization caused by bonding and the fact that the process solution is not contaminated by the enzyme while its catalytic effect is still felt. In addition, long half-lives, predictable decay rates, and the elimination of the preparation of enzyme solutions mean that immobilized enzymes can be used for analytical purposes.

Transfer rates of reactants from the bulk liquid to the outer surface and into the pores, if present, of the immobilized biocatalyst may be relatively slow and thereby cause a decrease in the reactant concentration at the catalytic site compared to that in the bulk liquid. This reduction in reaction rate may be expressed in terms of an effectiveness factor, which is the ratio of the reaction rate with and without mass transfer limitations present.

A hollow fibre reactor for the specific dehalogenation of 2-haloalkanoic acids has been developed by Diaz et al. (1989). The organism used was *Pseudomonas cepacia* MBA4 which utilizes monobromoacetate as a sole source of carbon and energy and produces a dehalogenase responsible for the cleavage of the bromo-carbon bond. It was observed that while the $V_{\text{max}}$ of the enzyme was decreased by immobilization,
the sensitivity of the reaction rates to temperature also decreased. This suggests that immobilization may be an effective method of biocatalyst entrapment. In addition, immobilization increased the \( K_{m(app)} \) of the dehalogenase, suggesting that microenvironmental parameters, acid production, and subsequent changes in the local \( pH \), influence the binding of the substrate to the enzyme. The stability of the immobilized enzyme in the hollow fibres was also studied. The half-life of the free enzyme at 300°C was 7 days. Similar decreases observed with other immobilized enzyme systems have been attributed to the differences between the real \( pH \) values in the reactor and those encountered by the free enzyme.

The efficiency of immobilized and soluble enzymes in organic solvents has been determined by their catalytic parameters (Ulbrich-Hofmann and Selisko, 1993). The application of organic solvents may cause the enzyme to be inactivated by denaturation due to the non-aqueous surroundings. In addition, the organic reaction may change the mechanism and the kinetics of the catalytic reaction. This work showed that immobilized enzymes had better long-term stability than soluble enzymes. In all solvents, \( K_m \) values were more affected than \( V_{max} \) values, suggesting that reversible denaturation plays an inferior role in activity losses.

In this project, the enzyme was immobilized for the benefits already detailed, to enable the catalyst to be reused and to protect the catalyst from the solvent interface, if one is present. Hopefully, this has improved the stability of the catalyst and made it more resistant to the effects of a change in conditions such as \( pH \) and temperature. Although the substrate was a haloalkane and poorly water soluble, the project has concentrated on the development of a system which dealt with an aqueous phase and not a two liquid phase system. A more readily water soluble substrate, 4-chlorobutanol, has also been studied for comparison.
1.8.2 Immobilization Techniques

There are five basic approaches to immobilization which may be classified as follows (Messing, 1975):
- the crosslinking of enzyme to enzyme without the benefit of a carrier.
- the crosslinking of enzymes within carriers or on the surface of carriers.
- covalent attachment to carriers.
- adsorption on or in carriers.
- encapsulation or entrapment.

1.8.3 Criteria for Selection

For successful immobilization, the following factors should be taken into account if possible (Trevan, 1980):

1 - The enzyme must be stable under the reaction conditions.
2 - Cross-linking reagents should react preferentially with chemical groups other than those at the active site.
3 - If the above condition cannot be met, then the cross-linking reagent should be as large as possible to prevent it from penetrating the active site.
4 - The active site of the enzyme should be protected in some way. This can sometimes be achieved by incorporating saturating concentrations of substrate into the reaction mixture.
5 - The washing procedure used to remove the uncross-linked enzyme from the preparation must not adversely affect the enzyme.
6 - Consideration should be given to the nature of the reaction before choosing the reaction method.
7 - The mechanical properties, in particular mechanical stability and physical form of the support material must be considered.

It is usually not possible to satisfy all these factors when choosing the best method, so compromise is vital.
In this study Eupergit C was chosen as the technique for immobilization. The oxirane groups of Eupergit C function as active components for the covalent binding of ligands containing amino, mercapto or hydroxy groups. Guisan (1988), showed that there were several reasons for choosing the amine groups for multipoint attachment. Firstly, most proteins have many lysine residues which are usually involved in the catalytic site. Secondly, amine groups are polar and they are usually exposed to the medium on the protein surface. Finally, when amino groups are unprotonated, they are very reactive as nucleophilic agents against atoms with +δ charge placed on the surface of the support.

1.8.4 Effects of Immobilization

When an enzyme is immobilized onto a support, enzymic activity may be lost in a number of ways. The reaction conditions for binding may cause denaturation, or the enzyme molecule may be immobilized relative to the support in such a way that prevents substrate access to the active site. Alternatively, a reactive group in the active site may be involved in binding to the support or the enzyme molecule may be held in an active configuration on binding (Wang et al, 1979).

1.8.5 Choice of Bioreactor

Reactor design and operation is a critical factor in immobilization technology. Two basic reactor configurations have been proposed. These are the continuous stirred tank reactor and the packed bed or plug flow reactor. The continuous stirred tank reactor is the least expensive to construct and has the advantage of easy access. Its effective mixing of enzyme and substrate reduces diffusional limitations to a minimum, even with low substrate flow rates. In general though, this type of reactor is unsuitable where high conversion reactions are needed, and will require the use of a larger amount of enzyme for a particular conversion. For a packed bed reactor, the main disadvantages are the high cost of fabrication and poor access to the matrix
which make it difficult to recharge with immobilized enzyme. Thus, it is not ideally suited for use with enzymes with a short operational half-life. However, it is capable of high substrate conversions, even in the presence of product inhibition, using less enzyme than a tank reactor (Trevan, 1980). When choosing this type of reactor, it is assumed that the reactor behaves as an ideal plug flow reactor (i.e. there is no variation of solvent flow rate across the column). If the reactor deviates from plug flow, the reactor efficiency will fall.

Chung et al. (1987), described the continuous production of rifamycin B using Nocardia mediterranei immobilized in a dual hollow fibre reactor. They showed that the productivity of the immobilized cells was 30 times that obtained in a comparable batch system. However, as the residence time of medium in the reactor increased, the pH of the effluent rose to an alkaline region that was outside its optimum condition and the yield and productivity decreased. This loss of activity due to a change in the pH of the system should be avoided wherever possible.

Carleysmith et al. (1980) immobilized penicillin acylase to carboxymethylcellulose and to the resin Amberlite XAD7. They showed that the reaction kinetics were affected by two microenvironmental effects. Diffusional limitation of access of substrate was a problem as was a reaction generated pH depression in the support particles. They found that the diffusional limitation of the reaction rate could be controlled by the conditions of preparation of the immobilized enzyme and that the pH depression could be reduced by the addition of pH buffering salts to the reactor.

Thus, in all cases, consideration must be given to the effects of parameters such as temperature conditions, flow rates, pH, ionic strength, substrate concentration and diffusional effects.
1.9 BIOREACTOR DESIGN

1.9.1 Introduction to Bioreactor Design

In order to choose the most efficient type of bioreactor for a particular biotransformation, it is necessary to fully understand the kinetics of the enzyme and of the different types of reactors. There are three possible types of reactor described in this section; batch stirred tank, continuous stirred tank and plug flow reactors.

1.9.2 Kinetics of Enzyme Reactors

1.9.2.1 Batch Stirred Tank Reactor

The equation describing the performance of a well-mixed batch stirred tank reactor for an irreversible reaction obeying Michaelis - Menten kinetics is as follows:

\[ XSO - \frac{K_m}{1 - X} \ln (1 - X) = \frac{kE t}{V} \]  
\[ \text{Equation 1.10} \]

where:

\[ X = \left( S_0 - \frac{S}{S_0} \right) \]

\[ S_0 = \text{initial substrate concentration} \]

\[ kE = \text{total enzyme activity} \]

\[ t = \text{residence time} \]

\[ V = \text{reactor volume} \]

1.9.2.2 Continuous Stirred Tank Reactor

The equation describing the performance of a well-mixed continuous stirred tank reactor is as follows:

\[ XS_0 + \frac{K_m}{1 - X} = \frac{kE}{q} \]  
\[ \text{Equation 1.11} \]

where:

\[ q = \text{flow rate} \]
1.9.2.3 Continuous Plug Flow Reactor

The equation describing the performance of a plug flow reactor is as follows:

\[ Xs_0 - K_m \ln (1 - X) = kE/q \]  
(Equation 1.12)

1.10 ENVIRONMENTAL APPLICATIONS OF DEHALOGENASES

Large scale industrial use of halogenated aliphatic compounds has led to widespread environmental pollution. Many of these compounds are toxic, carcinogenic, recalcitrant and potentially teratogenic. They enter the environment due to improper disposal, spillage or direct application.

The majority of trace contaminants produced by industry can be removed by trickle filters and activated sludge systems. However, it has been shown (Hall and Mercer, 1983) that, if one or more toxic or recalcitrant compounds are present, a different approach is required. This section discusses the removal of these compounds from contaminated waste water and groundwater by selective microorganisms.

1.10.1 Waste Water Contaminants

The characteristics of the contaminants can affect the rate and extent of degradation. The nature of the contaminant also determines whether it can be degraded aerobically or anaerobically. Microorganisms are known to exhibit sequential removal of contaminants when a mixture of contaminants is present in waste water. This effect is related to the structure and complexity of the organic molecule (Bradford and Krishnamoorthy, 1991). Traditionally, waste water treatment systems removed biochemical oxygen demands and nutrients such as nitrogen and phosphorus. More recently, treatment systems have begun to remove toxic organic chemicals from waste water. Pollution control engineers have found that the sequential exposure of microorganisms to anaerobic (no nitrite or nitrate and no dissolved oxygen), anoxic (nitrite or nitrate present and no dissolved oxygen) and aerobic environments
enhance the removal of a broad range of chemicals. For example, tetrachloroethylene, chloroform and hexachlorobenzene have been degraded in a two stage biofilm reactor consisting of an anaerobic column followed by a conventional aerobic column. Reductive dechlorination occurred in the anaerobic column and trichlorinated and dichlorinated products were formed. In the aerobic column, the less chlorinated intermediates were substantially transformed into carbon dioxide and non-volatile products (Zitomer and Speece, 1993).

Biofilm reactors for the treatment of halogenated polluted waste waters are advantageous in their ability to withstand shock loading. Transient higher concentrations may be toxic to the biomass and kill the outer surface of film while the underlayers are protected. Once a film is formed it can be used to treat halogenated organic pollutants at concentrations that do not support cell growth. The halidohydrolase of *Rhodococcus erythropolis* Y2 is a biocatalyst that has been shown to be effective at catalyzing dehalogenations in the laboratory. Hence this organism could be incorporated into biofilms to utilize the broad specificity of its enzyme system towards cometabolic compounds. Sallis *et al.* (1990) showed that this halidohydrolase exhibited activity towards many Red List compounds (Department of the Environment, 1988) including 1,3-dichloropropene and 1,1,1-dichloroethane. These activities indicate the potential of the use of this organism in the biotreatment of waste waters containing a mixture of haloalkanes. In addition, the haloalkane halidohydrolase producing strains already isolated can withstand considerably higher concentrations of substrate than the anaerobic methanogens and so may have particular applications in heavily polluted sites.

**1.10.2 Groundwater Contaminants**

The conventional system for the treatment of contaminated groundwaters is by air-stripping the volatile organic compounds from the groundwater and incinerating
them, but this approach is limited by new legislation. Biofilters have now been used for the treatment of groundwaters. Pflug and Burton (1988) described a continuous immobilized film bioreactor system that optimized the use of *Flavobacterium* sp. to treat groundwater contaminated with wood preserving waste. The bioreactor was capable of reducing PCP concentrations from 93,000 µg/L in effluent wellwater to 51 µg/L in the effluent stream.

Valo et al., (1990) described the biotreatment of chlorophenol containing groundwater by *Rhodococcus chlorophenolicus* and *Rhodococcus* sp. CP-2 immobilized on a polyurethane carrier. A column with the immobilized biomass was fed with chlorophenol contaminated water. The chlorophenol concentration of the column effluent was found to be 1/1000 - 1/10,000 of the influent concentration. The life-time of the immobilized rhodococci was several months, suggesting this as a suitable method for the removal of chlorophenols from groundwater.

Lackey et al., (1993) utilized a single-pass packed bed bioreactor to study the biodegradation of organic waste mixtures consisting of tetrachloroethylene, dichloroethylene and other short-chain chlorinated organics from groundwater. The bioreactor consisted of two columns, one packed with sand containing a microbial consortia enriched from a contaminated site. The other column provided a reservoir for oxygen and a carbon source of methane/propane that was recirculated through the reactor. Greater than 99 % degradation of trichloroethylene, approaching drinking water standards, was observed when the bioreactor residence time ranged from 1.9 - 3.2 days.

Strict regulations governing the release of any genetically modified microorganisms into the environment as well as the advantages of immobilization discussed earlier mean that immobilized cell bioreactors may be more widely used in the future.
1.10.3 Other Uses

It may also be possible to use *Rhodococcus erythropolis* Y2 in bioreactors for the treatment of leachate. A culture of *Rhodococcus erythropolis* Y2 may be temporarily stable enough with respect to concentrations and components to allow the selective growth of the organism, and selective pressure would help to ensure that the isolate was not outcompeted by naturally adapted strains.

Microbial dehalogenation reactions have also been used for the production of optically active compounds. For example, both the enantiomers of lactic acid can be prepared separately from racemic 2-chloropropionic acid by successive treatment with L-2-haloacid dehalogenase and D,L-2-haloacid dehalogenase (Motosugi et al., 1984). More recently, the microbial production of chiral glycerol derivatives such as optically active epichlorohydrin, 3-chloro-1,2-propanediol and glycidol has been studied. These compounds are important building blocks for the synthesis of chiral pharmaceuticals (Leisinger and Bader, 1993).

1.11 AIMS OF STUDY

Many halogenated aliphatic compounds used in industry are serious pollutants and there is therefore increasing interest in organisms and their enzymes for the detoxification of such chemicals. Whereas halidohydrolases acting on 2-haloacids and haloacetate have been widely reported, there are few detailed reports of this enzyme acting on haloalkanes. The organism *Rhodococcus erythropolis* Y2, capable of growth on a range of x-substituted haloalkanes, has been isolated and the single halidohydrolase produced by this organism has been characterized (Sallis et al., 1990).
Such use of enzyme systems is an effective short term solution for combatting the problems encountered in waste water treatment. Ideally, industry should try to eliminate these problems at source rather than removing them downstream.

The objective of this project is to demonstrate the production of the haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2 on a large scale to remove toxic haloalkanes from aqueous process streams. From the results of the effects of immobilization, the use of this halidohydrolase in an immobilized form will be examined. The enzyme isolation procedure will be scaled up to 1500 litres to produce adequate amounts of the enzyme for the study and to assess the effects of scale-up on the fermentation. The project will concentrate specifically on the enzymic route but this does not imply that the whole cell route is not a valid one. Once a production system is established it will hopefully be shown to be both reproducible and viable.

To achieve these objectives the following aspects were addressed:

- The scale-up of the fermentation and isolation procedures from shake flasks to 1500 L.
- The kinetics of the halidohydrolase and its stability under various conditions.
- Evaluation of the effect of enzyme immobilization and choice of the conditions for immobilization.
- Evaluation of reactor designs. The proposed options for the immobilized enzyme system were the continuous stirred tank reactor, the well-mixed batch reactor and the packed bed or plug flow reactor.
- Successful demonstration of the operation of a small-scale reactor for the dehalogenation of saturated solutions of haloalkanes and haloalcohols.
2.0 MATERIALS AND METHODS

2.1 ORGANISM

The bacterial strain used in this project was *Rhodococcus erythropolis* Y2, identified by the National Collections of Industrial and Marine Bacteria Limited, (NCIMB), Aberdeen, U.K.

2.1.1 Isolation

The organism was isolated using batch-enrichment culture by Sallis *et al.*, (1990) at the University of Kent, Canterbury, Kent, U.K. Soil samples were taken from an industrial site where haloalkanes had been widely used. 5 g samples were incubated in 250 mL screw-top bottles containing 20 mL defined growth medium (Slater *et al.*, 1979) at pH 7.0. The medium contained modified quantities of (g/L): Na$_2$HPO$_4$.12H$_2$O, 4.0; KH$_2$PO$_4$, 1.0; NaHCO$_3$, 1.0; yeast extract, 20 mg; CoCl$_2$.6H$_2$O, 100 µg; NiCl$_2$.6H$_2$O, 20 µg and H$_3$BO$_3$, 100 µg. 0.9 g/L of 1-chlorobutane was added as the sole carbon and energy source.

Batch enrichment cultures were incubated on a shaker (120 rpm) at 30 °C for 3 days. 1 mL samples of the culture were subcultured into 100 mL of the above medium in 300 mL bottles and incubated as before. After three consecutive subcultures, samples were streaked for single colonies on nutrient agar. Inocula from all of the separate colonies were tested for their ability to grow on the above liquid medium with 1-chlorobutane as the growth substrate. One isolate (Y2) was obtained which grew as an axenic culture.

2.1.2 Maintenance of the Bacterial Strain

Cultures were stored at -70 °C in 20 % (w/v) glycerol or as freeze dried vials at 4 °C. Cultures were maintained at 4 °C for short term storage on defined growth
medium agar slopes and plates, and subcultured at monthly intervals.

