

THE EFFECT OF GROWTH CONDITIONS UPON THE  
SOLVENT TOLERANCE AND AROMATIC OXIDATION ACTIVITY  
OF PSEUDOMONAS PUTIDA ML2

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

by

Richard Rhys Jones

Advanced Centre for Biochemical Engineering,  
Department of Chemical and Biochemical Engineering,  
University College London,  
Torrington Place,  
London, WC1E 7JE.

October 1993



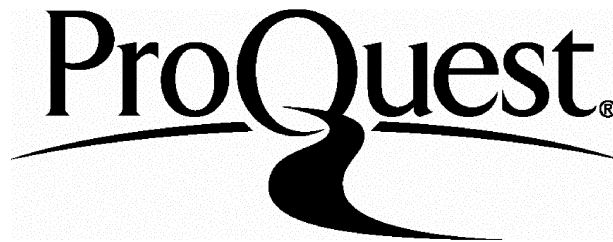
ProQuest Number: 10046216

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10046216

Published by ProQuest LLC(2016). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code.  
Microform Edition © ProQuest LLC.

ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

Ysgol Profiad

Rhoi i oes ddyfalbarhad - a dysgu,  
Drwy dasg, nid arholiad;  
Gweld her mewn camgymeriad,  
Ym mhoen y wers cael mwynhad.

yn 'Cadw Gwyl', Myrddin ap Dafydd  
Gwasg Carreg Gwalch, 1991.

The 'englyn' (Welsh poem written in a strict metre)  
translates as follows:-

He gives life perserverance,  
And learns by experience not examination,  
He regards every error as a challenge,  
And finds pleasure in every new lesson.

translation by Rev. Gwilym Tilsley,  
Past Archdruid of Wales.

## ACKNOWLEDGEMENTS

I would like to thank Professor Malcolm D. Lilly for his guidance, advice, criticisms and encouragement throughout the project.

I would also like to thank the following:-

- all the technical staff at the department for their assistance.
- fellow researchers for their useful comments and practical assistance.
- family and friends outside the department for their support and encouragement.

Finally I would like to thank the Science and Engineering Research Council and Shell Research Ltd for the funding of this project.

## ABSTRACT

Pseudomonas putida ML2 converts fluorobenzene to fluorocatechol, a compound of commercial value. The addition of a second immiscible solvent to the biotransformation could maintain low aqueous concentration of fluorobenzene (which inhibited growth and oxygen uptake of the bacterium). The impact of fermentation conditions on the solvent tolerance and aromatic oxidation activity was examined. Analytical techniques to quantify biocatalytic activity and solvent tolerance were developed. Growth of the bacterium at dissolved oxygen tensions (DOT) of 5 % air saturation resulted in specific aromatic oxidation activity increasing to approximately double that of cultures grown at 20 % DOT. Two phases to the growth phase were detected by online gas analysis and correlated to the changes in biocatalytic activity. A slow down in growth as a result of oxygen or iron limitation resulted in at least a doubling of aromatic oxidation activity, which was attributed to the change in the rate of growth. Fermentation samples lost biocatalytic activity within a few hours of harvesting, but shake flask cultures remained active for at least 24hr.

Solvent tolerance was examined using chloroxylenol as a test solvent. The bacterial oxygen uptake rate (OUR) decreased exponentially reaching a steady value at the end of the assay. An equation was fitted to the data obtained for chloroxylenol, octanol and other solvents allowing specific decay rates to be calculated. The solvent tolerance of the bacterium was highest at the end of the fermentations controlled at 5 and 20 % DOT with a specific decay rate constant below  $8 \text{ hr}^{-1}$ . A decrease in growth temperature from  $30^{\circ}\text{C}$  to  $26^{\circ}\text{C}$  resulted in a decrease in solvent biocompatibility when assayed at  $30^{\circ}\text{C}$ . At a growth temperature of  $34^{\circ}\text{C}$  the OUR was close to linearity signifying little loss of activity but bacterial growth at  $34^{\circ}\text{C}$  was slow and not reproducible. Addition of magnesium sulphate to the solvent tolerance assay increased tolerance of the bacterium to chloroxylenol, fluorobenzene and dichloroethane. A reduction in temperature of the solvent tolerance assay from  $30^{\circ}\text{C}$  to  $23^{\circ}\text{C}$  increased bacterial tolerance to dichloroethane. Significant improvement in solvent tolerance of the bacteria was dependent upon biotransformation conditions rather than changes in the growth conditions.

## CONTENTS

	Page
TITLE PAGE	1
ACKNOWLEDGEMENTS	3
ABSTRACT	4
LIST OF FIGURES	10
LIST OF TABLES	21
1 - INTRODUCTION	22
1.1 - Two liquid phase biocatalysis	22
1.1.1 - Biocatalysis	22
1.1.2 - Two liquid phase biocatalysis	27
1.2 - Solvent toxicity	31
1.2.1 - Toxicity of solvents below aqueous saturation	32
1.2.2 - Kinetic analysis of solvent toxicity	33
1.3 - Process set up	35
1.3.1 - Biocatalyst production	35
1.3.1.1 - Effect of fermentation conditions on enzyme activity	37
1.3.1.2 - Effect of growth conditions on solvent tolerance	39
1.4 - Experimental model system	44
1.4.1 - Commercial interest	44
1.4.2 - Enzyme system	44
1.4.2.1 - Microbial dioxygenase	47
1.4.2.2 - Benzene <u>cis</u> glycol dehydrogenase	48
1.4.3 - Fermentation conditions	48
1.4.4 - Substrate and product toxicity	48
1.5 - Objectives	50