2.1.3 Medium Preparation

The initial medium used was obtained from Sallis et al. (1990) and was as follows (g/L): Na$_2$HPO$_4$, 2.1; KH$_2$PO$_4$, 1.0; NaHCO$_3$, 0.5; MgSO$_4$, 0.2; (NH$_4$)$_2$SO$_4$, 0.5; yeast extract, 0.02; sodium succinate, 0.025 and 5 mL of a trace element solution. The solution contained (g/L): Na$_2$EDTA, 12.0; NaOH, 2.0; MgSO$_4$.7H$_2$O, 1.0; ZnSO$_4$.7H$_2$O, 0.4; MnSO$_4$.4H$_2$O, 0.4; CuSO$_4$.5H$_2$O, 0.1; H$_2$SO$_4$(conc), 0.5 mL; Na$_2$SO$_4$, 10.0; Na$_2$MoO$_4$.2H$_2$O, 0.1 and Fe$_3$SO$_4$.7H$_2$O, 2.0. The pH of the medium before and after sterilization was 6.8. 1-chlorobutane at a final concentration of 0.7 g/L was added as a growth substrate. This medium resulted in only low final bacterial concentrations of 0.7 g$_{dw}$/L.

After some modifications, the medium was changed to the following (g/L): Na$_2$HPO$_4$, 2.1; KH$_2$PO$_4$, 1.5; NaHCO$_3$, 0.5; MgSO$_4$, 0.2; (NH$_4$)$_2$SO$_4$, 0.5; yeast extract, 6.0; glycerol, 6.0 and 5 mL of the above trace element solution. The medium was then sterilized at 1 bar, 121°C for 20 minutes. The pH of the medium before and after sterilization was 6.5. This gave a final bacterial concentration of 5.9 g$_{dw}$/L (See Results).

To prepare a solid medium, 15 g/L of Bacto agar (Difco Laboratories) was added to the modified medium prior to sterilization at 1 bar for 20 minutes.

2.2 GROWTH CONDITIONS

All fermentations were performed at 30°C.
2.2.1 Growth in Shake Flasks

50 mL of the medium was inoculated with 50 μL of a 100-fold concentrated sample of Y2 in a 300 mL flask, sealed with a Suba-seal. The culture was incubated on a shaker (120 rpm) at 30 °C for 24 hours and 1 mL samples were withdrawn with a needle and syringe.

2.2.2 Growth in Batch Fermenters

On a small scale (2 L), an LH fermenter was used to optimize various growth conditions. 1.5 L of medium was sterilized in the fermenter placed in an autoclave at 1 bar, 121 °C for 20 minutes. A 1% (v/v) inoculum was used and the fermentation was run for 45 hours before being harvested.

On a larger scale (20 L), an LH fermenter was used. 14 L of medium was sterilized in situ at 1 bar, 121 °C for 35 minutes. A 1% (v/v) inoculum was used and the fermentation was run for 24 hours before being harvested.

A 1500 L Chemap fermenter was used for scale-up of the fermentation. 1000 L of medium was sterilized in situ at 1 bar, 121 °C for 45 minutes. A 1.4% (v/v) inoculum was used and the fermentation was run for 14 hours before being harvested. The conditions for the above fermentations are shown in Table 2.0.

Table 2.0  Fermentation Conditions at Different Scales

<table>
<thead>
<tr>
<th>Vessel Volume</th>
<th>2 L</th>
<th>20 L</th>
<th>1500 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Volume (L)</td>
<td>1.5</td>
<td>14</td>
<td>1000</td>
</tr>
<tr>
<td>Air Flow Rate (vvm)</td>
<td>0.33</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Stirrer Speed (rpm)</td>
<td>500</td>
<td>700</td>
<td>200</td>
</tr>
<tr>
<td>Initial pH</td>
<td>7.2</td>
<td>7.0 (constant)</td>
<td>7.0 (constant)</td>
</tr>
</tbody>
</table>
2.2.3 Criteria for Scale-up

Assuming geometrical similarity, constant impeller tip speed was used as the parameter for scale-up. The stirrer speed of the 1500 L vessel can be calculated as follows:

20 L impeller diameter = 6.75 cm
1500 L impeller diameter = 30 cm

Both vessels have 3 equally spaced Rushton turbine impellers

\[
\text{Tip speed} = \pi N D \tag{Equation 2.0}
\]

where: 
\(N\) = impeller speed (rpm)
\(D\) = impeller diameter (m)

For 20 L: \[\text{Tip speed} = \pi \times 700 \times 0.0675 = 148.44\]
For 1500 L: \[148.44/\pi \times 0.3 = 157.5 \text{ rpm}\]

In practice, an impeller speed of 200 rpm was used to ensure that the dissolved oxygen tension (DOT) remained above 40 %.

2.2.4 Data Determination During Fermentation

During fermentation, measurements were taken of chloride release using a chloride analyzer (Coming Medical and Scientific, Halstead, Essex) and from these data, the specific enzyme activity was calculated.

Initially, during model 20 L fermentations, exit gas analysis using a mass spectrometer was monitored using Bio-I (Biotechnology Computer Systems, Chiswick, U.K.) which determined OUR (oxygen uptake rate), CER (carbon dioxide evolution rate) and RQ (respiratory quotient) as well as monitoring the following fermentation parameters: air flow rate, DOT, pH, stirrer speed and temperature. For subsequent 20 L and 1500 L fermentations Bio-I was replaced by RTDAS version 2.0 (Data Acquisition Systems, Surrey, U.K.) which recorded readings every 3 minutes and produced the same fermentation profiles as before.
2.2.5 Determination of Bacterial Concentration

During fermentation, regular samples were withdrawn and readings of absorbance (600nm) were taken using a spectrometer. Using this information, the maximum specific growth rate was determined (See Equation 2.1) and a growth profile of absorbance against time was obtained.

The maximum specific growth rate ($\mu_{\text{max}}$) of *Rhodococcus erythropolis* Y2 was determined by inoculating 140 mL of an overnight culture into 14 L of medium to give an initial absorbance at 600 nm of 0.2 to 0.4. The absorbance of the culture was measured every hour. $\log_{10}$ absorbance was plotted against time and the $\mu_{\text{max}}$ calculated using the following equation:

$$\mu_{\text{max}} = \ln 2/ t_d = 2.303 \times \text{slope} \quad \text{(Equation 2.1)}$$

where $t_d$ is generation time and the slope is that obtained from the growth curve.

In order to determine the cell concentration during 20 L fermentations, 100 mL samples were withdrawn at hourly intervals. The absorbance of these samples was measured at 600 nm and the sample was then centrifuged (7500 rpm, 4 °C, 45 minutes), and the supernatant removed. The pellet was dried at 100 °C and the dry weight measured after 24 hours. The cell concentration in g_{dw}/L was then calculated. Cell concentration and absorbance against time were plotted as well as absorbance against bacterial concentration.

2.3 WHOLE CELL STUDIES

2.3.1 Assay for Dehalogenation in Whole Cell Suspensions

Washed whole cells were assayed in 5 mL of 100 mM glycine/NaOH buffer, pH 9.1, in 100 mL flasks with Suba-seals agitated on a shaker at 30 °C and 200 rpm. The reaction was started by the addition of 1-chlorobutane to a final
concentration of 10 mM. Samples were taken between 2-6 hours and the initial rate of halide release determined from plots of chloride analyzer (Corning Medical and Scientific, Halstead, Essex) reading against time.

2.3.2 Determination of Optimum pH for Dehalogenase Activity in Whole Cells

Whole cells were assayed in 100 mM glycine/NaOH buffer (using the method used by Bergmann & Sanik, 1957) over a range of pH values from 5-11.

2.4 PREPARATION OF CRUDE CELL FREE EXTRACTS

2.4.1 Disruption using the French Pressure Cell

On the small scale, the culture of *Rhodococcus erythropolis* Y2 (1600 mL) grown in four 2 L shake flasks was harvested during the late exponential phase by centrifugation (7500 rpm, 4 °C, 15 minutes). The pellet was washed and resuspended in 20 mL of 100 mM glycine/NaOH buffer, pH 9.1. The cell suspension was then passed through a French pressure cell (SLM Instruments Inc. Urbana, USA) five times at 1.38 x 10^8 Pa. Cell-free extracts were produced by removing the cell debris by centrifugation (45,000 rpm, 4 °C, 45 minutes). The supernatant was decanted and stored at -20 °C, at which temperature the enzyme activity remained stable for several months (Figure 3.32).

2.4.2 Disruption using the High Pressure Homogenizer

Depending on the scale of the fermentation and the sample size, different equipment was used for cell disruption.

2.4.2.1 Small Scale

In order to determine protein and enzyme activity profiles of the cell free extract
during a 20 L fermentation, it was necessary to take 40 mL samples of the fermentation broth at hourly intervals. These samples were passed five times through an APV Gaulin Micron Lab 40 homogenizer (12,000 bar) and enzyme and protein assays were performed after each pass.

2.4.2.2 Large Scale

On the larger scale, the culture (14 L) was harvested during the early stationary phase by centrifugation (45,000 rpm, 4 °C, 15 L/min) in a Pennwalt tubular bowl centrifuge, model 1P, and the pellet was resuspended in 2 L of 100 mM glycine/NaOH buffer, pH 9.1. The suspension was then passed five times through a Manton-Gaulin APV high pressure homogenizer, model 15M8BA (500 bar, 60 L/hr). The cell debris was removed by centrifugation in the 1P tubular bowl centrifuge under the same conditions as before. The supernatant was removed and stored at -20 °C for several months (Figure 3.32).

Hetherington et al. (1971) showed that the release of protein from bakers' yeast and several bacteria may be described by:

\[
\log \frac{R_m}{(R_m - R)} = KN
\]

(Equation 2.2)

where:

R_m = maximum amount of protein available for release
R = amount released after N passes through homogenizer
K = temperature and pressure dependent rate constant
N = number of passes
2.5 DEHALOGENATION DETERMINATION USING THE DEHALOGENASE IN THE CELL FREE EXTRACTS

Dehalogenase activity was measured in 10 mL glass vials with Suba-seals in the following reaction mixture: 5 mL 100 mM glycine/NaOH buffer, pH 9.1 and 4 μL of 1-chlorobutane (or 73 μL of 4-chlorobutanol). After equilibration of the mixture for 15 minutes at 30 °C on a shaker (120 rpm), the reaction was started by the addition of 200 μL of the cell-free extract. 1 mL samples were taken every 30 minutes and the Cl⁻ content was determined. The Cl⁻ content was measured by the method of Bergmann & Sanik, (1957) for 1-chlorobutane and on the chloride analyzer for 4-chlorobutanol. (See Section 2.7.1)

2.6 INDUCTION OF DEHALOGENASE ACTIVITY

The dehalogenase was inducible and active towards a greater range of halogenated aliphatic compounds than would support growth of the organism. Initially, 1-chlorobutane was used as an inducer (Sallis et al. 1990). After subsequent trials with many haloalkanes and haloalcohols a new less volatile inducer was chosen. This new inducer was 4-chloro-1-butanol and was used as the inducer in all of the 20 L and 1500 L fermentations.

2.7 ANALYTICAL PROCEDURES

2.7.1 Determination of Halide Ion Concentration

2.7.1.1 In Growing Cultures

The concentration of free halide ions was initially measured using a Marius Chlor-O-counter (F.T. Scientific Instruments Ltd; Tewkesbury Glos.). 1 mL samples were added to 20 mL of a base counting solution containing 92 mL glacial acetic acid and 8 mL concentrated nitric acid in 1.0 L of distilled water. 1 mL of an indicator solution, containing 600 mg powdered gelatin, 10 mg thymol and 10 mg thymol blue pH indicator in 100 mL of distilled water, was also added. Free halide ions
were titrated against silver ions generated electrocoulometrically and insoluble silver halide precipitates formed. The end-point of the titration was detected by silver electrodes which measured the appearance of free silver ions. The titration time was directly proportional to the halide ion concentration and was expressed as mM halide released per minute. The instrument was calibrated using a standard NaCl solution.

A second instrument, a chloride analyzer (Corning Medical and Scientific, Halstead, Essex) was also used to determine the chloride ion concentration. The principle was the same as for the Marius Chlor-O-counter. 0.5 mL of sample was added to 10 mL of solution and the reading obtained was expressed as mgCl/L.

2.7.1.2 In Cell Free Extracts using Bergmann-Sanik method

Chloride ion concentrations in cell-free extracts were determined by a modified method of Bergmann-Sanik (1957). To stop the enzyme reaction, 100 µL of acid reagent A (0.25 Fe(NH₄)(SO₄)₂.12H₂O in 9 M HNO₃) were added to 1mL samples of the assay mixture. Turbidity (protein and cellular precipitates) was removed by centrifugation for 5 minutes (MSB Microcentaur). A 1 mL sample was removed into a cuvette and 91 µL of reagent B (95 % ethanol saturated with Hg(SCN)₂) was added and vortexed. The absorbance (460 nm) was measured after 2 minutes.

This method uses a colorimetric reaction based on the formation of the orange ferric thiocyanate complex:

\[
2\text{Cl}^- + \text{Hg(SCN)}_2 + 2\text{Fe}^{3+} \rightarrow \text{HgCl}_2 + 2[\text{Fe(SCN)}^2+] \quad (\text{Equation 2.3})
\]

The chloride ion displaces thiocyanate from mercury (II) thiocyanate and in the presence of iron III a highly coloured complex of iron III thiocyanate is formed. The
intensity of the orange colour is proportional to the original chloride ion concentration. A standard curve was produced from standard solutions of NaCl (Figure 2.0). The absorbance reading does not begin at zero because a small amount of colour is formed in the absence of chloride ions.

Extreme care should be taken when using mercury (II) thiocyanate, and details of its use can be found in the Appendix.

2.7.1.3 In Cell Free Extracts using Florence and Farrar method

Chloride ion concentrations in cell-free extracts were also determined by a modified method of Florence and Farrar (1971). To stop the enzyme reaction, 100 μL of acid reagent C (15.1 g of Fe(NO₃)₃.9H₂O in 100 mL distilled water containing 45 mL of 72 % perchloric acid) was added to 1mL samples of the assay mixture. 100 μL of reagent B (95 % ethanol saturated with Hg(SCN)₂) was added and the solution was vortexed. Turbidity (protein and cellular precipitates) was removed by centrifugation for 5 minutes (MSE Microcentaur). A 1 mL sample was removed into a cuvette and the absorbance (460 nm) was measured after 2 minutes. The mercury (II) thiocyanate method, as before, involves the reaction of chloride with mercury (II) thiocyanate to form a chloromercurate (II) complex ion, with the liberation of thiocyanate ions which then react with iron (III) to give the familiar orange colour. A standard curve was produced from standard solutions of NaCl (Figure 2.1). This method was found to be 10 times more sensitive than the Bergmann-Sanik method and could detect chloride concentrations as low as 16 μg/L.

Extreme care should be taken when using perchloric acid, and details of its use can be found in the Appendix.
Figure 2.0 Standard curve for the Bergmann-Sanik enzyme assay to determine chloride release. The standard is obtained using different concentrations of NaCl solution.

\[ y = 0.26600 + 0.37800x \quad R^2 = 0.991 \]
Figure 2.1 Standard curve for the spectrophotometric determination of chloride using the mercury (II) thiocyanate method. The standard is obtained using different concentrations of NaCl solution.

\[ y = 5.2000 \times 10^{-3} + 0.45440x \quad R^2 = 0.999 \]
2.7.1.4 Chloride analyzer method

Chloride ion concentrations in cell-free extracts with 4-chlorobutanol as substrate were determined using the chloride analyzer as described in section 2.7.1.1. This method was used in preference to the spectrophotometric methods because 4-chlorobutanol is much more water soluble than 1-chlorobutane. Therefore, when using a saturated solution in the assay, more chloride ions are released and so a highly sensitive assay is not required. The chloride analyzer also has the advantage of being more accurate because there is less chance of human error and the reagents are less harmful.

2.7.2 Gas Chromatographic Analysis

Gas chromatography was used both to determine the saturation concentrations of 1-chlorobutane and 4-chlorobutanol and to determine the concentration of these compounds in solution.