3 - RESULTS	68
3.1 - Fermentation	68
3.1.1 - Carbon source selection	68
3.1.2 - Fermentation development	70
3.1.3 - Characterisation of the fermentation controlled at 20 % DOT and 30°C	74
3.1.3.1 - Exit gas analysis	75
3.1.3.2 - Biomass	82
3.1.3.3 - Acid control	82
3.1.3.4 - Carbon data analysis	86
3.1.4 - Fermentations under different DOT controlled conditions	92
3.1.4.1 - Fermentation controlled at 5 % DOT	92
3.1.4.2 - Fermentation with transition of DOT from 20 to 0 % aqueous saturation	98
3.1.4.3 - The effect upon the growth of cells of moving from 20 % DOT to a low $K_L a$ which results in oxygen limited growth	103
3.1.5 - Fermentations at different growth temperatures	107
3.1.5.1 - 26°C	107
3.1.5.2 - 34°C	113
3.1.5.3 - Comparison of the initial growth of the bacterium at different temperatures	115
3.1.6 - Carbon limited	121
3.2 - Fluorobenzene oxidation activity	125
3.2.1 - Assay development and characterisation of the enzyme activity	125
3.2.1.1 - Substrate concentration	128
3.2.1.2 - Cell concentration	135
3.2.1.3 - Stability of the aromatic oxidation activity	135
3.2.1.4 - Effect of propanol upon activity	135
3.2.1.5 - Product toxicity	139
3.2.1.6 - Fluorocatechol resistance	142
3.2.1.7 - Plasmid stability	146



3.2.2 - Fermentation controlled at 20 % DOT and at 30°C	148
3.2.2.1 - Stability of the aromatic oxidation activity	148
3.2.2.2 - Fermentation profile	150
3.2.3 - Aromatic oxidation activity of <u>P.putida</u> in shake flask cultures	152
3.2.4 - Changes in dissolved oxygen tension at 30°C	154
3.2.4.1 - Fermentation controlled at 5 % DOT	154
3.2.4.2 - Aromatic oxidation activity of the cells grown at 30°C and 20 % DOT with transition to zero DOT at the end of the growth phase.	157
3.2.4.3 - The effect upon the aromatic oxidation activity of moving from 20 % DOT to a low $K_L a$ during the growth phase	159
3.2.4.4 - Unstable DOT	159
3.2.4.5 - The aromatic oxidation activity of the cells from the fermentation at 20 % DOT with transition to zero DOT for a short period of time during the growth phase	162
3.2.5 - Effect of changing temperatures	165
3.2.5.1 - 26°C growth temperature	165
3.2.5.2 - 34°C growth temperature	165
3.2.6 - Carbon limited growth	168
3.2.7 - Iron limitation	170
3.3 - Solvent tolerance	175
3.3.1 - Assay development	175
3.3.2 - Fermentation at 20 % DOT and 30°C	195
3.3.3 - Fermentation at 30°C but at different oxygen concentrations	199
3.3.3.1 - Fermentation at 5 % DOT and 30°C	199
3.3.3.2 - Fermentation at 30°C and 20 % DOT with transition to 0%DOT at the end of the growth phase	202

3.3.3.3 - Fermentation at 30°C with transition of DOT from 20 % aqueous saturation to zero for a short period of time during the growth phase	202
3.3.4 - Fermentations at 20 % DOT but at different temperatures	205
3.3.4.1 - 26°C	205
3.3.4.2 - 34°C	205
3.3.5 - The tolerance of <u>P.putida</u> to a variety of solvents	210
3.3.6 - The effect of magnesium and EDTA upon the solvent tolerance of <u>P.putida</u> ML2	219
 4 - DISCUSSION	 226
4.1 - Fermentation	226
4.2 - Fermentation kinetics	229
4.3 - Aromatic oxidation activity	234
4.4 - The effect of growth conditions on aromatic activity	235
4.5 - Aromatic oxidation stability	241
4.6 - Solvent tolerance	246
4.7 - Effect of growth conditions on solvent tolerance	251
4.8 - The biotransformation reaction	254
CONCLUSIONS	261
REFERENCES	264
NOMENCLATURE	282

## LIST OF FIGURES

Figure name	Page
1.1 - The three geometrical isomers of dihydroxy benzene.	24
1.2 - The process flow sheet of a typical two liquid phase biotransformation.	36
1.3 - The structure of the outer membrane of a Gram - bacterium.	40
1.4 - The chemical structure of the lipopolysaccharide from the outer membrane of a Gram - bacterium.	41
1.5 - The biotransformation of fluorobenzene to fluorocatechol by <u>P.putida</u> ML2.	45
1.6 - The benzene degradation pathway of <u>P.putida</u> ML2	46
2.1 - Standard curve of the $A_{670}$ of the bacterial culture against dry cell weight.	58
3.1 - DOT and biomass profile of the fermentations with cells grown on glucose / fructose as carbon source.	69
3.2 - DOT profile of two fermentations with succinate as carbon source at different $K_L a$ values.	71
3.3 - The effect of changes in the inoculum upon the growth of <u>P.putida</u> ML2.	72
3.4 - OUR profiles of three fermentations controlled at 30°C with DOT controlled at 20 % aqueous saturation.	77

3.5	- CER and RQ profiles of three fermentations controlled at 30°C with DOT controlled at 20% aqueous saturation.	78
3.6	- Logarithmic plot of OUR and CER to determine exponential increase in activity.	79
3.7	- Totalised values for carbon dioxide evolved and oxygen consumed for the fermentation controlled at 30°C with DOT at 20% aqueous saturation. Data expressed on both a linear and logarithmic scale.	81
3.8	- Logarithmic plot of the biomass data to observe exponential increase in cell concentration.	83
3.9	- Relationship between the total carbon evolved as carbon dioxide, total oxygen consumed and the cell concentration of the fermentation controlled at 30°C and 20 % DOT.	84
3.10	- Relationship between the cell concentration and carbon evolved from the fermenter as carbon dioxide to the acid added to control the pH.	85
3.11	- Carbon balance and profile of the fermentation controlled at 30°C and 20% DOT.	88
3.12	- Relationship between cell concentration and concentration of carbon consumed.	91
3.13	- CER and RQ profiles of the fermentations at 30°C and DOT controlled at 20 and 5% aqueous saturation.	93
3.14	- Logarithmic profile of the CER and biomass data of the fermentation controlled at 30°C and 5 % DOT.	95

3.15 - Correlation between biomass concentration, carbon dioxide evolved and acid input to control pH.	96
3.16 - Carbon balance of the fermentation controlled at 30°C and 5 % DOT. The calculated carbon content of the supernatant and the carbon uptake by the cells are also shown.	97
3.17 - Correlation between total carbon uptake and cell concentration.	99
3.18 - Endogenous OUR, OUR due to ethanol consumption of the resuspended and washed samples of the fermentation at 30°C and 5 % DOT with the fermentation CER profile shown.	100
3.19 - DOT and CER profiles from 15 to 25 hr after inoculation to show the effect upon the CER of decreasing the DOT to 0. The CER profile is compared to the fermentation controlled at 20 % DOT throughout but with a 3 hr lag time subtracted.	101
3.20 - CER and DOT profile of the fermentation controlled at 20 % DOT and 30°C but near the end of the growth phase the $K_L a$ had been reduced.	104
3.21 - DOT, CER and OUR profile of the fermentation at low $K_L a$ after high biomass had been achieved.	106
3.22 - Comparison of the CER and biomass profile of the cells grown at 30°C and 26°C.	108
3.23 - Correlations between total carbon dioxide evolved from the fermenter, cell concentration and the acid added to the fermenter.	110

3.24 - Carbon balance of the fermentation at 26°C and controlled at 20 % DOT. The calculated carbon source concentration and uptake by the bacteria are also shown.	111
3.25 - Correlation between the calculated carbon source uptake and cell concentration of the cells grown at 26°C and DOT controlled at 20 % aqueous saturation.	112
3.26 - CER and RQ profiles of the two fermentations controlled at 34°C and 20% DOT	114
3.27 - Comparison of the carbon out of the fermentation at 30°C and 34°C. Profile of 30°C fermentation which was from 0 to 16.75 hr after inoculation was moved to 12.05 to 28.8 hr after inoculation so that end of growth phase was at the same time as the fermentation at 34°C.	116
3.28 - Biomass profile of the growth phase of the first fermentation at 34°C and 20% DOT	117
3.29 - Comparison of the initial activity of the cells in the fermentations at 26, 30 and 34°C with DOT at 20 % aqueous saturation. Activities were expressed as a profile of (100-DOT) against time	120
3.30 - The DOT profile of the carbon limited fermentations which had the oxygen transfer rates changed to determine its effect upon the growth of the cells.	122
3.31 - Total oxygen consumption and the total carbon dioxide evolved from the carbon limited fermentation.	124

3.32 - The effect of ethanol and propanol concentration upon the specific OUR of the cells.	129
3.33 - The effect of fluorobenzene concentration upon the specific OUR of the cells.	130
3.34 - Profiles of the DOT against time of three runs of a single sample with fluorobenzene added at different concentrations.	131
3.35 - The effect of fluorobenzene and benzene concentration upon the aromatic oxidation activity	133
3.36 - The effect of fluorobenzene concentration upon the growth of <u>P.putida</u> ML2 in succinate minimal medium.	134
3.37 - The effect of cell concentration upon the fluorobenzene oxidation activity.	136
3.38 - The stability of the fluorobenzene oxidation activity of cells from shake flask culture which were stored on ice.	137
3.39 - The effect of fluorocatechol upon the endogenous OUR and the specific aromatic oxidation activity of the bacteria expressed as a percentage of the control.	140
3.40 - The effect of fluorocatechol concentration upon the growth of <u>P.putida</u> ML2 in minimal medium.	141
3.41 - The initial growth of the bacteria in succinate minimal medium after shaking in buffer containing different concentrations of fluorocatechol.	143

3.42 - Growth of four different <u>P.putida</u> ML2 cultures in succinate minimal medium containing 0.5 gl <sup>-1</sup> fluorocatechol but had different inoculum development.	145
3.43 - Effect of storing the bacterium at 0°C on the specific fluorobenzene oxidation activity of cells from four different fermentations controlled at 30°C and 20% DOT, which were assayed after different storage times.	149
3.44 - Oxygen uptake rate profile of cells grown at 30°C and 20% DOT and the fluorobenzene oxidation activity of samples taken from the fermentation.	151
3.45 - Growth of <u>P.putida</u> ML2 in succinate minimal medium with normal and double amounts of buffer to analyse the effect of pH upon growth.	153
3.46 - Fluorobenzene oxidation activity of <u>P.putida</u> ML2 samples taken from fermentation controlled at 30°C and 5 % DOT, the fermentation OUR profile is also shown.	156
3.47 - OUR profile of fermentation controlled at 20 % DOT which is then dropped to zero and the resulting effect upon the fluorobenzene oxidation activity of the samples taken.	158
3.48 - The aromatic oxidation activity of the samples taken and the OUR of the fermentation initially at 20 % DOT but is taken to low oxygen transfer rates near the end of the growth phase.	160



- 3.49 - The DOT, specific aromatic oxidation activity and biomass profile from 15 to 25 hr after inoculation of the fermentation at 34°C and with oscillating DOT. 161
- 3.50 - The biomass and specific aromatic oxidation activity of the samples from the growth phase of the fermentation controlled at 20 % DOT which was reduced to 0, <sup>% DOT</sup> for a short period of time. 163
- 3.51 - OUR and DOT profile of the fermentation at 26°C and 20 % DOT and the fluorobenzene oxidation activity of the samples taken from the fermentation. 166
- 3.52 - Profile of the aromatic oxidation activity of samples taken from the two fermentation controlled at 20% DOT and 34°C. 167
- 3.53 - Profile of the cell concentration of the carbon limited fermentation controlled at 30°C and the aromatic oxidation activity of the samples taken. 169
- 3.54 - The specific aromatic oxidation activity and OUR profile of two fermentations which were initially limited by iron. Additional iron sulphate was added to remove the growth limitation. 171
- 3.55 - The specific aromatic oxidation activity and the OUR profile of the iron limited fermentation at 30°C and 20 % DOT. 172
- 3.56 - The OUR and biomass profile of the iron limited fermentation at 30°C and 20 % DOT expressed on a logarithmic scale to detect changes in metabolism. 174