**GC Details**

: SHIMADZU, JAPAN, GC-14A Gas Chromatograph

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injector temp.</td>
<td>200 °C</td>
</tr>
<tr>
<td>Detector temp.</td>
<td>200 °C</td>
</tr>
<tr>
<td>Column temp.</td>
<td>60 °C for 4-chlorobutanol</td>
</tr>
<tr>
<td></td>
<td>45 °C for 1-chlorobutane</td>
</tr>
<tr>
<td>Run time</td>
<td>12 minutes for 4-chlorobutanol</td>
</tr>
<tr>
<td></td>
<td>30 minutes for 1-chlorobutane (to minimise baseline)</td>
</tr>
<tr>
<td>Injection volume</td>
<td>2 μL</td>
</tr>
<tr>
<td>Detector type</td>
<td>flame ionization detector</td>
</tr>
<tr>
<td><strong>Column details</strong></td>
<td>:SCIENTIFIC GLASS ENGINEERING, AUSTRALIA.</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td><strong>Code</strong></td>
<td>: 25QC3/BP1 1.0</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td>: 25 m</td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td>: bonded phase</td>
</tr>
<tr>
<td><strong>Material</strong></td>
<td>: fused silica column</td>
</tr>
<tr>
<td><strong>Phase</strong></td>
<td>: BP1 (non-polar)</td>
</tr>
<tr>
<td><strong>Film thickness</strong></td>
<td>: 1.0 micron</td>
</tr>
<tr>
<td><strong>Max. temp.</strong></td>
<td>: 280 °C</td>
</tr>
<tr>
<td><strong>I.D</strong></td>
<td>: 0.33 mm</td>
</tr>
<tr>
<td><strong>O.D</strong></td>
<td>: 0.43 mm</td>
</tr>
</tbody>
</table>

### 2.7.2.1 Determination of Standard Curves

Standard curves were obtained with 1-chlorobutane in heptane and 4-chlorobutanol in ethyl acetate (See Figures 2.2, 2.3 and 2.4).
Figure 2.2  Standard for 1-chlorobutane in heptane with column temperature at 45 °C and substrate concentrations of 0-60 mM.

\[ y = -44.764 + 146.33x \quad R^2 = 0.990 \]
Figure 2.3  Standard from two experiments for 1-chlorobutane in heptane with column temperature at 45 °C and substrate concentrations of 0-5 mM.

\[ y = 30.426 + 139.34x \quad R^2 = 0.975 \]
Figure 2.4 Standard from two experiments for 4-chlorobutanol in ethyl acetate with column temperature at 60 °C and substrate concentrations of 0-60 mM.

\[ y = 166.13 + 177.32x \quad R^2 = 0.999 \]
### 2.7.2.2 Determination of Extraction Efficiency

In order to determine the percentage of substrate that was extracted into the carrier solvent, the extraction efficiency was determined in each case and thus the partition coefficient could be calculated (Equation 2.4). Solutions of 0-60 mM 1-chlorobutane and 4-chlorobutanol in 100 mM glycine/NaOH buffer, pH 9.1 were made up and then 0.5 mL of each solution was extracted and vortexed with 0.5 mL of heptane and ethyl acetate respectively. After centrifugation, the top layer was removed and run through the gas chromatograph. Extraction efficiency curves were obtained for 1-chlorobutane in heptane (Figure 2.5) and 4-chlorobutanol in ethyl acetate (Figure 2.6).

(Equation 2.4)

\[
\text{% extraction efficiency} = \left( \frac{\text{slope of standard curve}}{\text{slope of extraction curve}} \right) \times 100
\]

**1-chlorobutane**

\[
\text{% extraction efficiency} = \frac{136.27}{146.33} \times 100 = 93.12 \%
\]

Partition coefficient = 0.93

**4-chlorobutanol**

\[
\text{% extraction efficiency} = \frac{170.16}{177.32} \times 100 = 95.96 \%
\]

Partition coefficient = 0.96

**Injection volume**

Experiments were carried out with the highest possible injection volume (8 µL) in order to increase the peak areas, but several problems were incurred. As a result, 2 µL was chosen as a suitable injection volume. These problems included:

- Peak distortion - where the peak is not the correct shape and tails off.
- Leading Peaks - where the peak increases gradually and not sharply.
- Excess material remaining in column
Figure 2.5  Extraction efficiency curve for 1-chlorobutane in heptane

\[ y = -27.385 + 136.27x \quad R^2 = 0.997 \]
Figure 2.6  Extraction efficiency curve for 4-chlorobutanol in ethyl acetate

\[ y = 375.13 + 170.16x \quad R^2 = 0.991 \]
2.7.2.3 Determination of Saturation

Using a Lewis cell, saturated solutions of 1-chlorobutane and 4-chlorobutanol were prepared over several days in distilled water at pH 7. The peak area which was obtained on an undiluted sample solubilized in a Lewis cell and then extracted into heptane was found to be 857 (Figure 2.7). This corresponded to a concentration of 6.64 mM. When the extraction efficiency was taken into account, 100 % saturation of 1-chlorobutane in water was found to be at a concentration of 7.13 mM.

A similar experiment was performed with 4-chlorobutanol to determine its solubility. The concentration at 100 % saturation was found by comparing the value obtained with a diluted sample of the saturated solution (Figure 2.8) with the standard curve and multiplying by the extraction efficiency (or by comparing with the extraction efficiency curve directly) and the dilution factor. This gave a value that corresponded to a concentration of 315 mM for 100 % saturation.
Figure 2.7  Gas chromatograph trace of saturated solution of 1-chlorobutane in water
Figure 2.8  Gas chromatograph traces of a diluted saturated solution of 4-chlorobutanol in water.
2.7.3 Determination of Protein Concentration in Cell Free Extracts

The method used was that of Bradford (1976). 1.0 mL of assay solution [100 mg Coomassie Brilliant Blue G250 (Biorad, Richmond, USA) dissolved in 50 mL of 95 % ethanol and 100 mL of 85 % w/v phosphoric acid and made up to 1 L with distilled water], was added to 0.1 mL samples containing 10-100 μg of protein and vortexed. Absorbance at 595 nm was measured after 2 minutes and before 1 hour. The method relies on the fact that Coomassie Blue exists in two forms. The red anionic form is converted into a blue form when the dye binds to the amino groups of proteins. The Bradford protein assay has a sensitivity of less than 0.5 mg/mL. The reaction is quick and can be used as a microassay. The protein concentration was determined by reference to a standard curve using bovine serum albumin (Sigma, 96 - 99 % albumin) as the reference standard (Figure 2.9).

2.7.4 Chemicals and Reagents

All chemicals were obtained from Aldrich Chemical Co. (Gillingham, Dorset). Some biochemical reagents were obtained from Sigma (Poole, Dorset).
Figure 2.9  Standard curve for protein determination in cell free extracts using the Bradford protein assay. The curve was obtained using bovine serum albumin as the reference standard.

\[ y = 7.1429 \times 10^{-2} + 7.8714 \times 10^{-3}x \quad R^2 = 0.999 \]

[Graph showing absorbance at 595nm against protein concentration (μg protein/100μL)]
2.8 IMMOBILIZATION

Eupergit C was chosen as a suitable matrix for immobilization of the clarified cell-free extract.

2.8.1 Procedure for Immobilization on Eupergit C

The standard procedure for immobilizing enzymes on a small scale is as follows:

a) Add the enzyme (10 - 200 mg) to 5 mL of 1 M potassium phosphate buffer at pH 7.5 (or pH 6 or pH 9), whatever pH is nearest to the pH-activity optimum of the enzyme.

b) Add 1 g of Eupergit C and mix the suspension by gentle shaking. Seal the flask and allow to stand at room temperature (23 °C) for 72-96 hours without additional shaking.

c) Collect the beads on a sintered-glass filter (porosity 2) and drain off the solution by vacuum.

d) Wash the beads thoroughly on the filter with 50 mL of 0.1 M potassium phosphate buffer pH 7.5. (Divide the total volume of the washing fluid into 3 aliquots. Use one aliquot for each of three repetitive washing steps.)

e) Store the immobilized enzyme in a buffer solution containing a preservative, e.g. 0.1 M potassium phosphate buffer pH 7.5 containing 500 ppm ethyl p-hydroxybenzoate and 2 % 2-propanol.

The standard procedure was followed although it was necessary to optimize the immobilization procedure by assessing factors such as the amount of enzyme, the pH and ionic strength of the binding buffer, and the immobilization time.

2.8.2 Determination of Immobilization Conditions

2.8.2.1 pH Value of the Binding Buffer

The oxirane groups of Eupergit C can react with proteins over a wide pH range
(from 0-12). Therefore, the binding reaction can take place in the acidic pH range (e.g. in HCl/NaCl buffer at pH 1-3 or in acetic acid/acetate buffer at pH 4-6) as well as in the alkaline pH range (e.g. in K₂HPO₄ solution at pH 9). If required, this medium can be made even more alkaline by the addition of KOH. Within the range of pH 5-9, any distinct pH can be adjusted by appropriate mixing of aqueous K₂HPO₄ and KH₂PO₄ solutions.

An enzyme should be immobilized at the pH optimum of its stability, provided that at this pH the enzyme remains reasonably active. In this case a range of alkaline pH values were tested under conditions of constant temperature, ionic strength and immobilization time.

2.8.2.2 Ionic Strength of the Binding Buffer
In many cases, the activity of an immobilized enzyme depends on the molarity. Most enzymes should preferably be immobilized at a high ionic strength, e.g. in a 0.5-1 M potassium phosphate buffer. In this case a potassium phosphate buffers at 0.1-1 M were tested.

2.8.2.3 Amount of the Enzyme
The ratio of the protein/matrix has to be optimized for any individual enzyme in order to obtain the optimum activity per volume of the biocatalyst without an unreasonable loss of the enzyme. Amounts of clarified cell extracts between 5-250 mL per gram of Eupergit C were tried under conditions of constant pH, temperature, ionic strength and immobilization time.

2.8.2.4 Immobilization Time
When immobilizing any enzyme, the binding yield, the activity yield and the
operational stability of the immobilized enzyme all depend upon the immobilization time. Stable enzyme preparations should be immobilized for 72-96 hours to guarantee a maximum binding yield and activity. In some cases, such a long term procedure will result in a much higher operational stability of the immobilized enzyme due to increased multipoint attachment. The enzyme preparations were incubated for between 24 and 96 hours and their activity was determined.

2.8.2.5 Determination of the Binding Yield/Activity Yield and of Operational Stability.

The determination of the binding yield was achieved by measuring the remaining enzymatic activity in the supernatant. For estimation of the activity yield, the activity of the immobilized enzyme itself was determined. This was done by removing a 5 mL sample from the 25 mL/gEupergit preparation. The beads were removed from the solution as before and then used directly in the enzyme assay (See section 2.5).

For judging the feasibility of the biocatalyst in an industrial application, the operational stability of activity was estimated. This was achieved by applying the same sample of the immobilized enzyme to a number of repetitive activity determinations.

2.9 DEVELOPMENT OF A SMALL SCALE REACTOR SYSTEM

In order to validate the reactor design, a small scale plug flow reactor was constructed and run under various conditions with saturated solutions of 1-chlorobutane and 4-chlorobutanol. A small scale batch reactor was also used with immobilized enzyme and clarified cell free extract for comparison.

2.9.1 Setting up the Plug Flow Reactor

A Pharmacia chromatography column (10 mm I.D, 30 cm length) was used as the
reactor and solutions of 1-chlorobutane and 4-chlorobutanol were pumped from a reservoir kept at 30 °C in a water bath. The column temperature was regulated via tubing wrapped around the outside with water being constantly pumped through (Figure 2.10).

2.9.2 Measurement of Column Parameters
In order to assess the efficiency of the column and validate the predictions made in the bioreactor design, several experiments were carried out to determine the percentage of saturated substrate that was converted at different flow rates through the column. Samples of the inlet and outlet solutions were taken at various time intervals and the concentrations were determined using gas chromatography (see section 2.7.2).

2.9.2.1 Determination of Bed Volume
In order to calculate the residence time of the liquid in the column, it was necessary to determine the bed volume. Using this value, it was possible to estimate the amount of time for steady state to be reached between flow rates. The column was packed with immobilized Eupergit C beads and a saturated solution of 1-chlorobutane at pH 7 was continuously pumped through the column for 30 minutes at 1.75 mL/min. The flow rate was then increased to 10 mL/min and samples of the outlet solution were taken every 15 seconds for over 2 minutes. The concentration of the outlet solutions was measured using gas chromatography (see section 2.7.2) and a profile of percentage conversion against volume collected was obtained (see Figure 2.11). From this the bed volume was estimated as the volume in which steady state was achieved. The percentage conversion plateaued between 10 - 20 mL and so at least 25 mL was collected after each change in flow rate to ensure the system had reached steady state.
Figure 2.10  Diagram to show the model plug flow reactor system

1- Water bath and pump
2- Saturated substrate reservoir
3- Centrifugal pump
4- Column packed with immobilized enzyme
5- Water jacket
6- Product stream
On that basis the minimum times to reach steady state and collect 25 mL were as follows:

<table>
<thead>
<tr>
<th>Flow rate (mL/min)</th>
<th>Time (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.75</td>
<td>857</td>
</tr>
<tr>
<td>3.0</td>
<td>500</td>
</tr>
<tr>
<td>5.0</td>
<td>300</td>
</tr>
<tr>
<td>7.0</td>
<td>214</td>
</tr>
<tr>
<td>10.0</td>
<td>150</td>
</tr>
</tbody>
</table>

2.9.2.2 Determination of Bed Flow Characteristics

The column was tightly packed with inactive Eupergit C beads i.e with no enzyme loading, and a 1 % solution of acetone in water was pumped through the column at 3 mL/min. Samples were continually collected in 5 mL glass vials every 20 seconds. 0.2 mL of each sample was diluted with 0.8 mL of distilled water and the absorbance was measured in a quartz cuvette at 260 nm. The resulting profile (Figure 2.12) indicates the degree of plug flow. Although every effort was made to minimize mixing in the feed line and outlet of the column, this could explain some of the deviation from plug flow observed.

2.9.2.3 Determination of percentage conversion vs flowrate

The column was packed with 6 g (dry weight) of immobilized Eupergit C beads. Saturated solutions of 1-chlorobutane and 4-chlorobutanol in distilled water at pH 7 and pH 9 were passed through the column at flow rates between 1.75 - 10.0 mL/min. After steady state had been reached, samples were taken at the inlet and outlet and the concentration of these solutions was determined using gas chromatography.
2.9.3 Use of a Mini Batch Reactor

In order to compare the performance of the plug flow reactor and a batch reactor, two 100 mL stirred tank batch reactors were run with 45 mL of a saturated solution of 1-chlorobutane in distilled water at pH 7. The temperature was kept constant by satnding the reactors in a water bath at 30 °C. 1 g of immobilized enzyme was added to one reactor and 5 mL of crude enzyme was added to the second batch reactor. 1 mL samples were taken every 30 minutes and the concentration of each sample was determined using gas chromatography.
Figure 2.11  Bed volume profile showing percentage conversion of a saturated solution of 1-chlorobutane at pH 7 vs volume through packed column.
Figure 2.12 Plug flow profile showing the outlet absorbance of a 1% acetone solution through a packed bed.
3.0 RESULTS

3.1 FERMENTATION DEVELOPMENT

In order to make the enzyme production system more industrially favourable, it was necessary to intensify the bioprocess by increasing both the bacterial concentration and the enzyme activity. This was done by developing the fermentation medium and the fermentation, induction and disruption conditions.

3.1.1 Medium Development

Initial experiments using the medium from Sallis et al. (1990) produced a final cell concentration of 0.7 \( g_{dw}/L \) in a 2 L fermenter. Subsequent medium changes increased the final cell concentration to 4.5 \( g_{dw}/L \) in a 2 L fermenter (Figure 3.0), 5.9 \( g_{dw}/L \) on a 20 L scale and 9.28 \( g_{dw}/L \) on a 1500 L scale.

In the search for an improved medium, many combinations were tried. Glycerol and yeast extract were established as the main carbon and energy sources and the amounts were adjusted to obtain the maximum bacterial concentration and specific growth rate. The results of these experiments are presented in Table 3.1 with the corresponding medium components shown in Table 3.0.

Figure 3.0 clearly illustrates the increase in cell concentration on medium B and the reduction in fermentation time to achieve this increased concentration. The yield on glycerol and yeast extract on a 20 L scale was 0.5 g/g and 0.375 g/g on a 2 L scale. The main disadvantage of this rich medium was the increased possibility of contamination, which was not a major problem when the sole carbon source was 1-chlorobutane. Batches of highly contaminated yeast extract caused problems which were overcome by increasing the sterilization holding time at 121 °C from 20 to 35 minutes.
<table>
<thead>
<tr>
<th>Component</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>-</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.2</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.1</td>
<td>2.1</td>
<td>4.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Na succinate</td>
<td>0.025</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.025</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>6.0</td>
<td>-</td>
<td>6.0</td>
<td>10.0</td>
<td>3.0</td>
<td>1.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.02</td>
<td>6.0</td>
<td>0.04</td>
<td>12.0</td>
<td>20.0</td>
<td>6.0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace Elements(mL)</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 3.1 Comparison of bacterial concentration and specific growth rates obtained on the media shown in Table 3.0 on a 2 L scale.

<table>
<thead>
<tr>
<th>Media</th>
<th>Maximum Specific Growth Rate (h⁻¹)</th>
<th>Final cell concentration (g dw/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.085</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>0.13</td>
<td>4.5</td>
</tr>
<tr>
<td>C</td>
<td>0.066</td>
<td>0.5</td>
</tr>
<tr>
<td>D</td>
<td>0.073</td>
<td>3.7</td>
</tr>
<tr>
<td>E</td>
<td>0.070</td>
<td>4.1</td>
</tr>
<tr>
<td>F</td>
<td>0.070</td>
<td>3.8</td>
</tr>
<tr>
<td>G</td>
<td>0.068</td>
<td>2.9</td>
</tr>
<tr>
<td>H</td>
<td>0.067</td>
<td>3.3</td>
</tr>
<tr>
<td>I</td>
<td>0.100</td>
<td>3.1</td>
</tr>
</tbody>
</table>

In order to increase further the bacterial concentration, the amounts of both glycerol and yeast extract were increased to 12 g/L. On a 1500 L scale, this had the effect of increasing the cell concentration by a third (Section 3.4), but little difference was noticed on a 20 L scale. This difference in bacterial concentrations with increasing scale will be discussed later (Section 4.1).
Figure 3.0  Comparison in cell concentration against fermentation time for a culture of Y2 grown on a 2 L scale under the same conditions with two different media. A 10 % (v/v) inoculum was used in both cases. Medium A was developed by Sallis et al. (1990) and medium B was as shown above in Table 3.0.
For practical purposes when scaling-up, the percentage inoculum used during the fermentation was reduced from 10% to 1%. A 20 L fermentation was carried out to determine whether this reduction would have an effect on the final cell concentration and the fermentation time. The specific growth rate remained the same (0.13 h⁻¹) and the final cell concentration (5.9 g_dw/L) was greater than that obtained on a 2 L scale with a 10% inoculum (4.5 g_dw/L).