- 3.57 - The effect of chloroxylenol upon the oxygen uptake of P.putida ML2. (Chloroxylenol was added in 25 $\mu$ l of propanol). 182
- 3.58 - The (DOT<sub>0</sub>-DOT<sub>t</sub>) profile obtained after cells are subjected to 0.2 gl<sup>-1</sup> reactor chloroxylenol concentration. (DOT<sub>0</sub>-DOT<sub>t</sub>) = (801/19.06) . (1-exp<sup>-19.06.t</sup>) was fitted to the datapoints 186
- 3.59 - The effect of changing the number of datapoints analysed upon the curve profile obtained after fitting to the equation (DOT<sub>0</sub>-DOT<sub>t</sub>) = (OUR<sub>0</sub>/c) . (1-exp<sup>-ct</sup> ). 187
- 3.60 - Re-examination of the data shown in Figure 3.59 but with data fitted to the equation (DOT<sub>0</sub>-DOT<sub>t</sub>) = ((OUR<sub>0</sub>-OUR<sub>f</sub>)/c) . (1-exp<sup>-ct</sup>) + (OUR<sub>f</sub>.t) 190
- 3.61 - The effect of cell concentration upon the calculated values of C, OUR<sub>0</sub> and OUR<sub>f</sub>. 192
- 3.62 - The effect of chloroxylenol concentration upon C, OUR<sub>0</sub> and OUR<sub>f</sub>. 194
- 3.63 - The effect of chloroxylenol concentration upon the solvent tolerance of the cells obtained from the end of the growth phase of a fermentation controlled at 20 % DOT and 30°C. 196
- 3.64 - The fermentation profile for cells grown at 30°C and 20 % DOT and the calculated C value obtained for cells at two chloroxylenol concentrations. 197

- 3.65 - The fermentation profile for cells grown at 198  
30°C and 20 % DOT for the second fermentation  
analysed with the calculated C value obtained for  
cells at a reactor chloroxylenol concentration of  
0.067 gl<sup>-1</sup>.
- 3.66 - The fermentation profile for cells grown at 200  
30°C and 5 % DOT and the calculated C value  
obtained for cells at a chloroxylenol  
concentration of 0.067 gl<sup>-1</sup>.
- 3.67 - Comparison of the specific OUR due to ethanol 201  
consumption, biomass concentration in the reactor  
and the calculated C value.
- 3.68 - The fermentation profile and calculated C 203  
value for cells initially grown at 20 % DOT with  
transition to zero at the end of the growth phase.
- 3.69 - The biomass profile and calculated C value for 204  
the bacteria from the fermentation controlled at  
20 % DOT with transition to zero for a short  
period of time during the growth phase.
- 3.70 - The calculated C value for solvent tolerance 206  
of the bacteria from the fermentation controlled  
at 26°C and 20 % DOT which is compared to the  
fermentation OUR profile.
- 3.71 - Comparison of the specific oxygen uptake rate 207  
due to ethanol consumption, biomass concentration  
in the reactor and the calculated C value of the  
bacteria from the fermentation at 26°C and 20 %  
DOT.

3.72 - Comparison of the OUR profile of the fermentation at 26°C and 20 % DOT to the calculated value of $OUR_0$ / OUR before chloroxylenol addition.	208
3.73 - The drop in DOT after the addition of chloroxylenol to the reactor of the bacterium sampled from the fermentation at 34°C.	209
3.74 - Octanol toxicity upon the OUR due to ethanol consumption as demonstrated by changes in the DOT profile.	211
3.75 - The relationship between the calculated C values obtained from the profiles in Figure 3.74 and octanol concentration.	212
3.76 - Octanol toxicity at 0.82 $gl^{-1}$ indicating non-exponential decay in OUR as compared to fitted curve.	213
3.77 - The effect of 0.083 % (v/v) of decanol, hexane, nonanol, heptanol and octanol upon the OUR due to ethanol consumption.	215
3.78 - DOT profiles of <u>P.putida</u> ML2 against time demonstrating fluorobenzene inhibition of the OUR due to ethanol consumption.	216
3.79 - The relationship between the calculated C values obtained from the profiles in Figure 3.78 and fluorobenzene concentration.	217
3.80 - DOT profiles of <u>P.putida</u> ML2 against time demonstrating ethyl, butyl and pentyl acetate inhibition of the OUR due to ethanol consumption.	218

3.81 - The effect of K <sub>2</sub> EDTA and MgSO <sub>4</sub> upon the tolerance of <u>P.putida</u> ML2 towards chloroxylenol.	220
3.82 - The effect of various concentrations of MgSO <sub>4</sub> upon the tolerance of <u>P.putida</u> ML2 towards chloroxylenol.	221
3.83 - The effect of various concentrations of MgSO <sub>4</sub> upon the tolerance of <u>P.putida</u> ML2 towards fluorobenzene.	222
3.84 - The effect of K <sub>2</sub> EDTA and MgSO <sub>4</sub> upon the tolerance of <u>P.putida</u> ML2 towards dichloroethane.	224
3.85 - The effect of a decrease in reactor temperature and MgSO <sub>4</sub> upon the tolerance of <u>P.putida</u> ML2 towards dichloroethane.	225
4.1 - Comparison of the measured total carbon dioxide evolved data to the theoretical value obtained by subtracting the carbon content of the fermentation broth from the initial carbon content of the medium.	233
4.2 - Comparison of the aromatic oxidation activity of the samples taken from the fermentations controlled at 5 and 20 % DOT (30°C).	236

## LIST OF TABLES

1.1	-	Examples of the biocatalytic reactions available	25
1.2	-	Examples of two liquid phase biocatalytic reactions	28
1.3	-	The effect of different carbon sources upon the yield of biomass and catechol production	44
4.1	-	Growth characteristics at four different fermentation conditions.	227

## 1 - INTRODUCTION

### 1.1 - Two liquid phase biotransformation

#### 1.1.1 - Biocatalysis

Biocatalysis is the biological conversion of one chemical compound to another. The catalyst may be a micro-organism, plant or animal cell, whether growing or resting, a cell extract or a purified enzyme.