3.1.2 Effect of pH on Specific Growth Rate
Shake flask experiments were carried out to assess the effect of pH on the specific growth rate of the organism. Figure 3.1 shows that the maximum specific growth rate was obtained at pH values between 6-7. If the pH was below pH 6, the growth rate was severely affected. This was confirmed by a fermentation where the pH was decreased to pH 4 and the organism stopped growing. Above pH 7, the organism still grew but at a much reduced growth rate. It was decided, therefore, to maintain the pH at 7.0 during fermentation and then to resuspend the harvested cells in a glycine buffer at a pH of 9-10, the pH optimum for enzyme activity before disruption. Acid was produced and, in the fermentations, the pH was kept constant at pH 7 using 4 M NaOH solution. 50 mL of alkali was added over a 24 hour period to a broth volume of 14 L. It was not necessary to add acid (H₃PO₄) unless an excess of alkali had been added by the automatic alkali addition pump.

3.1.3 20 L Fermentation Data
Six successful 20 L fermentations were carried out and were found to be reasonably reproducible. The final cell concentration was always between 5.1 - 6.1 g_dw/L and this was achieved during 20 - 24 hours of fermentation depending on the state of the inoculum. The 140 mL inoculum was usually left on a shaker for 24 hours after which time the OD was 2.1. In all 20 L fermentations, the OUR and CER increased.
exponentially up to fifteen hours as expected, then there was a dip in the OUR and CER where a switch in the main nutrient source from yeast extract to glycerol occurred. In the fermentations shown in Figure 3.2 and Figure 3.3, the OUR and CER increased to a maximum of between 16-17 mmol/Lhr and 13.5-14 mmol/Lhr respectively. There were no problems in these fermentations of oxygen limitation, as the DOT did not drop below 80% of air saturation. The absorbance and cell concentration were measured at intervals throughout these fermentations and a typical profile can be seen in Figure 3.4. The relationship between the absorbance and the bacterial concentration is a linear one (Figure 3.5).
Figure 3.1 Results of shake flask experiments to show the effect of pH on the specific growth rate of *Rhodococcus erythropolis* on medium B.
Figures 3.2 OUR, DOT and CER profiles for two successive 20 L fermentations of *Rhodococcus erythropolis* Y2. The medium was inoculated with 140 mL of culture (OD = 2.1), 1.5 hours after the start of monitoring. The culture was induced with the addition of 4-chlorobutanol after 4 hours of fermentation (OD = 0.656). After 11 hours, (OD = 1.985), 2.0 g/L of glycine were added to weaken the cell wall.
OUR and CER profiles for two successive 20 L fermentations of *Rhodococcus erythropolis* Y2. The medium was inoculated with 140 mL of culture (OD = 2.1) and induced with the addition of 4-chlorobutanol after 4 hours of fermentation (OD = 0.656). After 11 hours, (OD = 1.985), 2.0 g/L of glycine were added to weaken the cell wall.
Figure 3.4 Plot of cell concentration, and absorbance at 600 nm, against fermentation time for a typical 20 L fermentation of *Rhodococcus erythropolis* Y2.
Figure 3.5  Relationship between absorbance at 600 nm and bacterial concentration during a 20 L fermentation of *Rhodococcus erythropolis* Y2.
3.2 INDUCTION

As stated earlier, the dehalogenase was inducible and active towards a greater range of halogenated aliphatic compounds than would support growth of the organism. Initially, 1-chlorobutane was used as the inducer (Sallis et al. 1990). After subsequent trials with chlorobutane and a range of haloalcohols, a less volatile was chosen. Experiments with the amount and type of inducer and the induction time showed that if 0.87 g/L of 4-chlorobutanol was added when the cell concentration was 0.8 gdw/L, the specific enzyme activity increased to 0.16 U/mg protein compared with 0.05 U/mg protein on 1-chlorobutane.

3.2.1 Choice of Inducer

4-chlorobutanol was chosen from a range of haloalkanes and haloalcohols that were known to induce the enzyme (Table 3.2). 4-chlorobutanol produced the highest enzyme activity (0.158 U/mg protein) and was less volatile and more soluble than 1-chlorobutane. Figure 3.6 shows a typical profile of chloride release against time for a 20 L fermentation of *Rhodococcus erythropolis Y2*. The culture was induced with 4-chlorobutanol (0.87 g/L) after 4 hours of fermentation (cell concentration = 0.8 gdw/L) and a slight increase in chloride concentration was noticed, due to HCl impurities in the 4-chlorobutanol. Three samples were taken each time to confirm the accuracy of the chloride analyzer. The differences in the results obtained for these samples show the slight deviations in the readings obtained using the chloride analyzer, which are negligible. An increase in the chloride concentration of 220 mM was observed over a 10 hour period as some of the inducer was converted to 1,4-butanediol with the release of a chloride ion. The background reading of approximately 470 mM came from the chloride in the medium and this decreased slightly as the organism started to grow and consume the nutrients. In order to
reduce the background reading, a low salt, low chloride containing yeast extract (Bovril Foods) was found and used in subsequent fermentations.

3.2.2 Amount of Inducer

Figure 3.7 shows the results of a shake flask experiment to determine the optimum amount of inducer needed to give the highest enzyme activity. The linear slopes of each plot show that the steepest gradients were obtained with 0.87-1.31 g/L of 4-chlorobutanol. Toxicity also had to be considered so 0.87 g/L was chosen as the amount of inducer that gave the maximum enzyme production for the least amount of 4-chlorobutanol added. In this case, the background reading was approximately 13 mM.

3.2.3 Induction Time

Figure 3.8 shows the results of a shake flask experiment to determine the optimum time to induce the culture. Chloride concentration (mM) is plotted against time for induction times between 0-23 hours. In the early stages of the fermentation, the time of induction did not have a profound effect on enzyme production as long as the culture was induced before the OD was 1.0 (approximately 12 hours in a shake flask) and ideally when the cell concentration was 0.8 g_{dw}/L. The addition of the inducer twice during the fermentation was also studied but only negligible increases in activity were noticed and, as there was an increased risk of contamination through a second addition, this option was not pursued.
<table>
<thead>
<tr>
<th>Inducer</th>
<th>Solubility</th>
<th>B.P (°C)</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Chlorobutane</td>
<td>7.13 mM</td>
<td>77-78</td>
<td>0.044</td>
</tr>
<tr>
<td>1-Chlorobutanol</td>
<td></td>
<td></td>
<td>0.035</td>
</tr>
<tr>
<td>4-Chlorobutanol</td>
<td>315 mM</td>
<td>84-85/16 mm</td>
<td>0.158</td>
</tr>
<tr>
<td>1,3-dichloro-2-propanol</td>
<td>174</td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>2-Chloroethanol</td>
<td>129</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>3-Chloro-1-propanol</td>
<td>160-162</td>
<td></td>
<td>0.042</td>
</tr>
</tbody>
</table>
Figure 3.6  Plot of three simultaneous readings of chloride release against time for a 20 L fermentation of *Rhodococcus erythropolis* Y2. The culture was induced after 4 hours with 4-chlorobutanol.
Figure 3.7 Chloride concentration (from Chlor-o-counter) against fermentation time for various amounts of inducer. The inducer was 4-chlorobutanol and was added at the beginning of the fermentation. The following amounts of inducer were added (g/L): 0, 0.22, 0.44, 0.87 and 1.31.
Figure 3.8 Results using a Chlor-o-counter showing chloride concentration against time for cultures of *Rhodococcus erythropolis* Y2 induced at different times with 0.87 mg/L of 4-chlorobutanol. The induction times (hours) are as follows: 0, 5, 9, 12, 23, and 25.
3.3 CELL DISRUPTION

*Rhodococcus erythropolis* is a Gram-positive organism and is therefore difficult to disrupt. The two main factors that have been found to influence protein release during disruption are the addition of glycine to the fermentation and the number of passes through the homogenizer.

### 3.3.1 Effect of Addition of Glycine to the Fermentation

Glycine has been shown to weaken cell walls and aid protein release (Miyashiro *et al.*, 1980). Figure 3.9 shows the results of a shake flask experiment to determine enzyme activity against the amount of glycine added. The cultures were induced after 9 hours (OD at 600 nm = 0.45) and amounts of glycine between 0.2 and 10.0 g/L were added to the flasks after 21 hours of fermentation. The bacteria were harvested during the late exponential phase by centrifugation (7500 rpm, 4 °C, 15 minutes), the pellet was washed and resuspended in 20 mL of 100 mM glycine/NaOH buffer, pH 9.1. The cell suspension was then passed through a French pressure cell five times at 1.38 x 10^8 Pa. After recentrifugation, the enzyme activity of the cell free extract was determined by the method used by Bergmann & Sanik, (1957). The results show that the addition of 2.0 g/L of glycine in the late exponential phase gave the highest enzyme activity. A 20% increase in protein release was also observed. Using the Bradford protein assay, the maximum protein concentration in cell free extracts was found to be 2.2 g/L.

Once glycine had been shown to aid protein release, it was necessary to determine the best time to add it. Several shake flask experiments indicated that the highest chloride release and hence enzyme release was achieved when the OD was 1.9, just before the fermentation reached the stationary phase (Figure 3.10). In a shake flask, the final OD was 2.1. It would have been preferable if both the glycine and the
inducer could be added at the same time. Since the ability to have only one addition would reduce the possibility of contamination and would be more convenient, especially on a large scale. Thus, in 20 L and 1500 L fermentations, 2.0 g/L of glycine was added in the late exponential phase. However, it was not possible to add the glycine at the same time as the inducer, as the larger scale fermentations grew much faster. It was therefore necessary to induce the cultures much earlier than it was desirable to add the glycine.

3.3.2 Effect of Number of Passes on Protein Concentration

At the end of a 2 L fermentation, the cells were harvested and the pellet was resuspended in 24 mL of 100 mM glycine/NaOH buffer, pH 9.1. These small volumes (24 mL) were put through the French pressure cell. At the end of a 20 L fermentation, the cells were harvested and resuspended in 2 L of 100 mM glycine/NaOH buffer, pH 9.1. This larger volume (2 L) was put through a high pressure homogenizer. When taking 40 mL samples from a 20 L or 1000 L fermenter during fermentation, the samples were passed directly through a Lab 40 homogenizer. All protein concentrations were measured using the Bradford protein assay.

Experiments were performed to assess the effect that the number of passes had on the protein release from cells in a French pressure cell (Figure 3.11) and in a high pressure homogenizer (Figure 3.12). As expected, the protein concentration increases with the number of passes through either a French pressure cell or a high pressure homogenizer and it reaches over 80 % of the total released after 5 passes through the French pressure cell. However, Figure 3.12 shows that, after four passes through the high pressure homogenizer, the enzyme activity begins to decrease despite an increase in protein concentration and this is probably the case in the French pressure cell as the enzyme may start to be denatured in the presence of
increasing protease concentration. For subsequent experiments, the cell suspension was passed 5 times through the French pressure cell and 4 times through the high pressure homogenizer.

3.3.3 Effect of Harvest Time on Protein Concentration in Cell Free Extracts

During a 20 L fermentation of *Rhodococcus erythropolis* Y2, 40 mL samples of the culture broth were taken in the late exponential phase and passed five times through a Lab 40 homogenizer (12,000 bar). Using this information and noting the shape of the OUR and CER trace at the point of maximum concentration it was possible to determine the best time to harvest the cells and thus predict in subsequent fermentations when the culture should be harvested. In this fermentation, the maximum protein concentration was obtained after 18 hours of fermentation when the OD at 600 nm was 4.2 (Figure 3.13). The maximum OD obtained on a 20 L scale was 5 but the fermentation was in its stationary phase at this point and enzyme and protein release are understood to decrease in this phase as the cell wall becomes stronger (Keshavarz et al., 1987).
Figure 3.9 Results of shake flask experiments to determine the final enzyme activity in cell free extracts (CFEs) for the amount of glycine added after 21 hours to a fermentation of *Rhodococcus erythropolis* Y2. The amounts of glycine are 0.0, 0.2, 0.5, 1.0, 2.0 and 10.0 g/L.
Figure 3.10 Effect of addition time of glycine (and OD at that time) on chloride release. The culture of *Rhodococcus erythropolis* Y2 was induced with 4-chlorobutanol and 2.0 g/L of glycine was added to the culture at various times throughout the fermentation. The absorbances (600 nm) at those times were: 0.12 — , 0.14 — , 0.30 — and 1.90 — .

![Graph showing chloride concentration over time](image-url)
Figure 3.11  Relative protein concentration against number of passes in a French pressure cell at 1.38 x 10^8 Pa (SLM Instruments Inc. Urbana, USA).

100 % protein concentration corresponds to 2.0 g protein/L.
Figure 3.12  Relative protein concentration and enzyme activity against number of passes in the APV Manton-Gaulin high pressure homogenizer. The pressure in the homogenizer was maintained at 500 bar, the flow rate through the homogenizer was 60 L/hr and the temperature of the homogenate was 4 °C. The cell concentration in the 100 mM glycine/NaOH buffer suspension, pH 9.1, was 4.13 g_{dw}/L. 100 % protein concentration corresponds to 2.2 g protein/L. Relative protein concentration — □— , relative enzyme activity — •—.
Figure 3.13 Protein concentration profile of 5 th pass cell free extracts against fermentation time. 40 mL samples were taken at intervals from a 20 L fermentation of *Rhodococcus erythropolis* and passed five times through a Lab 40 homogenizer operated at 1290 bar.
3.4 EFFECT OF SCALE-UP ON THE FERMENTATION

In order to produce sufficient amounts of enzyme for the project and to study the effects of scaling-up the fermentation, a 20 L (14 L working volume) fermentation was used as the inoculum for a 1500 L run (1000 L working volume). Medium B was used in both fermentations with 12 g/L of glycerol and yeast extract instead of 6 g/L as used previously.

3.4.1 Data from 20 L and 1500 L Fermentations

The operating conditions and fermentation data are summarized in Table 3.3, the conditions were decided by the scale-up criteria, assuming geometrical similarity (section 2.8.1). The inoculation time was decided by consulting the OUR/CER profile for the 14 L fermentation (Figure 3.15) and thus the seed culture was inoculated into the 1500 L vessel in the late exponential phase (section 3.3). Dry weight measurements were taken throughout each fermentation and these growth curves (Figure 3.14) demonstrate the much higher cell concentration and the faster growth rates obtained at the large scale. The 20 L fermentation in Figure 3.14 was used as the inoculum for the 1500 L fermentation and therefore does not reach a final maximum cell concentration. A typical 20 L fermentation will reach a maximum final cell concentration of between 5.1 - 6.1 g_{dw}/L (Figure 3.4). Hence, a 30 % increase in the final cell concentration was observed on a 1500 L scale and the growth rate was nearly three times that on a 20 L scale. The final OD on a 20 L scale was 5 and on a 1500 L scale was 10.5. A similar increase was observed when scaling-up the fermentation from 2 L to 20 L.

Using the data logging system, RTDAS, fermentation profiles were obtained during the 20 L and 1500 L fermentations (Figures 3.15 and 3.16 respectively). The 20 L profile is as expected, reaching slightly higher OUR and CER values due to an improved inoculum. It is also possible to note the diauxic lag, where there is a drop
In the 20 L profile the organism switches from using yeast extract as the energy source to using glycerol.

Table 3.3  Fermentation Conditions and Results

<table>
<thead>
<tr>
<th></th>
<th>20</th>
<th>1500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Volume (L)</td>
<td>20</td>
<td>1500</td>
</tr>
<tr>
<td>Working Volume (L)</td>
<td>14</td>
<td>1000</td>
</tr>
<tr>
<td>Stirrer speed (rpm)</td>
<td>700</td>
<td>200</td>
</tr>
<tr>
<td>Air flowrate (L/min)</td>
<td>7</td>
<td>500</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>OUR$_{\text{max}}$</td>
<td>26 (23hrs)</td>
<td>35 (9hrs)</td>
</tr>
<tr>
<td>CER$_{\text{max}}$</td>
<td>19 (23hrs)</td>
<td>28 (9hrs)</td>
</tr>
<tr>
<td>Final cell concentration (g$_{\text{dw}}$/L)</td>
<td>6.09</td>
<td>9.28</td>
</tr>
<tr>
<td>Specific growth rate (h$^{-1}$)</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>Medium</td>
<td>B</td>
<td>B</td>
</tr>
</tbody>
</table>
Figure 3.14 Bacterial concentration against fermentation time for two fermentations of *Rhodococcus erythropolis* Y2.1500 L fermentation with medium B — — , 20L inoculum fermentation with medium B — — .
Figure 3.15 Fermentation profile showing OUR, \( \bullet \) and CER, \( \square \), for a 20 L fermentation of *Rhodococcus erythropolis* Y2 which was used as the inoculum for the 1500 L fermentation (Figure 3.16).
Figure 3.16  Fermentation profile showing OUR, and CER, for a 1500 L fermentation of *Rhodococcus erythropolis* Y2.
3.5 ENZYME KINETICS

In order to design a bioreactor for the biotransformation of 1-chlorobutane and 4-chlorobutanol catalyzed by the halidohydrolase produced by *Rhodococcus erythropolis* Y2, it was necessary to understand fully the kinetics of the enzyme and its stability under various conditions. This section reports on the effects of substrate concentration, amount of cell free extract, temperature and pH on the enzyme activity and also investigates the possibility of substrate and/or product inhibition.

3.5.1 Experiments with 1-Chlorobutane

1-chlorobutane is very poorly water soluble and this affects the reactor design as does the low substrate concentration of the saturated solution as will be discussed later (Section 4.7).

3.5.1.1 Effect of Substrate Concentration on Activity

Substrate concentration profiles were obtained at two pH values, pH 7 and pH 9, the most likely pH of the effluent stream and the optimum pH for enzyme activity respectively (Figures 3.17 and 3.18). From these plots, it is possible to estimate $V_{\text{max}}$ as the following: $V_{\text{max}} = 0.067$ U/mg protein at pH 7 and 0.095 U/mg protein at pH 9. At pH 7 the maximum rate is 30% less than at pH 9 and this is as expected from the pH profile of the enzyme (Figure 3.30).

3.5.1.2 Lineweaver-Burk Plots

The substrate concentration profiles show some deviation from Michaelis-Menten kinetics. In order to determine the kinetic constants, Michaelis-Menten kinetics were assumed and thus a slight error has been incurred in the determination of $V_{\text{max}}$ and $K_m$. From Figures 3.17 and 3.18 it is possible to obtain Lineweaver-Burk plots of $1/\text{rate}$ against $1/\text{substrate concentration}$ (Figures 3.19 and 3.20). $1/K_m$ and hence $K_m$
can be determined where the slope hits the x-axis. The $K_m$ for this system at pH 7 is 1.54 mM and at pH 9, is 0.84 mM. Thus the $K_m$ at pH 7 is nearly twice the value at pH 9 but these are both higher than the value obtained by Sallis et al. (1990) of 0.26 mM.

All data points shown in this section on 1-chlorobutane are the mean of two measurements and they show a reproducibility of ± 5 %.
Figure 3.17 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 7.
Figure 3.18  Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9.
Figure 3.19  Lineweaver-Burk plot showing \(1/\text{rate} vs 1/\text{substrate concentration}\) for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 7. The data were obtained from the Figure 3.17.
Figure 3.20  Lineweaver-Burk plot showing $1/r$ vs $1/\text{substrate concentration}$ for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9.
3.5.2 Experiments with 4-Chlorobutanol

4-chlorobutanol was used as a comparison to 1-chlorobutane as it is more water soluble (0.315 M), and less volatile. However, a saturated solution of 4-chlorobutanol is highly corrosive to stainless steel and this needed to be considered when designing a small scale reactor to demonstrate the degradation of 4-chlorobutanol.

3.5.2.1 Effect of Substrate Concentration on Activity

Substrate concentration profiles were obtained at two pH values, pH 7 and pH 9 (Figures 3.21 and 3.22). From these plots it is possible to estimate $V_{\text{max}}$ as the following: $V_{\text{max}} = 0.5$ U/mg protein at pH 7 and 1.5 U/mg protein at pH 9, which are both in the order of magnitude of 10 times higher than those obtained on 1-chlorobutane but the solubility of 4-chlorobutanol is in the order of 50 times the solubility of 1-chlorobutane in water.

3.5.2.2 Lineweaver-Burk Plots

From Figures 3.21 and 3.22 it is possible to obtain Lineweaver-Burk plots of 1/rate against 1/substrate concentration (Figures 3.23 and 3.24). Although it is possible to estimate a value of $K_m$, it very difficult to estimate the actual value, as the profiles, especially in Figure 3.24, do not cross the x-axis at the same point. This is not surprising in view of the low activities and the fact that it is a reciprocal plot so any errors are magnified. The $K_m$ may also be estimated from Figure 3.22 as the value of the substrate concentration at $1/2 \ V_{\text{max}}$. Thus the $K_m$ for this system at pH 7 is 66 mM and at pH 9, is 110 mM. The $K_m$ at pH 9 is almost twice the value at pH 7 and between 40 - 130 times higher than the values obtained on 1-chlorobutane.

All data points shown in this section on 4-chlorobutanol are the mean of two measurements and they show a reproducibility of ± 3 %.
3.5.3 Effect of the Amount of Cell Free Extract on Halidohydrolase Activity

The rate versus the amount of cell free extract used in the enzyme assay was determined at two pH values, pH 7 and pH 9 with 1-chlorobutane as the substrate (Figures 3.25 and 3.26). Both of these profiles show an almost linear increase in the rate with enzyme concentration as was expected. The activity at pH 7 was 70% of the activity at pH 9.
Figure 3.21 Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 7.
Figure 3.22  Rate vs substrate concentration profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9.
Figure 3.23  Lineweaver-Burk plot showing $1/r$ vs $1/\text{substrate concentration}$ for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 7.
Figure 3.24  Lineweaver-Burk plot showing 1/rate vs 1/substrate concentration for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9.
Figure 3.25  Activity vs amount of cell free extract for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 7.
Figure 3.26  Activity vs amount of cell free extract for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9.
3.5.4 Effect of Temperature on Halidohydrolase Activity

In order to assess the effect of temperature on the halidohydrolase activity, the enzyme assay was performed at a range of temperatures with saturated solutions of 1-chlorobutane and 4-chlorobutanol as substrates. The solubility of 1-chlorobutane and 4-chlorobutanol varied slightly with temperature and this caused slight inaccuracies in the results, as not all of the solutions were completely saturated. However, the results did enable the operating limits of the enzyme and the optimum conditions in the column to be established.

Figure 3.27 demonstrates a linear increase in enzyme activity towards 1-chlorobutane with temperature. Above 40 °C, the enzyme activity drops sharply. This could be due to enzyme denaturation at higher temperatures as indicated by the reaction profiles which were curved at higher temperatures and linear below 40 °C (Figure 3.28). Above 40 °C, the activity drops sharply, therefore to get the most efficient use of the enzyme, the column should be operated at temperatures between 30 - 40 °C. Another serious constraint is the volatility of the substrates which evaporate at higher temperatures. In practice, outlet waste streams would probably be at ambient temperature or above depending on the temperature of the initial process and the heat produced during that reaction, usually at a temperature between 20 - 30 °C, which is within the limits of the enzyme’s capability.

Similarly, with 4-chlorobutanol as the substrate, the temperature increases linearly and reaches a maximum at 40 °C (Figure 3.29). Thus, the column should be operated between 30- 40 °C, whether the substrate is 1-chlorobutane or 4-chlorobutanol.
Figure 3.27  Activity vs temperature profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9. 100 % corresponds to a rate of $8.61 \times 10^{-4}$ μmoles/mL.
Figure 3.28  Chloride concentration profile during enzyme assay performed at 30 °C, 40 °C, 50 °C, and 65 °C, for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9.
Figure 3.29 Activity vs temperature profile for the halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9. 100% corresponds to a rate of 0.0173 μmoles/mL.
3.5.5 Effect of pH on Halidohydrolase Activity

Studies on pH have confirmed the findings of Sallis *et al.*, (1990) that the dehalogenase retained more than 50% of its maximum activity over the range pH 7.5-11.0 (Figures 3.30 and 3.31). The pH optima for the dehalogenase on 1-chlorobutane and 4-chlorobutanol have been determined (Figures 3.30 and 3.31).

On 1-chlorobutane, the pH optimum for activity was at pH 9, which would be the best pH to operate a packed column. However, the most likely pH of a waste stream would be pH 7 and the enzyme would retain 50% of its activity at this pH.

On 4-chlorobutanol, the enzyme exhibited a broad pH range. Thus, the enzyme could be used between pH 5-10 as the activity does not drop below 50% over this range. The pH optima for the enzyme on 4-chlorobutanol is at pH 8 but it retains 90% of its activity between pH 7-9.
Figure 3.30  pH profile of dehalogenase activity towards 1-chlorobutane in clarified cell free extracts of *Rhodococcus erythropolis* Y2 grown on 1-chlorobutane. Activities are relative to that at pH 8 (0.044 U/mg protein).
Figure 3.31  pH profile of dehalogenase activity towards 4-chlorobutanol in clarified cell free extracts of *Rhodococcus erythropolis* Y2 grown on a rich yeast extract/glycerol medium (B). Activities are relative to that at pH 8 (0.085 U/mg protein).
3.5.6 Substrate Inhibition
There was no evidence of substrate inhibition on 1-chlorobutane, as the rate versus substrate concentration profiles remained constant after 100 % saturation was reached (Figure 3.18) at 7.13 mM. If substrate inhibition was present, the profile would be expected to decrease as 100 % saturation was exceeded. However, the Lineweaver-Burk plots may be indicative of substrate inhibition as will be discussed (Section 4.5).

3.5.7 Product Inhibition
When assays were performed with 1-chlorobutane as the substrate and in the presence of butanol (7.13 mM) and chloride ions (7.13 mM NaCl), there was no detectable decrease in the rate of reaction. Thus, there was assumed to be no product inhibition.

3.5.8 Stability of Halidohydrolase Activity in Cell Free Extracts
The enzyme preparation was stored at 30 °C, 4 °C, and - 20 °C for 300 days. Samples were taken at regular intervals and the activity measured (Figure 3.32). These results show that the enzyme remains highly active and can be stored at - 20 °C for over a year. At 4 °C, the enzyme will retain over 75 % of its activity for several months. However, when the enzyme is stored at 30 °C, it rapidly loses activity and the enzyme should therefore be stored at - 20 °C whenever possible.
Figure 3.32 Stability of halohydrolase activity in clarified cell free extracts of *Rhodococcus erythropolis* Y2 grown on a rich yeast extract/glycerol medium (B) towards 1-chlorobutane. Pre-incubated for various durations at 30 °C, 4 °C, and -20 °C.
3.6 RESULTS OF ENZYME IMMOBILIZATION

The reasons for immobilizing an enzyme have been discussed in section 1.6 and the advantages of doing so will be discussed later (Section 4.4). Thus, once the kinetics of the isolated halidohydrolase had been established, the enzyme was immobilized onto Eupergit C beads in order to determine the performance of a packed bed immobilized enzyme reactor. Eupergit C was chosen as a suitable matrix for immobilization because of the following: its known effectiveness at immobilizing a wide range of proteins; its high binding capacity; operational stability and no diffusional limitations. This section describes the results obtained from experiments to find the optimum conditions for immobilization of the isolated enzyme. The enzyme has been purified by Sallis et al. (1990) but in this case the enzyme was not purified as this was thought that too many steps were involved for it to be economical for a waste treatment process.

3.6.1 Immobilization Conditions

Experiments were performed with different immobilization parameters which included the pH of the binding buffer (Figure 3.33), the molarity of the binding buffer (Table 3.5), the immobilization time (Figure 3.35), and the amount of cell free extract per gram of beads (Figure 3.36). Figure 3.34 indicates the mechanism of immobilization of proteins onto Eupergit C via free amino groups and the characteristics of the matrix are outlined in Table 3.4.

There was a linear increase in the amount of activity between pH 7 and pH 9. The highest activity was obtained when the binding buffer was at pH 9, which is the pH optimum of the enzyme on 1-chlorobutane.

The molarity of the binding buffer also had a large effect on the activity of the immobilized enzyme. The highest activity was obtained when the molarity of the phosphate buffer was 0.5 M, and the activity dropped sharply when the molarity was increased or decreased.
The immobilization time experiment showed that the highest activity of the immobilized enzyme occurred when the enzyme preparation had been left at room temperature for 72 hours. The activity increased with immobilization time and the activity in the supernatant decreased correspondingly as more enzyme was bound. There was also some activity lost due to denaturation but this was only noticed after 48 hours.

The activity increased linearly with the amount of cell free extract available to be bound. As the graph does not plateau, the binding capacity of the beads was not reached. The highest activities and protein concentrations (Figures 3.36 and 3.37 respectively) were obtained with the largest amounts of cell free extract (200 - 300 mL/g beads). However, in order to get the most efficient use of the enzyme, it was necessary to look at the percentage of the total protein absorbed as well. The best usage was obtained when 80 - 100 mL of enzyme was used per gram of beads (Figure 3.37).

The conditions for immobilization were 100 mL of extract per gram of beads in 0.5 M phosphate buffer at pH 9, left at room temperature for 72 hours. A 50 % decrease in enzyme activity was noticed on immobilization.

3.6.2 Kinetics of the Immobilized Enzyme

In order to assess how the kinetics of the immobilized enzyme differed from those of the free enzyme, activity against substrate concentration profiles were obtained on 1-chlorobutane (Figure 3.38) and 4-chlorobutanol (Figure 3.39) both at pH 9. From these plots, it is possible to estimate \( V_{\text{max}} \) at pH 9 for 1-chlorobutane as 0.0185 U/gdry and for 4-chlorobutanol as 0.5 U/gdry. The profiles are similar to the ones obtained with the free enzyme. For the immobilized enzyme, the ratio of \( V_{\text{max}} \)
on 1-chlorobutane to 4-chlorobutanol is 1 : 27, and for the free enzyme the ratio of $V_{\text{max}}$ on 1-chlorobutane to 4-chlorobutanol is 1 : 15. This may suggest that the activity against 1-chlorobutane is depressed more on immobilization. Alternatively, the increased solubility of 4-chlorobutanol may lead to better diffusion into the immobilized enzyme particle.
Figure 3.33 Activity of immobilized enzyme against the pH of the binding buffer.
The molarity of the binding buffer was 0.5 M and the enzyme preparation was left at room temperature for 3 days. The assays were performed at pH 9.1.
Figure 3.34  Immobilization of proteins onto Eupergit C via free amino groups

![Reaction diagram]

Table 3.4  Characteristics of the Eupergit C matrix

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main composition:</td>
<td>Methacrylamide</td>
</tr>
<tr>
<td>Content of oxirane groups (μmol/g(dry))</td>
<td>&gt; 600</td>
</tr>
<tr>
<td>Binding capacity:</td>
<td>50 mg human albumin/g(wet)</td>
</tr>
<tr>
<td></td>
<td>35 mg human IgG/g(wet)</td>
</tr>
<tr>
<td>Pressure (kPa):</td>
<td>up to 3 x 10⁴</td>
</tr>
<tr>
<td>Particle size (μm):</td>
<td>150</td>
</tr>
<tr>
<td>Exclusion limit of molecular weight (kDa):</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 3.5  Activity of immobilized enzyme against the molarity of the binding buffer. The binding buffer was at pH 9 and the enzyme preparation was left at room temperature for 3 days.

<table>
<thead>
<tr>
<th>Molarity of binding buffer (M)</th>
<th>Activity (U/gdry)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.028</td>
</tr>
<tr>
<td>0.5</td>
<td>0.039</td>
</tr>
<tr>
<td>1.0</td>
<td>0.011</td>
</tr>
</tbody>
</table>
Figure 3.35 Activity against immobilization time profile showing the activity loss due to denaturation ———, the activity of the immobilized enzyme ———, the activity remaining in the supernatant, ——— and the total activity ———, which is the sum of the immobilized activity and the activity in the supernatant. The molarity of the binding buffer was 0.5 M at pH 9 and the enzyme preparation was left at room temperature for 0 - 3 days.
Figure 3.36  Activity against 1-chlorobutane of the immobilized enzyme against the amount of cell free extract per gram of beads. The molarity of the binding buffer was 0.5 M at pH 9 and the enzyme preparation was left at room temperature for 3 days.
Figure 3.37 Protein concentration absorbed ——, and percentage of total protein absorbed ——, against the amount of cell free extract per gram of beads. The molarity of the binding buffer was 0.5 M at pH 9 and the enzyme preparation was left at room temperature for three days.
Figure 3.38 Activity vs substrate concentration profile for the immobilized halidohydrolase from *Rhodococcus erythropolis* Y2 towards 1-chlorobutane solution at pH 9.
Figure 3.39  Activity vs substrate concentration profile for the immobilized halidohydrolase from *Rhodococcus erythropolis* Y2 towards 4-chlorobutanol solution at pH 9.
3.7 BIOREACTOR DESIGN

In order to design a small scale system for the removal of halogenated aliphatic compounds from aqueous process streams, it is necessary to know something about the characteristics of the biotransformation. Thus the properties of the substrate, product, biocatalyst and the interaction between them as well as the the nature of the reaction for the removal of 1-chlorobutane are discussed. Using this information and the kinetic data already obtained, it is possible to calculate the amount of saturated solutions of 1-chlorobutane and 4-chlorobutanol that could be processed through a small scale packed bed reactor. The predicted values obtained are compared with experimental data in Section 3.8.

3.7.1 Characteristics of the Biotransformation

-Substrate properties:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>1-chlorobutane</th>
</tr>
</thead>
<tbody>
<tr>
<td>m.p</td>
<td>-123 0C</td>
</tr>
<tr>
<td>b.p</td>
<td>77-78 0C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>poorly water soluble (7.13mM)</td>
</tr>
</tbody>
</table>

-Product properties:

<table>
<thead>
<tr>
<th>Products</th>
<th>butanol (&amp; Cl-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m.p</td>
<td>-90 0C</td>
</tr>
<tr>
<td>b.p</td>
<td>117.7 0C</td>
</tr>
<tr>
<td>Water solubility</td>
<td>soluble</td>
</tr>
</tbody>
</table>

-Reaction characteristics:

The equation for the reaction is as follows:
\[ \text{CH}_3(\text{CH}_2)_3\text{Cl} + \text{H}_2\text{O} \rightarrow \text{CH}_3(\text{CH}_2)_3\text{OH} + \text{HCl} \quad (\text{Equation } 3.0) \]

Acid/base production: 1 mole HCl produced per mole of 1-chlorobutane degraded.