Biocatalysis (or biotransformation as it is also known) overcomes two major problems of the traditional chemical synthesis. First of all it can produce a pure isomer instead of a racemic mixture and secondly biocatalysis may involve a short synthesis reaction instead of a long chemical synthesis which results in low yields. Replacement of chemical catalysts with enzymes has been slow but industrial uses of enzymes include brewing, baking and detergents (Godfrey & Reichelt, 1983). Their initial value is in their ambient temperature and pressure requirements but now the production of specific high priced chemicals allows a greater use of a variety of enzymes. They have also been used to degrade unwanted isomers in a racemic mixture therefore leaving the purified product. Enzymes can be used in the whole process to produce a single product or may be used as a single step in a chemical reaction.

Enzyme specificity includes the production of geometrical and/or stereoisomers. Stereoisomers differ in the positioning of four different molecules on a chiral carbon centre; one isomer is the mirror image of the other. These stereospecific reactions are useful in the food industry to produce the natural isomer (e.g. L-aspartic acid) to be used as a food additive to improve the quality of the food. It can be produced in two ways, either the fumarate molecule can be converted to L-aspartate by aspartase (Tosa et al, 1973), or an acylase can convert the acyl L amino acid

into L aspartate with the acyl D amino acid being racemised into acyl L amino acid (Chibata et al,1976). Due to the range of enzymes available it is possible to select the reaction to produce the pure isomer required. A strain of Aspergillus niger can preferentially produce the ethyl (R) hydroxybutanoate from ethyl 3-oxobutanoate where as another strain can produce the S form of the isomer from the same substrate (Bernardi et al,1984).

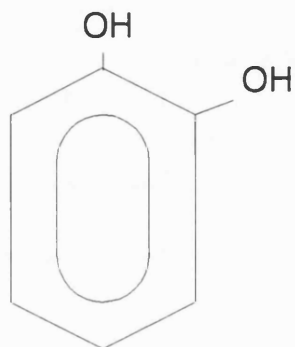
Geometrical isomers are different by the location of chemical <sup>groups</sup> on the carbon backbone, for example catechol, resorcinol and hydroquinone have two hydroxyl groups positioned at different locations on a benzene ring. These compounds are shown in Figure 1.1, but in the biocatalytic conversion of benzene by Pseudomonas putida, only the catechol is produced (Dagley,1986). P.putida produces the catechol from benzene via the benzene cis glycol, where the enzyme benzene dioxygenase shows both geometrical and stereospecificity (Dagley,1986). A number of examples <sup>have</sup> been given above to explain enzyme specificity, but the range of chemical groups which can be produced <sup>have</sup> not <sup>been</sup> discussed. A variety of enzymes and organisms can be used, and Table 1.1 illustrates some of the possible reactions.

Biocatalysis can produce many high priced products, but their purpose may not be obvious. Generally they can be split into three areas; pharmaceutical, food additives and other high priced chemicals. Obvious pharmaceuticals are the steroid conversions (Steinert et al,1987), but fluorocatechol (Cherry et al,1985 ) and glycidol (Philippi et al,1987) are also starting <sup>for pharmaceutical production.</sup> materials. Food additives include the production of tryptophan (Ribeiro et al,1987), aspartame (Oyama,1987) and natural flavour esters (Gillies et al,1987). High priced chemicals include benzene cis glycol which can be converted into pinatol (Ley et al,1987) and long chained polyphenylene (Ballard et al,1983).

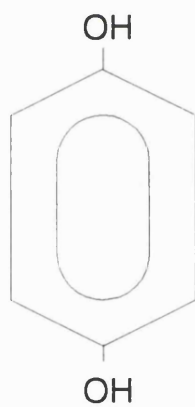


Figure 1.1 - The three geometrical isomers of dihydroxy benzene.

Catechol



Hydroquinone



Resorcinol

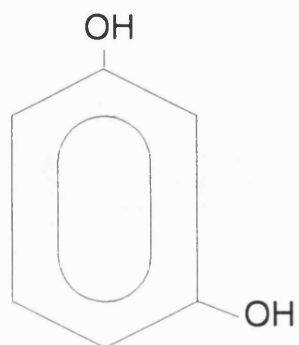
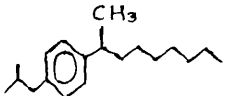
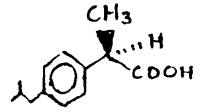
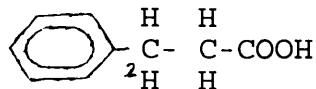
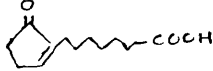
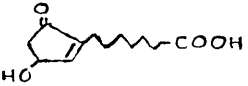
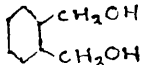
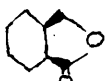



Table 1.1 - Examples of the biocatalytic reactions available

TYPE OF ORGANISM	NAME OF ORGANISM	REACTION TYPE	SUBSTRATE (S)	PRODUCT	REFERENCE
G+ Bacterium	<u>Rhodococcus</u>	Oxidative Degradation			Sugai & Morai (1984)
G- Anaerobic	<u>Clostridium Kluyveri</u>	Hydrogenase and Radioactive Labelling	Cinnamic Acid and Water		Bartl et al (1977)
G- Bacterium	<u>Pseudomonas</u>	Dehalogenase	$\text{CH}-\underset{\text{Cl}}{\text{CH}}-\text{COOH}$	L form COOH HO + H CH	Motosugi et al (1984)
G- Bacterium	<u>Xanthomonas citri</u>	Protease (Proteolytic Bond Formation)	6-aminopenicillamic acid + D-phenylglycine methyl ester	Ampicillin	Kato et al (1980)

25

Table 1.1 - Examples of the biocatalytic activity available (cont)