Water consumption: 1 mole of water consumed per mole of 1-chlorobutane degraded.

Gaseous consumption: none

-Biocatalyst characteristics:

Single/multiple enzyme: single dehalogenase induced, although an oxygenase may also be induced.

Requirement for cofactors: no cofactors or oxygen required.

Location of enzyme activity: The halidohydrolase is hydrophilic and so the reaction can be assumed to be occurring in the aqueous phase.

Biocatalyst requirement for water: \( 1\text{H}_2\text{O} \)

-Interactions between biocatalyst, substrate and product:

Substrate inhibition: none

Product inhibition: none

Activity:
- dependence on substrate concentration (Figure 3.18)
- dependence on pH (Figure 3.30)
- dependence on temperature (Figure 3.27)

Substrate toxicity: not toxic at saturation

Product toxicity: not toxic
3.7.2 Enzyme Production Target

The following assumptions were made to allow an estimation of the enzyme production target to be made:

1. Rhodococcus erythropolis grown to 9.8 gDW/L
2. Cellular protein = 0.53 g/g bacteria
3. Haloalkane dehalogenase = 3.9 % of cellular protein

Enzyme concentration in fermentation broth:
\[ = 9.8 \times 0.53 \times 0.039 = 0.203 \text{ g/L} \]

Estimated loss on extraction assuming use of clarified cell free extracts, 4 steps:

1. harvesting
2. cell disruption
3. clarification
4. resuspension and reclarification

If step yield is 90 % then overall yield = 0.94 \times 100 = 65.61 \% = 66 \%
\[ = 0.66 \times 0.203 = 0.134 \text{ g/L fermentation broth} \]

If a 1500 L is used, fermenter then can produce 133.7 g/batch

The immobilized enzyme retained 50 % of its activity:
\[ = 66.9 \text{ g/batch} \]

Assume total time per batch = 15 hrs = 900 mins

total reaction time = 13 hrs = 780 mins

If two batches are run per day for 300 days per year then they can produce:
\[ 2 \times 300 \times 80.4 = 40.1 \text{ kg/annum} \]
What is the percentage conversion required?

- E.E.C directives for similar compounds: 3 μg/L in drinking water
  3 mg/L in river water
- 100% saturation in water of 1-chlorobutane is 7.13 mM (0.66 mg/L)
- 100% saturation in water of 4-chlorobutanol is 315 mM (34.2 mg/L)

Therefore, if we assumed that the waste streams are saturated with 1-chlorobutane and 4-chlorobutanol, the information above suggests values of 99.5% conversion of 1-chlorobutane (to drinking water standards) and approximately 91% conversion of 4-chlorobutanol (to river water standards) or 99.99% conversion of 4-chlorobutanol (to drinking water standards).

3.7.3 Design of Mini-Reactors

Mini immobilized enzyme packed bed reactors were designed using the kinetic data obtained. The information was used to determine how much effluent could be processed in the model reactors assuming plug flow and assuming saturated solutions of 1-chlorobutane and 4-chlorobutanol respectively were being pumped through them.

3.7.3.1 Design of Mini-reactor with 1-Chlorobutane as Substrate

For plug flow reaction that obeys Michaelis-Menten kinetics:

Rate equation (Wang et al., 1979):

\[ XS_0 - K_m \ln (1 - X) = kE/q \]  

(Equation 3.1)

where:

X = fraction conversion = 0.995

S_0 = Initial substrate concentration = 7.13 mM

K_m = 0.84 mM
\[ 0.995 \times 7.13 + 0.84 \times 5.3 = \frac{kE}{q} \]

\( k = \text{specific activity} = 0.1 \, \mu\text{mol/min/mg} \)
\( E = \text{amount of enzyme} = 66.9 \, \text{g/batch for 1000 L and 0.94 g/batch for 14 L} \)
\( q = \text{flow rate (L/min)} \)

\[ \frac{kE}{q} = 11.54 \, \text{mM} \]

\[ E = \frac{11.54q}{10^{-4}} \]

If \( E = 0.94 \, \text{g} \) then,

\[ q = 0.94 \times 0.1/11.54 = 0.008 \, \text{L/min} = 0.008 \times 60 \times 24 = 11.52 \, \text{L/day} \]

If we take \( X = 0.90 \) then,

\[ \frac{kE}{q} = 8.35 \, \text{mM} \]

\[ q = 0.94 \times 0.1/8.35 = 0.011 \, \text{L/min} = 0.011 \times 60 \times 24 = 15.84 \, \text{L/day} \]

### 3.7.3.2 Design of Mini-reactor with 4-Chlorobutanol as Substrate

For plug flow reaction that obeys Michaelis-Menten kinetics:

Rate equation:

\[ XS_o - K_m \ln (1 - X) = \frac{kE}{q} \quad \text{(Equation 3.2)} \]

\( X = \text{fraction conversion} = 0.9999 \)
\( S_o = \text{Initial substrate concentration} = 315 \, \text{mM} \)
\( K_m = 110 \, \text{mM} \)

176
0.9999 \times 315 + 110 \times 9.21 = \frac{kE}{q} \\

k = \text{specific activity} = 1.5 \mu\text{mol/min/mg} \\
E = \text{amount of enzyme} = 0.94 \text{ g/batch} \\
q = \text{flow rate (L/min)} \\

k\frac{E}{q} = 1328 \text{ mM} \\

If E = 0.94 \text{ g then,} \\

q = 0.94 \times 0.1/1328 = 7 \times 10^{-5} \text{ L/min} = 0.1 \text{ L/day} \\

If we take X = 0.91 then, \\

k\frac{E}{q} = 551.5 \text{ mM} \\

q = 0.94 \times 0.1/551.5 = 1.7 \times 10^{-4} \text{ L/min} = 0.25 \text{ L/day} \\

3.8 RESULTS FROM SMALL SCALE TRIALS OF TWO MODEL SYSTEMS 

A small scale packed bed plug flow reactor and a batch stirred tank reactor were set up and run with saturated solutions of 1-chlorobutane and 4-chlorobutanol as described in section 2.9. The results show the data obtained when the reactors were run under different conditions. 

3.8.1 Packed Bed Plug Flow Reactor 

The percentage conversions of saturated solutions of 1-chlorobutane and 4-chlorobutanol against the flow rate through the reactor were determined. Samples
3.8.1.1 Percentage Conversion vs Flow rate Profiles with Saturated 1-Chlorobutane

Figure 3.40 shows the percentage conversions obtained when the packed column with 75 mg of immobilized enzyme was run with saturated 1-chlorobutane at pH 7, three times over a ten hour time period. In between experiments, the column was left running continuously at 1.75 mL/min. This plot clearly illustrates that when the flow rate is increased to 10 mL/min and then decreased again to 1.75 mL/min, the percentage conversion returns to the initial value obtained. This confirms the reproducibility of the reaction over a range of flow rates, although, a slight decrease in the overall percentage conversion was noticed over the 10 hour time period. If there is a loss of activity, it would be much less obvious at conversions approaching 100 %. The percentage conversion of saturated 1-chlorobutane with immobilized enzyme beads in the packed column was very high (between 97 - 100 %). This high conversion rate made it difficult to obtain a broad range of profiles as it was not possible to increase the flow rate above 10 mL/min because the pressure drop across the column would be too great and may cause the column to explode. In an attempt to extend the conversion range, the column was run with 50 % and 25 % of immobilized enzyme, the rest of the column being filled with inert beads (Figure 3.41). Under these conditions the lowest percentage conversion achieved was 80 % which is still very high. When these results are corrected for the total amount of enzyme in the column, then the curves obtained in Figure 3.41 are almost superimposable (Figure 3.42) as would be expected from Equation 3.1. It should also be noted that the percentage conversions obtained in Figure 3.41 with 100 % beads are lower than those obtained in Figure 3.40 with the same column. This is
because the immobilized enzyme in Figure 3.41 had been in use for over 30 hours and had been stored at 4°C for two weeks when not in use.

3.8.1.2 Percentage Conversion vs Flow rate Profiles with Saturated 4-Chlorobutanol

The highest percentage conversion obtained with a saturated solution of 4-chlorobutanol at pH 7 was only 20% (compared to 100% with 1-chlorobutane) (Figure 3.43). This is due to the much greater solubility of 4-chlorobutane in water without a corresponding increase in enzyme activity on 4-chlorobutanol. In order to achieve 100% conversion, a much smaller flow rate than was possible would be needed as was predicted in the bioreactor design (section 3.7).

3.8.2 Mini Batch Reactor

The percentage conversions of a saturated solution of 1-chlorobutane at pH 7 in a small scale batch stirred tank reactor over a reaction period of over 3 hours were determined. Two experiments were performed, one with the free enzyme (Figure 3.44) and the other using the immobilized enzyme (Figure 3.45). The conversion increased linearly with the free enzyme to over 45% conversion. If the experiment could have been run for 7 hours, this could allow the free enzyme system to achieve a possible 100% conversion. By contrast, the batch reactor run with immobilized enzyme reached 100% conversion after 210 minutes and followed the predicted profile. The differences in using the immobilized or free enzyme in a batch stirred tank reactor will be discussed later as will the comparison between a batch or plug flow system (Sections 4.4 and 4.6).
Figure 3.40 Percentage conversion against flow rate of a saturated solution of 1-chlorobutane at pH 7 through a plug flow reactor packed with immobilized enzyme after 0 hours — , 6 hours — , and 10 hours — .

% conversion

Flow rate (mL/min)
Figure 3.41 Percentage conversion against flow rate of a saturated solution of 1-chlorobutane at pH 7 through a plug flow reactor packed with the following percentages of immobilized enzyme; 100 % , 50 % , and 25 % .
Figure 3.42  Percentage conversion against (flow rate of a saturated solution of 1-chlorobutane at pH 7) / (total amount of enzyme in the column).

Data obtained from Figure 3.41.
Figure 3.43  Percentage conversion against flow rate of a saturated solution of 4-chlorobutanol at pH 7 through a plug flow reactor packed with immobilized enzyme.
Figure 3.44  Percentage conversion against reaction time in a batch stirred tank reactor with 5 mL of free enzyme in 45 mL of a saturated solution of 1-chlorobutane at pH 7.
Figure 3.45  Percentage conversion against reaction time in a batch stirred tank reactor with 1 g\textsubscript{bw} of immobilized enzyme beads in 45 mL of a saturated solution of 1-chlorobutane at pH 7.
4.0 DISCUSSION

This project has studied the production and utilization of a haloalkane halidohydrolase from *Rhodococcus erythropolis* strain Y2. The discussion will deal with the main elements of the project, namely the large scale production of the enzyme, the state of the biocatalyst and the kinetics of the enzyme. Several reactor configurations were considered for a small scale biotransformation using the immobilized enzyme for the removal of halogenated aliphatic compounds from aqueous process streams.

4.1 LARGE-SCALE PRODUCTION OF THE HALIDOHYDROLASE FROM RHODOCOCCUS ERYTHROPOLIS Y2

The small scale growth of *Rhodococcus erythropolis* Y2 on short chain chloroalkanes was demonstrated by Sallis *et al.* (1990). They concluded that growth on these compounds was due to the presence of a halidohydrolase. The halidohydrolase showed no activity towards terminally halogenated C\textsubscript{14}, C\textsubscript{16}, and C\textsubscript{18} alkanes but could use these substrates for growth. The biochemical characteristics of the halidohydrolase were very similar to dehalogenases from *Corynebacterium* sp. m-15-3 (Yokota *et al.*, 1987), and *Arthrobacter* sp. HA1 (Scholtz *et al.*, 1987) and much information was already known about the characteristics of such dehalogenases.

An n-alkane monooxygenase from *Rhodococcus erythropolis* which allowed growth on n-alkanes of carbon chain length C\textsubscript{7} - C\textsubscript{18} was also found, but was not studied in this project.
presence of 1-chlorohexane at levels above 3 mM. Thus, in order to reduce the toxic
effects and increase the bacterial concentration, the organism was grown on a new
medium. Several different carbon and nitrogen sources were tested using
information from previous *Rhodococcus* papers (Armfield et al., 1990; Buckland et
al., 1974; Kurane et al., 1986; Nagasawa et al., 1988; Takeda et al., 1991; Watanabe et
al., 1987). A rich glycerol and yeast extract medium was preferred because, as
expected, much higher bacterial concentrations were obtained. The main problem
with this medium was the increased risk of contamination due to the variable degree
of contamination between different batches of yeast extract. Thus, a sterilization
protocol was established for a normal batch of yeast extract with a sterilization time
that killed off contaminants but left most of the nutrients and vitamins intact.
However, highly contaminated batches of yeast extract may need a longer
sterilization time which may have a detrimental effect on the available nutrients. The
overall idea of the new medium was to use a cheaper non-toxic medium to produce
an increased biomass and then induce the synthesis of larger quantities of the
enzyme.

*Rhodococcus* has often been used as a flocculating agent, an emulsifier and for
whole cell dehalogenation. Previously, *Rhodococcus erythropolis* and other species
of *Rhodococcus* have been grown on a relatively small scale, up to 50 L and usually
over a number of days, producing lower bacterial concentrations than were achieved
in this study (5.9 g<sub>dw</sub>/L on a 20 L scale). Rapp et al., (1979) grew *Rhodococcus
erythropolis* on a yeast extract and mineral salts medium in a 50 L batch fermenter to
a maximum concentration of 2.1 g<sub>dw</sub>/L. *Rhodococcus rhodochrous* was grown on a
basal medium containing benzonitrile and glucose in a 2 L shake flask and reached a
final cell concentration of 2.2 g<sub>dw</sub>/L in 76 hours (Nagasawa et al., 1988). Takeda
et al., (1991) used *Rhodococcus erythropolis* to produce a protein bioflocculant. The
culture was incubated for 4 - 5 days in a rich glucose and mineral salts medium to a final cell concentration of 3.75 g<sub>dw</sub>/L. Watanabe et al, (1987) showed the effects of organic nutrients on the growth and enzyme production of *Rhodococcus* sp. N-774 (Table 4.1). The basal medium contained the following (g/L): glucose, 10; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5; KH<sub>2</sub>PO<sub>4</sub>, 0.5; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5; FeSO<sub>4</sub>.7H<sub>2</sub>O, 0.2.

Table 4.1 Effects of Organic Nutrients on the Cell Growth and Enzyme Production by *Rhodococcus* sp. N-774. (Watanabe et al, 1987).

<table>
<thead>
<tr>
<th>Organic nutrients</th>
<th>(%)</th>
<th>Growth (g&lt;sub&gt;dw&lt;/sub&gt;/L)</th>
<th>pH</th>
<th>Nitrile hydratase Activity (U/mg dry cell wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>0.1</td>
<td>1.57</td>
<td>6.52</td>
<td>58.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5</td>
<td>3.40</td>
<td>6.70</td>
<td>60.2</td>
</tr>
<tr>
<td>Meat extract</td>
<td>0.5</td>
<td>1.90</td>
<td>7.12</td>
<td>12.6</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.1</td>
<td>0.20</td>
<td>7.34</td>
<td>-</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.1</td>
<td>1.24</td>
<td>6.53</td>
<td>49.8</td>
</tr>
<tr>
<td>-Thiamine.HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamino acid</td>
<td>0.1</td>
<td>0.12</td>
<td>7.90</td>
<td>-</td>
</tr>
<tr>
<td>Casamino acid</td>
<td>0.1</td>
<td>4.21</td>
<td>7.90</td>
<td>53.7</td>
</tr>
<tr>
<td>-Thiamine.HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casamino acid</td>
<td>0.5</td>
<td>5.45</td>
<td>7.94</td>
<td>51.4</td>
</tr>
<tr>
<td>-Thiamine.HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn Steep Liquor</td>
<td>1.0</td>
<td>3.01</td>
<td>6.51</td>
<td>55.9</td>
</tr>
<tr>
<td>Corn Steep Liquor</td>
<td>2.0</td>
<td>3.59</td>
<td>7.37</td>
<td>47.4</td>
</tr>
</tbody>
</table>
As in this example and with the growth of *Rhodococcus erythropolis* Y2, yeast extract was found to be the most favourable for cell growth and enzyme formation from amongst the organic nutrients tested. Different amounts of casamino acids were tested in this study but no significant effect was noticed on the growth of *Rhodococcus erythropolis* Y2 or on the production of the halidohydrolase.

It can also be seen that doubling the amounts of yeast extract does not necessarily double the final cell concentration. A similar observation was made, and has been discussed, in the 20 L fermentation. The final medium therefore contained sufficient concentrations of yeast extract and glycerol to produce high cell concentrations. It was also considered to be very economical, especially on a large scale where supplies could be bought in bulk.

A 1500 L (1000 L working volume) fermentation of *Rhodococcus erythropolis* was successfully grown and induced and the product was recovered downstream. Using this information it was possible to assess the industrial prospects of such a process and also to produce sufficient amounts of enzyme for the study. The 1000 L fermentation confirmed that the enzyme could be easily and reproducibly produced on a large scale without any significant problems or losses in activity. Unexpectedly, the culture grew faster and to a much higher final concentration on a large scale. There are several possible explanations for this observation. First, the state of the inoculum may have been at a more appropriate stage, thus reducing the initial lag phase from 10 hours in the 14 L fermenter to 4 hours in the 1000 L fermenter (Figures 3.14, 3.15 and 3.16). The inoculum for the 14 L fermenter was prepared in a shake flask and grown for 24 hours so that the inoculum was well into the stationary phase. For the 1000 L fermentation, the 14 L inoculum which obviously had better oxygen, temperature and pH regulation than in a shake flask, was inoculated into the 1000 L fermentation broth in the late exponential growth
phase. Another reason for the increase could be due to differences in the batch sterilization procedure at different scales. Fewer nutrients may have been destroyed on the larger scale and the use of a low salt yeast extract on the smaller scale may have reduced the amount of mineral salts available. Although this organism is not thought to be particularly shear sensitive, reduced shear was experienced on the larger scale.