TYPE OF ORGANISM	NAME OF ORGANISM	REACTION TYPE	SUBSTRATE (S)	PRODUCT	REFERENCE
Yeast	<u>Saccharomyces cerevisiae</u>	Condensation	Acylain	D - ephedrine	Rose (1961)
Fungus	<u>Aspergillus niger</u>	Hydroxylation			Kurozumi et al (1973)
Mammalian	Rat liver/ Monoamine oxidase	Decarboxylase	Tyrosine + <sup>2</sup> H <sub>2</sub> O	<sup>2</sup> H-tyramine	Belleau & Burba (1960)
Mammalian	Horse Liver/ Dehydrogenase	Oxido - Reduction	meso diol 	Lactone 	Jakovac et al (1982)
Plant	Almond Seeds	Flouroprotein D - oxynitrilase	RCHO + HCN	R cyanohydrin H R-C-CN OH	Becker & Pfeil (1966)
Plant	Horse Radish/ Peroxidase	Oxidation	Phenol		Schwartz & Hutchinson (1981)

### 1.1.2 - Two Liquid Phase Biotransformation

The potential use of biocatalysts in organic synthesis could increase with the recent US Food and Drug Administration (FDA) policy to apply a more vigorous justification of enantiomeric mixtures as chiral pharmaceuticals (Faber & Franssen, 1993). Many biotransformations of industrial interest involve substrates and products which have low aqueous solubilities and may demonstrate toxicity or inhibition to microorganisms (Kawakami & Nakahara, 1994). However, their low aqueous solubility may result in preferential partitioning into a solvent phase from the aqueous phase thereby reducing these deleterious effects. A liquid substrate can act as the second liquid phase itself as long as it is not toxic to the biocatalyst. A recombinant E.coli containing the P.oleovorans gene for conversion of octane to octanoic acid was shown to become tolerant to the substrate allowing the biotransformation to occur in a medium containing 20 % octane (Favre-Bulle et al, 1991). These reactions as well as others listed in Table 1.2 indicate the range of two liquid phase biotransformations possible. The second liquid phase may also act as a reservoir for water insoluble solids (e.g. steroids) so allowing easy transfer to the aqueous phase (Pinheiro & Cabral, 1991). Biocatalytic production of alkene oxide which is toxic to Mycobacteria involved two gaseous substrates (propene and oxygen) which are poorly water soluble (Tramper & Vermue, 1993). A second liquid phase may act as a reservoir for these compounds which in aqueous solutions would be difficult to solubilise and control by mass transfer. It is possible to use either isolated enzymes or whole microorganisms for such reactions, depending on the complexity of the reactions and the stability of the catalyst in two phase systems. The organic solvent, itself, may also damage the microorganism and one of the aims of this study was to examine this aspect.

Substrate inhibition towards Pichia pastoris was observed when benzyl alcohol concentration was increased from 28.7 gl<sup>-1</sup> (to 36.3 and 46.2 gl<sup>-1</sup>), this was overcome by adding solvents to the biotransformation (Kawakami & Nakahara, 1994). Here the substrate partitioned favourably into the solvent (benzene, toluene, xylene

Table 1.2 - Examples of two-liquid phase biocatalytic reactions

Type of organism	Name of organism	Reaction type	Reference
Gram - bacterium	Recombinant <u>E.coli</u> (gene from <u>P.oleovorans</u> )	Alkane oxidation	Favre-Bulle et al (1991)
Gram + bacterium	<u>Athrobacter simplex</u>	Steroid dehydrogenation	Pinheiro & Cabral (1991)
Yeast	<u>Pichia pastoris</u>	Alcohol oxidation	Kawakami & Nakahara (1994)
Fungal	<u>Aspergillus ochraceus</u>	Steroid hydroxylation	Ceen et al (1987)
Enzymic	Pig liver esterase	Ester hydrolysis	Williams et al (1987)
Enzymic	<u>Candida rugosa</u> lipase	Acid/alcohol esterification (low water reaction)	Sjursnes et al (1992)

and cyclohexane) so maintaining aqueous concentrations below an inhibitory level. Likewise the capacity of the solvent to partition the product benzaldehyde from the aqueous phase allowed higher reactor yield to be obtained before reaching the inhibitory aqueous concentration of 5 gl<sup>-1</sup>. Further examples of inhibitory substrates include benzene toward Pseudomonas (Van den Tweel et al,1987) and tetralin toward Arthrobacter (Tramper & Vermue,1993), these biotransformations were improved by inclusion of a solvent to maintain low aqueous substrate concentrations.

Many biotransformations are limited by product inhibition resulting in maximum product yield which cannot be overcome in a conventional aqueous biotransformation. This can make the process uneconomic if the product is not removed from the aqueous phase before cessation of activity. Hexadecane which acted as a second liquid phase was shown to partition 1,2-epoxyalkanes from the aqueous phase before they reached a level inhibitory to the biocatalyst Nocardia corallina (Kawakami,1990). Economic assessment of industrial ethanol production indicated a major reduction of cost upon addition of a second immiscible solvent (Daugulis et al,1991). The estimated economic cost of butanol production was reduced by 20 % upon addition of a solvent mixture (50% oleyl alcohol in decane) to the fermentation broth (Roffler et al,1991). Ethanol and butanol are highly water soluble and many other biotransformations involve a substrate of low solubility which is converted to a highly water soluble product which may not partition favourably into a second solvent phase. Catechol has a solubility 600 times greater than benzene (Windholz,1983), its production by P.putida was improved upon removal of the inhibitory product from the reaction medium with activated charcoal (Robinson et al,1992). In situ product removal may be carried out by a number of methods and the value of the addition of a second immiscible solvent must be considered along side these other techniques (Freeman et al,1993).

Developments in this field have included classification of the various processes available, use of novel bioreactors and changes to the biotransformation medium. Novel bioreactors

considered include the liquid-impelled loop reactor, which allows the continuous removal of solvent and is particularly useful for shear sensitive organisms (Tramper & Vermue,1993). Changes in reaction medium have included the use of carbon dioxide as solvent by performing the reaction under high pressures (Isenschmid et al,1992). Changes in temperature and pressure lead to major changes in the physical properties of the fluid which maybe advantageous for the biotransformation. Major aspects of two liquid phase biotransformations analysed in detail include the rate of transfer of components between phases and the biocompatibility of a biocatalyst to a set of solvents (Woodley & Lilly,1992).