Another suggestion for the increases could be due to a reduction in the inhibitory metabolites which were carried over from the inoculum. Alternatively, any increased production of amino acids in the early stages of the fermentation may have had the effect of increasing the organism's ability to use the yeast extract as an energy source. Both of these possibilities are advantageous to the fermentation.

A similar increase in the bacterial concentration was noticed whenever the scale was increased. Growth was faster in a 2 L fermenter than in a shake flask and a higher final bacterial concentration was achieved. In a shake flask, it was not possible to regulate the pH of the culture which has been shown to have a marked effect on the specific growth rate. There is also poorer oxygen transfer in a shake flask as air is not being continuously sparged into the system as in a fermenter. The final cell concentration also increased on a 20 L scale and then, as already mentioned, a larger increase was noted on a 1500 L scale (Figure 3.14).

The fact that the 1000 L fermentation reached its maximum OUR and CER after 9 hours, and not 23 hours as in the case of the 14 L fermentation, means that with a turnaround time of 3 hours, 2 fermentations may be carried out per day. Each fermentation could produce enough enzyme to be immobilized and packed into plug flow reactors which would be capable of processing approximately 850 L of a saturated solution of 1-chlorobutane to EEC standards per day. Assuming that all of
the enzyme was immobilized, 1700 L of a saturated process stream could be processed per day with two fermentations.

4.2 ENZYME INDUCTION

The haloalkane halidohydrolase in common with malic dehydrogenase and mannose dehydrogenase from *Rhodococcus erythropolis* is an inducible enzyme, unlike isocitrate dehydrogenase which is constitutive (Finnerty, 1992). A wide choice of inducers were available and the choice was made mainly on the inducer that gave the highest enzyme activity of those tested. 4-chlorobutanol was chosen as a suitable inducer and produced much higher activities than on 1-chlorobutane (0.16 U/mg protein compared to 0.05 U/mg protein previously). This value is still fairly low when compared to other similar organisms. Sallis *et al.* (1990) noted that the $K_m$ values of the dehalogenases from *Xanthobacter autotrophicus* (Verschueren *et al.* 1993c), *Corynebacterium* sp. m-15-3 (Yokota *et al.*, 1987), and *Arthrobacter* sp. HA1 (Scholtz *et al.*, 1987) all fell in the range 0.06-0.40 mM and with activities of approximately 1.5 U/mg protein. The main problem with 4-chlorobutanol as the inducer was one of cost. 1-chlorobutane was a relatively cheap inducer (£10/L) whereas 4-chlorobutanol was nearly 20 times more expensive. However, the advantages of having a less volatile inducer that produced higher activities with small concentrations of substrate, caused a decision to use 4-chlorobutanol as the inducer in all of the batch fermentations.

The results from the chloride analyzer (Figure 3.6) enabled the production of the enzyme throughout the fermentation to be monitored. This information was confirmed by the results of the enzyme assays performed on cell free extracts taken at different times in the late exponential phase of the fermentation (Figure 3.13). Thus, it was possible to determine the best time to harvest the culture and, coupling
this information with the on-line OUR and CER readings meant that the optimum harvest time could be predicted in future fermentations.

4.3 ENZYME ISOLATION
The halidohydrolase from *Rhodococcus erythropolis* is an intracellular enzyme and therefore it needs to be recovered once the cells have been harvested and resuspended. Rhodococci are gram positive and, like other similar bacteria, they are difficult to disrupt. Buckland *et al.*, (1974) described the large scale isolation of intracellular cholesterol oxidase from Nocardia. Figure 4.1 shows the relationship between the first order rate constants for enzyme and protein release from various organisms and the operating pressure of the homogenizer. The disruption rate constant obtained for *Rhodococcus erythropolis* Y2 at 500 kg f/cm² in a Manton-Gaulin homogenizer was estimated to be 0.072. This is over eight times the value for Nocardia but three times less than the values for *E.coli* and bakers' yeast. This is a relatively high value for this organism and this may be due to the weakening of the cell wall and the knowledge on protein release with the number of passes through the homogenizer (Figure 3.12).

Experiments with different homogenizers and different homogenization regimes meant that, once the culture had been harvested, the resuspended pellet could be processed under the most suitable disruption conditions. The addition of 2.0 g/L of glycine to the culture in the late exponential phase appeared to aid protein release (Figure 3.9). Although the exact mechanism is not known, glycine appears to alter the cell wall structure in such a way as to make protein excretion easier. A high concentration of glycine is known to interfere with the synthesis of the cell wall and also to depress the production of protease in the culture broth, thus producing higher protein concentrations (Hara *et al.*, 1983). Hishinuma *et al.*, (1971) observed that the primary site of action of glycine appeared to be the inhibition of cell wall
biosynthesis because glycine, D-amino acids or penicillin, induced spheroplast formation. Strominger and Birge, (1965) noted that glycine induced in Staphylococcus aureus the accumulation of uridine diphosphate acetylamino sugar compounds, which were percursors of the cell wall. In addition, Miyashiro et al, (1980) showed that protein synthesis was not inhibited by glycine but that the addition of glycine to the culture media made the cells more sensitive to lysozyme and caused a large decrease in the alanine content of the cell wall.

One of the main aims of this project, to demonstrate the production of the halidohydrolase from Rhodococcus erythropolis on a large scale, has been successfully achieved.
Figure 4.1 The relationship between the first order rate constants (k) at 5 °C for the release of beta-galactosidase from *E. coli* O, and protein from bakers' yeast △, ▲ and Nocardia □, and the operating pressure (P) of a Manton-Gaulin/APV homogenizer.
4.4 STATE OF THE BIOCATALYST

A decision had to be made on the form of the biocatalyst and this was determined partly by what was most favourable and partly by the nature of the project. The first choice was between using the free enzyme from *Rhodococcus erythropolis* or using the whole cell. As extensive studies had been done by a number of researchers using the whole cell, studying the enzyme system would allow for comparison of these two approaches. Thus, whether the enzyme should be used in a free or immobilized form will be discussed and the outcome of this discussion will then allow for a comparison with a whole cell system in either a free or immobilized form. All of these decisions will affect the type of reactor chosen.

Research had not previously been carried out on the immobilization of a dehalogenase from *Rhodococcus erythropolis*. This project performed studies with both the free and immobilized enzyme and although a 50% loss of activity was noted on immobilization, there were many advantages to immobilization. The ability of the immobilized enzyme to be packed into a plug flow reactor and used continuously was a great advantage over the free enzyme, as will be discussed in section 4.6. The immobilized enzyme could be removed from the system and reused many times, unlike the free enzyme which could only be used once unless it was recovered by membrane filtration. This means that the lifespan of the immobilized enzyme particles is greater than with the free enzyme, which is very significant for a low cost waste treatment process. In practice, when used in a batch reactor, the free enzyme showed much slower reaction rates for the same amount of enzyme as with the immobilized enzyme (Figures 3.43 and 3.44). Once the effectiveness of the enzyme in the immobilized form had been established, it became clear that use of the free enzyme would be too costly.
The stability of the enzyme increased on immobilization. The free enzyme showed a significant loss in activity when stored above 4 °C, but this was not the case with the immobilized enzyme which was used continuously at 30 °C for several days without any significant loss in activity. This increased stability may be due to the covalent bonding of the enzyme to the immobilized support. Alternatively, the enzyme may be protected from proteases which have either bound to the support or have been washed off in the process stream.

Immobilization also appeared to increase the $K_m$ of the enzyme, suggesting that the microenvironmental parameters may have a significant effect on the binding of the substrate to the enzyme (Carleysmith et al., 1980). Goldstein et al. (1964) proposed the idea that a different environment exists around an immobilized enzyme, especially when the support has a net charge and is at low ionic strengths. If the support has a net positive charge, as in this case, the pH activity profile of the immobilized enzyme shifts towards the acidic region. At high ionic strengths, however, the pH activity profile would shift back toward that of the free enzyme. The use of a porous support may also protect the enzyme against physical abrasion and reduce the risk of denaturation by reagents that are toxic to the enzyme. This ability is especially important if the preparation is to be subjected to shock loading or sudden changes in the pH of the solution.

The main problem with immobilization of the enzyme was the possibility of diffusional limitations, which would slow down the rate of transfer of the substrate to the enzyme and thus reduce conversion of the harmful substrate. This is not thought to be a problem in this case, as the Eupergit C beads were not thought to be subject to diffusional limitations. However, the presence of any diffusional limitations would have severely affected the activity because of the low substrate
concentration and the low value of \( [S_0/K_m] \). The presence of any diffusional limitations can be calculated using the Thiele modulus and by referring to a plot of the effectiveness factor against the Thiele modulus.

The Thiele modulus was calculated using the following equation:

Equation 4.0 (Wang et al., 1979)

\[
\text{Thiele modulus} = \frac{R}{V_{max} / D_e K_m}
\]

where:
- \( R \) = particle radius
- \( D_e \) = effective diffusivity
- \( V_{max} \) = maximum activity per unit volume of support
- \( K_m \) = constant for immobilized enzyme

By assuming a value of \( 4 \times 10^{-4} \) cm\(^2\)/min for the effective diffusivity, the effectiveness factor was estimated as approaching 1.0, which confirms that diffusional limitation was not a problem in this case.

A comparison between the use of the immobilized enzyme or the whole cell also confirms the immobilized enzyme system as the most effective and elegant solution. The main problem with a whole cell system is that the cells need to be maintained and, thus, nutrients will need to be added. In a batch operation, the cells would need to be grown in one fermenter, then transferred into a second vessel and then recovered at the end of the biotransformation. In a continuous stirred tank reactor (CSTR) with whole cells, a large micropore membrane would be needed to prevent the cells from escaping and this would be prone to blockage. Alternatively, the exit stream could be processed by a centrifuge and the cells recovered and reused. Both of these methods are expensive and impractical.
If the cells were to be immobilized and used in a biotransformation, the most usual method is by growing the cells on a biological film. The main problem with this option is that the cells tend to slough off, and as a result, the cells need to be regenerated by adding extra nutrients and any free cells need to be removed by clarification. The other disadvantage of using a whole cell system in a free or immobilized form is the possibility that, as the cells lyse, they will release harmful cellular endotoxins.

By contrast, the immobilized enzyme system requires no aftercare and there are no cellular fragments or chances of blockage. However, there is the additional expense of equipment and operating costs to break open and clarify the cell extract and the additional losses in activity due to immobilization. These costs would not be such a problem for a large company with existing equipment which could produce large batches of immobilized enzyme on demand. However, for the immobilized enzyme system to be the best option, the stability of the system has to be high. Obviously, the activity of the bed will decrease after some time and there are several ways in which this can be minimized. Firstly, the pH and temperature of the inlet stream can be adjusted to increase the stability once the optimum conditions for operational stability have been assessed. By increasing the temperature of the column, the rate of the reaction may be increased, but this is only a temporary measure as the activity will eventually decrease due to thermal effects (Woodley and Lilly, 1994). The life of the column can also be prolonged by increasing the depth of the catalyst packing. Alternatively, there may be a decrease in stability due to entrained droplets which have not been properly removed in the organic phase separation process. These droplets may get stuck at the beginning of the bed or they may be removed with a coalescing filter or similar removal system (Menon et al., 1983). If the packed bed is being operated as a continuous flow process, the flow rate may be reduced to
maintain a fixed conversion, but this may prove difficult with an industrial outlet stream.

Nevertheless, as the activity of a packed bed of immobilized enzyme declines, the substrate conversion will fall. If the reactor is operating close to 100 % conversion then the conversion will only fall slowly despite loss of activity by the packed bed. One way of minimizing changes in conversion, as has been used with glucose isomerase, is to have a series of plug flow reactors with the oldest column at the beginning which is then replaced by the second reactor and a new reactor is added to the end. This extends the life of the overall system and allows the flow rate and required conversion to be maintained (Figure 4.2). The use of the enzyme in an immobilized form is therefore advantageous as long as the inlet stream is not contaminated with microorganisms or toxic elements which could block the reactor and kill the enzyme respectively.

4.5 IMPLICATIONS OF THE ENZYME KINETICS

Verschueren et al. (1993c) described the method of binding of the halide ion to the active site of the dehalogenase from Xanthobacter autotrophicus GJ10. They found that the hydrogen atoms bound to the ring nitrogens of the two tryptophans, Trp-125 and Trp-175, in the cavity of the dehalogenase have a slightly positive charge which facilitates positioning of the negatively charged halide ion between the two nitrogen atoms. In addition, the bound anion may be stabilized by non-polar interactions in the cavity. The interaction of the halogen atom with the tryptophans might lower the transition state energy and thus facilitate the formation of a covalently bound intermediate. This may also be an explanation for the increased stability caused by binding on immobilization of the halidohydrolase from Rhodococcus erythropolis onto Eupergit C.
It was necessary in this case to obtain substrate concentration versus activity profiles and, from these, the $K_m$ and $V_{\text{max}}$ of the enzyme could be estimated using Lineweaver-Burk plots. This information allowed for the complete design of a biotransformation. For comparison of a very poorly water soluble and a fairly water soluble substrate, these data were obtained using 1-chlorobutane and 4-chlorobutanol, respectively. The experiments were also carried out at two different pH values, pH 7 and pH 9, so that the effects of pH on the reactor may be more fully understood and taken into account when designing the reactor.

The immobilized enzyme when used in a plug flow reactor was not subjected to large changes in pH during the reaction. Only a very slight increase in the pH of the system was noticed between the inlet and outlet of the aqueous stream, which was not buffered.

The results showed that the experiments were reproducible, allowing for a loss in enzyme activity over time. The fact that, with both substrates, the kinetics deviate slightly from Michaelis-Menten kinetics means that only approximate values have been obtained for $K_m$ and $V_{\text{max}}$. The Lineweaver-Burk plots may show an indication of substrate inhibition, as they are not completely linear, although none was detected in the experiments. This may have been due however, to evaporation of the volatile substrate into the headspace. The possible presence and effects of substrate inhibition will be discussed later (Section 4.6).
Figure 4.2  Flow rate vs operation time for one continuous flow reactor compared to three continuous flow reactors in series. (Oestergaard and Knudsen, 1976).
At low substrate concentrations, the reaction in both cases is a first order reaction, as indicated in the substrate concentration profiles (Figures 3.17, 3.18, 3.21 and 3.22). The activity of the enzyme was greater with both substrates at pH 9 than at pH 7. The solubility in water, $K_m$ and $V_{max}$, are all much greater in the case of 4-chlorobutanol compared to 1-chlorobutane. The results also indicated that, with 1-chlorobutane, the enzyme will retain its highest activity at its saturation point and this will not reduce significantly until the substrate concentration has reduced to approximately 60% of this value. The enzyme will still retain approximately 75% of its activity at 30% of its saturation value (Figures 3.17 and 3.18). With 4-chlorobutanol, the decrease in activity with decreasing substrate concentration is steeper and therefore less favourable. At pH 9, (Figure 3.22) the activity decreased sharply below 65% of the saturation value. At 30% of the saturation value, the enzyme retained only 50% of its activity. This means that the immobilized enzyme is used much more efficiently with 1-chlorobutane as the substrate, as it can continue to achieve high conversions as the substrate concentration decreases.

4.6 COMPARISON OF REACTOR TYPES FOR DEGRADATIVE BIOTRANSFORMATIONS

A variety of reactor types and configurations have been used to carry out biotransformations in a degradative mode. Several researchers have immobilized cells into calcium alginate beads and used them in column reactors. Bernet et al. (1987), used *Brevibacterium* sp. R312 to hydrolyze acrylamide into acrylic acid. The biotransformation was carried out in a fluidized bed reactor which was specially designed to obtain good contact conditions between the cells entrapped into small calcium alginate beads and low concentration acrylamide solutions (10-40 g/L). *Pseudomonas* sp. US1 ex was used in a similar way to dehalogenate a mixture of isomeric monochlorobenzoates and 2,4-dichlorophenoxyacetic acid (Sahasrabudhe et al., 1991). Hollow fibre reactors have also been used to carry out other
Chung et al. (1987) showed rifamycin B production by *Nocardia mediterranei* immobilized in a dual hollow fibre reactor for the continuous production of rifamycin B. Packed bed reactors have also been used. Vaughan et al. (1989) demonstrated the conversion of 3-cyanopyridine to nicontinic acid by *Nocardia rhodochromus* immobilized in calcium alginate beads and used in a packed column bioreactor. More recently, a single-pass packed bed bioreactor was utilized to study the biodegradation of organic waste mixtures consisting of tetrachloroethylene and trichloroethylene (Lackey et al., 1993). In all of the above cases, the whole cell is being used in an immobilized form thus incurring all of the disadvantages already mentioned. Diaz et al. (1989) demonstrated the performance of a 2-haloalkanoate dehalogenase from *Pseudomonas cepacia* MBA4 immobilized in a hollow fibre reactor for the specific dehalogenation of 2-haloalkanoic acids. They confirmed the effectiveness of such an immobilized enzyme system for dehalogenation. However, hollow fibres are fairly costly, so a more cost effective, equally efficient approach was sort.