Five main types of two liquid phase biocatalytic reactions are observed, two involve freely suspended biocatalysts with either the aqueous or organic phase as bulk medium, two involve immobilised biocatalysts and the last involves a reaction with minimal amount of water (Lilly et al,1987). The thermal tolerance of a lipase was shown to increase upon removal of virtually all of the water which generally acts as a denaturant at high temperatures (Cowan & Plant, 1992). Enzymes dissolved in a aqueous solution within a reversed micelle were shown to be protected from solvent damage by the surfactant layer (Sanchez-Ferrer et al,1992). Removal of water from a reaction may result in a shift of the equilibrium towards bond formation as in the esterification of butanoic acid and butanol by a lipase (Sjursnes et al,1992). Selection of the system to be followed is dependent upon the location of the substrates and products in the reaction; it is important to consider the need for a bulk aqueous phase for water soluble nutrients such as carbon sources essential for cofactor regeneration. However, microorganisms lose their activity rapidly under conditions of high solvent concentration and it is necessary therefore to maintain a discrete aqueous phase. The main problem of a two liquid phase biotransformation is the incompatibility of the biocatalyst to solvents commonly used in industrial processes. The alteration of bacterial growth to alleviate solvent toxicity effects was investigated in this project.

## 1.2 - Solvent toxicity

The microbial epoxidation of propene and 1-butene in the presence of water immiscible solvent was analysed to develop a method for optimising the selection of solvents (Brink & Tramper, 1985). They had concluded that a solvent with a low polarity and a high molecular weight would lead to high retention of the biocatalytic activity. This method of selecting solvents was superseded by analysing the Log P value of the solvents which is a measure of their hydrophobicity (Laane et al, 1985).

The Log P values are calculated by taking the logarithm of the partition coefficient of the compound between octanol and water. Laane <sup>et al (1985)</sup> had shown that solvents with a Log P lower than 2 inactivated the biocatalyst whereas solvent with a Log P greater than 4 had shown minimal inactivation of the biocatalyst. Harrop (1990) had also observed that the Log P value of a solvent indicates strongly its compatibility when the hydroxylation of naphthalene by P.putida was considered. It was also shown that an increase in the solvents interfacial area by increasing stirrer speed resulted in a faster inactivation of the bacterium. This could be due to two possible reasons, the first being that the bacterial <sup>cells</sup> have a greater opportunity to come into contact with the interphase. This could result in an increase in physical inactivation of the bacterium or an increase in the solvent uptake by the bacterium. The second reason is that the solvent is transferred into the aqueous phase at a faster rate therefore allowing the bacterium to take it up at a faster rate. Athrobacter simplex was inactivated by concentrations of solvents below aqueous saturation, indicating that the inactivation was not due to the second phase alone (Hocknull, 1989). The biocompatibility of A.simplex was shown to be different to P.putida with the bacterium being less tolerant to solvents between a Log P of 4 and 10 (Harrop et al, 1989).



### 1.2.1 - Solvent toxicity at concentrations below aqueous saturation

Hocknull (1989) had shown that solvent could be toxic without the presence of the solvent interphase. The profile obtained by Bar (1988) for inactivation of S.cerevisiae against the Log P of the solvent was similar to that obtained by Harrop (1990). The growth medium had been saturated with alkanes or alcohols and the growth, glucose consumption and ethanol production had been analysed. The solvents with a Log P below 4 were toxic where as those above 5 were biocompatible.

Various primary alcohols at different concentrations below aqueous saturation were analysed to determine their effect upon the 11  $\alpha$ -hydroxylase activity of Rhizopus nigricans (Osborne et al,1990). It was shown that the log (aqueous concentration of solvent) to bring about full loss of activity was directly related to the Log P in that the higher the hydrophobicity of the alcohol the lower the aqueous concentration to bring about full inactivation. Octanol (Log P = 2.92) was shown to be more toxic than heptanol (Log P = 2.39) at the same aqueous concentration. They had shown that the addition of the second phase to the reaction vessel had increased the toxicity of the solvents above a Log P of 4 which were not toxic at a concentration below aqueous saturation. The anaesthetic potential of long chain alcohols upon tadpoles (Alifimoff et al,1989) had been shown to have a similar profile to that obtained for 11  $\alpha$ -hydroxylase activity of Rhizopus nigricans (Osborne et al,1990). They had monitored the aqueous concentrations of the compounds and shown that dodecanol did not have anaesthetic potential which was not due to its depletion from the aqueous environment.

The profiles observed with alcohols were similarly observed with 2,4 dichlorophenol derivatives having n-alkyl side chain of various sizes (Blackman et al,1955). A number

of aromatic compounds were analysed for their effect upon methanogenic activity and had shown a linear relationship between Log P of compounds in the same homologous series and the log of the concentration of the compound to inhibit activity (Sierra-Alvarez & Lettinga,1991). It is therefore clear that Log P can indicate the inhibitory activity of the compounds but can be variable between different groups which would indicate different modes of activity.

### 1.2.2 - Kinetic analysis of solvent toxicity

Two liquid phase biotransformations have been inhibited by solvents with a Log P lower than 4. Analysis of solvents at a concentration below aqueous saturation indicated that higher toxicity was observed with octanol (Log P = 2.92) than with alcohols of lower Log P (Osborne et al,1990). Controlling the transfer of solvents of low Log P to the aqueous phase may prevent their toxicity towards the biocatalyst. The Log P concept provides a basis to compare the actions of different solvents upon the biocatalyst but it does not indicate the way that a single solvent inactivates the biocatalyst.

Solvent toxicity was directed towards the membrane of the biocatalyst and there was a 'critical membrane concentration' for the solvents at which all activity was lost (Osborne et al,1990). This work correlates well with other work which had analysed the effect of alcohols upon tadpoles (Alifimoff et al,1989) and interaction with synthetic biological membrane (Westerman et al,1988).The primary site of solvent damage was the bacterial membranes, they can also affect the overall cell morphology, cell transport mechanisms and cell metabolism, these effects have been reviewed in detail by Harrop (1990).