This project concentrated on the small-scale removal of 1-chlorobutane and 4-chlorobutanol from saturated process streams. The fact that the 1-chlorobutane is very poorly water soluble meant that it was very difficult to obtain accurate results at lower saturation levels and problems were also incurred due to the volatile nature of the substrate. In the case of 4-chlorobutanol, a glass reactor with inert seals needed to be used because of the extreme corrosive properties of the substrate when in a saturated solution.

The temperature of any type of reactor must be regulated as the enzyme is very sensitive to large temperature changes. Although, in industry, it may not always be possible to control the temperature of the inlet stream, this would normally be at ambient temperature or above. A heat exchanger upstream would be sufficient to
maintain a constant temperature within acceptable limits of the enzymes' capabilities (Figures 3.27 and 3.29).

Similarly, the pH of the solution also has an effect on the conversion. In both cases, the activity of the immobilized enzyme in the reactor increases with increasing pH (Figures 3.30 and 3.31). EEC limits dictate that any emissions into rivers have to be at a pH value of between pH 6-9 and therefore the enzyme would be able to cope with any changes in pH. If the pH was at pH 6, the activity on 1-chlorobutane would be severely reduced. However, as has been seen in the results, the column was extremely efficient at removing 1-chlorobutane, due to its low solubility. For example, from an initial value of $kE/q$ of 13.5 mM (approaching 100 % conversion), a 50 % reduction in the enzyme activity would still produce high conversions of the haloalkane (over 80 %) (Figure 4.3). Thus, for a constant flow rate, the conversion will remain high until the enzyme activity falls sharply, however, the graph also illustrates how the percentage conversion can be maintained by decreasing the flow rate ($q$).
Figure 4.3  Percentage conversion of a saturated aqueous feed of 1-chlorobutane against (the total enzyme activity of the haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2)/ (flow rate through the reactor).
A packed bed immobilized enzyme reactor was set up experimentally to test the proposed design. The ideal ratio of particle diameter to internal diameter of the reactor was known to be 1:30 (Smith, 1988). In this case the ratio was approximately 1:67, which was over twice the recommended ratio and had the effect of increasing the pressure drop across the reactor. It was noticed that the direction of flow also had an effect on the system. The reactor was initially set up with flow in a downwards direction. This approach gives a stable bed that will not fluidize or lift out of the reactor. It also minimizes catalyst attrition and potential entrainment of catalyst fines. However, the main disadvantages of downward flow are the tendency of the bed to compress itself and the gravitation of catalyst fines down through the bed. Both phenomena may lead to increased pressure drop and channelling or maldistribution of the flow. The Eupergit C beads used have a small particle diameter (150 \mu m) and both channelling and an extremely high pressure drop were noticed. It was therefore necessary to flow upwards through the column, and only use low flow rates up to 10 mL/min. Upflow has the advantage of lifting catalyst fines or fragmented particles and reduces channelling and the pressure drop. By ensuring that the bed was very tightly packed, fluidization was avoided and the flow pattern approached plug flow (Figure 2.12). Another disadvantage of fixed bed reactors is the fact that the minimum particle size that can be used is restricted by the pressure drop across the bed, as in this case. The smaller the particle, the more efficiently the internal area is used but the greater the pressure drop.

On a large scale operation, one of the major disadvantages of a fixed bed reactor is the difficulty associated with catalyst regeneration or replacement. Thus, the most successful fixed bed operations are those where the catalyst activity does not decline significantly over long periods of time.
The extent of the reaction is determined by the amount of enzyme in the reactor and different reactors require different amounts of enzyme to achieve the same conversions. Lilly and Sharp, (1968) showed that for a first order reaction and with very small substrate concentrations, as in this case, \((S_0 = 10^{-3}-10^{-6} \text{ M})\), approximately 20 times the amount of enzyme was required for the same high conversion in a CSTR as compared to a CPFR. For a batch reactor, the amount of enzyme required was shown to be higher than for a CPFR because of the downtime between batches. They showed that if \(\vartheta\) is the proportion of time for batch operation, the amount of enzyme required to give a productivity equal to the CPFR will be \(E_{PF}/\vartheta\). Thus, CSTRs are rarely used to carry out biotransformations unless several CSTRs are used in series where they approximate to a plug flow pattern. This solution is often very expensive. Thus, it is very difficult to achieve 100% conversion in a CSTR because the outlet concentration is equal to the concentration in the reactor which is being constantly supplied with more substrate. However, it is very possible, as has been shown, to achieve a substrate conversion approaching 100% in a CPFR.

The kinetics are more favourable in a CPFR than in a CSTR for Michaelis-Menten kinetics. For a first order reaction at a low \(S_0/K_m\), the value of \(t\) (time) for any value of \(x\) (proportion of substrate converted) is almost independent of \(S_0/K_m\). At low values of \(S_0/K_m\), high values of \(x\) will be obtained at much slower flow rates in the CSTR than in the plug flow system. For example, an increase in \(x\) from 0.95 to 0.98 when \(S_0/K_m = 0.1\) would require a 61% decrease in the flow rate through a CSTR and a corresponding 23% decrease in flow rate through a CPFR (Wang et al., 1979). To meet EEC regulations, percentage conversions of up to 99.9% may be required and at this very low substrate concentration, the activity with both
substrates is nearly zero. Thus, the more favourable kinetics obtained in a CPFR are preferred.

Although substrate inhibition has not been confirmed in this case, the Lineweaver-Burk plots obtained from the substrate concentration profiles suggest that there might be some evidence of it. When substrate inhibition is present, the Lineweaver-Burk plots are not linear at high substrate concentrations, the profile at these higher concentrations may flatten and then start to increase again. This effect has been shown to some extent with both 1-chlorobutane and 4-chlorobutanol as substrates (Figures 3.19, 3.20, 3.23 and 3.24). Substrate inhibition causes more serious effects in a plug flow reactor than in a CSTR because, in a CSTR, the enzyme is operating at the same substrate concentration as in the product stream. In a batch reactor, substrate inhibition may be reduced by feeding the substrate and in a plug flow reactor by feeding the substrate at points along the reactor. In a CSTR, the substrate inhibition may be reduced by using several small reactors in series which are continuously fed with the product (Wang et al., 1979).

Overall, the plug flow reactor was considered more suitable because of the advantages already discussed and because it is possible to have a higher concentration of solid catalyst with little potential for mechanical damage to the catalyst.

4.7 EFFECTS OF THE SUBSTRATE ON REACTOR OPERATION

The substrate versus concentration profiles highlighted the differences in the kinetics of the two substrate reactions and these differences explain how the packed bed reactor performance differs for each substrate. As has already been shown, very high percentage conversions were obtained with 1-chlorobutane as the substrate and much lower conversions with 4-chlorobutanol as the substrate. These values are as
predicted for 1-chlorobutane, i.e a flow rate of 8 mL/min was predicted to achieve a conversion of 99.5%. The actual values for 4-chlorobutanol are almost twice as good as the predicted values, i.e, in the model reactor, a flow rate of 2 mL/min produced a conversion of 20% whereas the predicted flow rate for a 20% conversion was 1.07 mL/min.

Due to the higher solubility of 4-chlorobutanol, more reaction needs to be carried out and, although the enzyme is more active, it is not of the same order of magnitude as the difference in the solubilities. Thus, with 4-chlorobutanol as the substrate, instead of 1-chlorobutane, several changes may be made to the system to increase the percentage conversions. The reactor could be increased in size, so that the bed would be extended. Alternatively, more enzyme could be loaded onto the beads or the flow rate through the reactor could be decreased.

Thus the decision to use a packed bed immobilized enzyme system for both substrates, 1-chlorobutane and 4-chlorobutanol, was the best choice for reactions requiring high conversions from a low substrate concentration feed stream.

In the design of an industrial biotransformation process for the dehalogenation of chlorinated aliphatic compounds, several reactor configurations have been considered using the information obtained on the kinetics of the halidohydrolase under various conditions and with 1-chlorobutane and 4-chlorobutanol as substrates. The project has also addressed several problems which might arise with an immobilized enzyme packed bed including any changes that could be made to extend the life of the packed bed. Given the details of a process stream, including the pH, temperature, degree of saturation and volume of effluent, it would now be possible to design a complete process for a particular percentage conversion of the substrate dependent on legislative requirements. The size of the reactor, the amount of
immobilized catalyst and the flow rate through the reactor could all be determined and used to build a fully functional dehalogenating system. The development of an accurate, user-friendly, fast assay using gas chromatography for the determination of the substrate concentration means that any decreases in the percentage conversion could be easily detected and corrective measures taken immediately. This knowledge could then be extended to other substrates once their particular kinetics were understood, and units could be constructed to order, to degrade a mixture of haloaliphatic compounds.
5.0 CONCLUSIONS

1. The enzyme has been produced on a large scale and the improved medium formulation has increased the bacterial concentration thirteen-fold.

2. Scale-up of the fermentation from 20 L to 1500 L resulted in an increase in the final cell concentration and a three-fold increase in the specific growth rate.

3. The enzyme was inducible and the specific enzyme activity in cell extracts has been significantly increased by changing the inducer and the induction conditions.

4. The enzyme was intracellular and its release was affected by the number of passes through a particular homogenizer, the type of homogenizer and the addition of 2.0 g/L of glycine to the fermentation in the late exponential phase.

5. The kinetics of the enzyme showed a slight deviation from Michaelis-Menten kinetics. The $V_{\text{max}}$ and $K_m$ obtained on 4-chlorobutanol were much higher than those obtained on 1-chlorobutane.

6. Immobilization of the enzyme on Eupergit C resulted in a 50% loss in activity of the free enzyme. This loss was compensated by the many advantages of immobilization, mainly reuse of the enzyme.
7. The kinetics of the immobilized enzyme differed to those of the free enzyme. The ratio of $V_{\text{max}}$ on 1-chlorobutane to 4-chlorobutanol was 1:27 for the immobilized enzyme and 1:15 for the free enzyme.

8. An immobilized enzyme plug flow reactor has been shown to be more efficient than a batch reactor at removing haloalkanes and haloalcohols.

9. For a saturated solution of 1-chlorobutane passed through a 30 mL immobilized enzyme packed bed reactor, a flow rate of 0.5 L/hr will result in a 99.5% conversion to meet EEC standards.

10. For a saturated solution of 4-chlorobutanol passed through a 30 mL immobilized enzyme packed bed reactor, a flow rate of 0.004 L/hr will result in a 99.99% conversion to meet EEC standards.
6.0 FUTURE RESEARCH

Further research should be done on the following areas of work in order to develop the project into a commercially viable option:

Downstream Processing:
- The effect of large scale downstream processing on *Rhodococcus erythropolis*

Biotransformation:
- Scale-up of the immobilized enzyme biotransformation to pilot plant scale

Immobihzed Enzyme Reactor System:
- The long term stability of the immobilized enzyme
- The effects of shock loading and toxicity on the immobilized enzyme
- The effects of sudden large shifts in pH on the efficiency of the reactor
- The effects of a legislative need to reduce emission levels

Overall Process Improvement:
- Gene cloning or genetic manipulation to increase intracellular levels
- Assessment of the effect of enzyme purification on the immobilized enzyme

Other Possible Uses of the Immobilized Enzyme System
- Removal of more than one substrate from an aqueous process stream
- Removal from a two-phase system
- Treatment of volatilized materials i.e after air-stripping by passing them through a biofilter

Execution of a sensitivity analysis to confirm the choice of fermentation process and immobilization technique to assess whether such a system would be commercially viable.
7.0 REFERENCES


8.0 APPENDIX

8.1 Safety Aspects of Hazardous Chemicals

The following hazardous chemicals were used in this project and guidelines for their safe use are given:

**ALCOHOLS**

No general statement can be made due to wide variations in toxic effects.
Dangerous fire hazard when exposed to heat or flame.

**CHLORINATED HYDROCARBONS, ALIPHATIC**

The substitution of a chlorine (or other halogen) atom for a hydrogen greatly increases the anaesthetic action of the aliphatic hydrocarbons and increases the range of their systemic effects. In many cases, the chlorine derivative is quite toxic. They are often experimental carcinogens of the liver, lung, skin, and blood-forming tissues. In general, the unsaturated chlorine derivatives are more narcotic but less toxic than the saturated derivatives. In the saturated group, the narcotic effect is proportional to the number of chlorine atoms. This relationship is not true for toxicity.

In dealing with these chlorinated hydrocarbons, it must be remembered that a toxic action may result from repeated exposure to concentrations which are too low to produce a narcotic effect, and which, consequently, are too low to give warning of danger. Individual susceptibility varies widely but, in general, reactivity decreases with greater substitution of halogen for hydrogen atoms.

**4-CHLORO-1-BUTANOL**

An experimental neoplastigen. Moderately toxic by ingestion. Mutagenic data. When heated to decomposition it emits toxic fumes of Cl⁻.
GENERAL INFORMATION:
Molecular weight: 108.57
Soluble in water (315 mM)
Boiling point: 84-85 °C/16 mm
Density at 20 °C relative to water at 4 °C: 1.088
Flash point: 36 °C
Refractive index at 20 °C: 1.4550
Flammable liquid
Irritant
Readily absorbed through skin

TOXICITY DATA:
mutation in microorganisms-Salmonella typhimurium 20 μmol/plate
intraperitoneal-mouse -Lowest published toxic data:3650 mg/kg/8W-I:NEO
oral-mouse - Lethal dose 50 % kill: 990 mg/kg

2-CHLORO-1-PROPANOL
COMPARABLE TOXICITY DATA:
oral-rat - Lethal dose 50% kill - 218mg/kg (c.f. 990mg/kg oral-mouse)
Poison by ingestion. Moderately toxic by inhalation and skin contact. A skin and severe eye irritant. Flammable when exposed to heat, flame or powerful oxidizers.
When heated to decomposition it emits toxic fumes of Cl⁻.

When comparing the warnings given for these two halo-alcohols nothing is mentioned about 4-chloro-1-butanol being toxic either by inhalation or skin contact (unlike 2-chloro-1-propanol). However, the bottle does state that it is readily absorbed through the skin and is an irritant. 4-chloro-1-butanol is not poisonous but
only moderately toxic when ingested and a large amount would consciously need to be ingested if it were to be toxic. The only other possible danger is that perhaps, as was mentioned earlier, a toxic action may result from repeated exposure to concentrations which are too low to produce a narcotic effect, and which, consequently, are too low to give warning of danger. However, the supplier, Aldrich, was contacted and they confirmed that they had no evidence of repeated exposure being a problem or of any problems associated with inhalation of 4-chloro-1-butanol. In addition, there was no mention of 4-chloro-1-butanol in the list of suspected carcinogens.

Gloves must always be worn when handling halo-alcohols. Fume cupboards must be used, and aerosol production should be kept to a minimum by ensuring that the liquid level covers the needle when 4-chloro-1-butanol is injected into a culture. As long as good laboratory practice is maintained, there should be no abnormally high risks associated with using 4-chloro-1-butanol.

MERCURY
In humans it is readily absorbed via the respiratory tract (elemental mercury vapour, mercury composed dusts), intact skin and G.I tract, although occasional incidental swallowing of metallic Hg is without harm. Spilled and heated elemental Hg is particularly hazardous. A number of mercury compounds cause skin irritation and can be absorbed through the skin. They are strong allergins; common air contaminants.

Acute Toxicity:
Soluble salts have violent corrosive effects on skin and mucous membrane; severe nausea, vomiting, abdominal pain, bloody diarrhea, kidney damage, death usually within 10 days.
**MERCURY (II) THIOCYANATE**

Odourless powder. When crystalline, usually in radially arranged needles. Poisonous! When heated, it swells up to many times its original volume, decomposing finally into mercury, nitrogen etc. at about 165 °C. Slightly soluble in cold water, more soluble in boiling water with decomposition. Soluble in dilute HCl, in solutions of alkali cyanides, chlorides. On decomposition, toxic fumes of mercury are emitted.

When using Mercury (II) thiocyanate, gloves and a face mask must always be worn. All reactions must be carried out in a fume cupboard.

**PERCHLORIC ACID**

Perchloric acid is an extremely hazardous chemical. Its hazardous properties are due chiefly to it being a powerful oxidizing agent and, as such, will react violently with a large number of carbonaceous materials, e.g., acetic acid, alcohols, cellulose, charcoal, ethyl ether, glycol ethers, ketones, paper, and wood. It is also a fire and explosion hazard in contact with finely divided metals.

The acid has been known to react with explosive force when in contact with wood (especially shavings and sawdust), rubber and cork.

The following precautions should be taken when handling perchloric acid:

- Spillages should be mopped up immediately and thoroughly by washing with large quantities of water.
- Do not use organic chemicals in hoods employed for perchloric acid digestion.
- Wear safety glasses and work behind a safety screen as necessary.
Perchloric acid with strong dehydrating agents, e.g. conc. H$_2$SO$_4$, P$_2$O$_5$, give rise to the explosive anhydrous perchloric acid. Avoid the use of such reagents whenever possible.

Where digestion of perchloric acid in fume cupboards takes place, special arrangements to prevent acid fumes from entering the duct must be made. Hoods and ducts should be of chemically inert materials and capable of being thoroughly washed with water.

It is important not to stand bottles of perchloric acid on wooden or plastic surfaces. They must stand on an inert surface, e.g., glass.

Perchloric acid must be kept in a cool environment and on no account be subjected to heat.

The quantity of perchloric acid stored in a laboratory should be an absolute minimum to accommodate the work being undertaken.