The site of action of these solvents seem to be the membranes but the rate of action is not discussed. The rate

of action of most of the alcohols was shown to be rapid and had a distinct cut-off point at a Log P around 4 (Harrop,1990;Bar,1988). No mathematical expression for the inactivation profile had been obtained making quantitative analysis difficult. Inactivation of steroid dehydrogenation (A.simplex) by buffer saturated with solvent indicated an exponential decrease in activity and half life values for the loss of activity had been obtained (Hocknull,1989). Exponential decreases in viability have been observed with disinfectants (Chick,1908). This mathematical expression could be suitable starting point for a more detailed quantitative analysis of solvent toxicity. In these expressions a second organic phase had been avoided so that the results were independent of the interphase mass transfer characteristics of the system.

### 1.3 - Process Set Up

The two liquid phase biocatalytic process can be divided into four main units as shown in Figure 1.2, recovery of the biocatalyst and solvent may be essential to decrease process costs.

The biochemical engineer must consider all aspects of the process since the problems created in either the biocatalyst production or the biotransformation will have to be solved during the downstream processing. Possible problems faced include the separation of an emulsion from a two liquid phase biotransformation. The growth of P.oleovorans upon octane had resulted in an emulsion which was stable for at least 4 hr after stirring was stopped (Witholt et al,1990). This is one of the problems that need to be considered when optimising the process. This study was based on optimising the fermentation conditions so that optimal activity during the biotransformation was obtained. Therefore biocatalyst production and the biotransformation will be considered further.

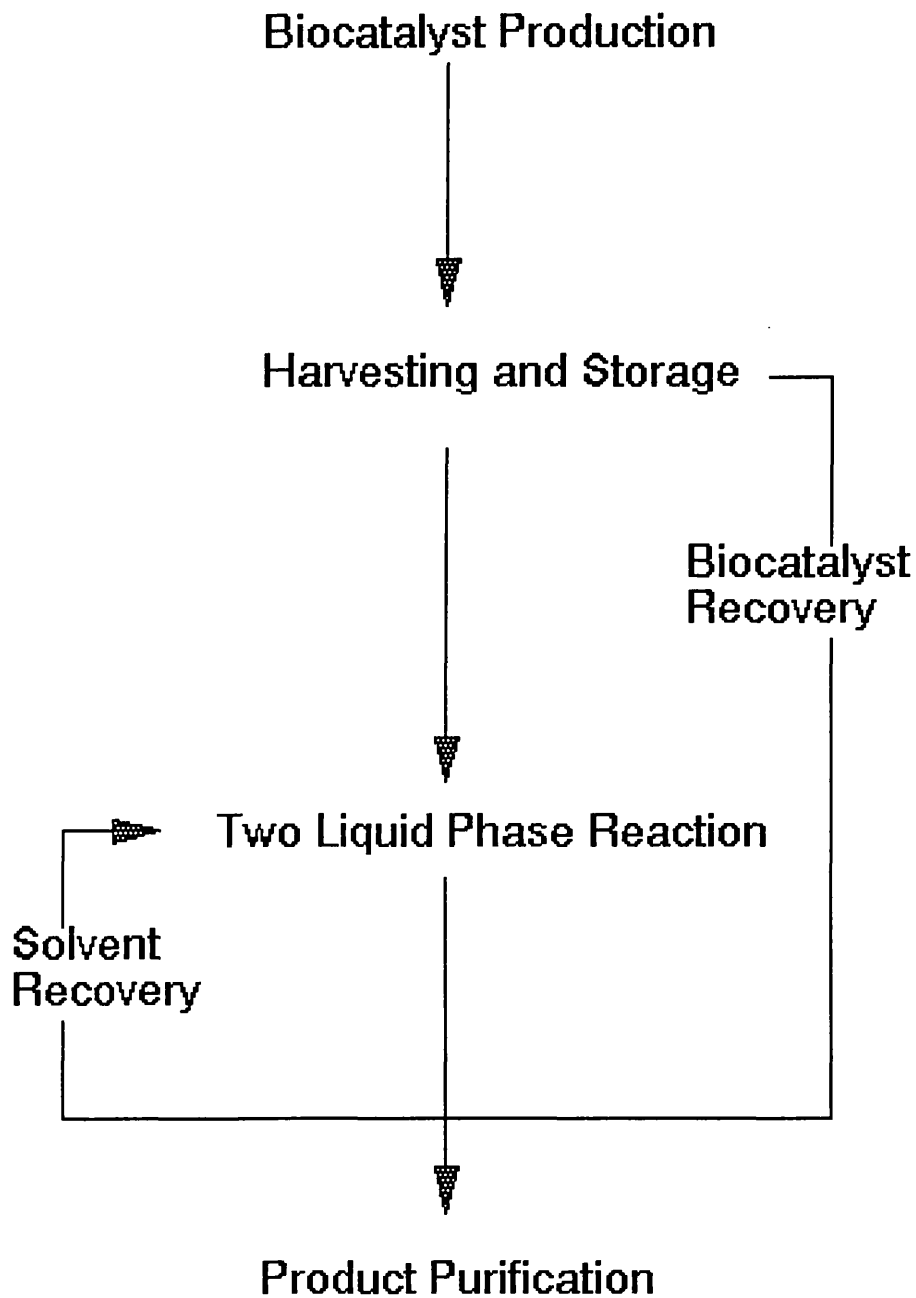
#### 1.3.1 - Production of biocatalyst

This work has focussed upon a fermentation process in which metabolically active whole cells are the biocatalyst rather than a purified enzyme. The biocatalytic activity of the bacterium must therefore be maintained so that the biotransformation can last for as long as possible. The major costs involved in a fermentation will be:-

- media
- labour
- aeration and agitation
- heating and cooling

A wide range of media have been used for biochemical engineering processes (Atkinson & Mavituna,1983), many contain byproducts of other processes such as corn steep

Figure 1.2 - The process flow sheet of a typical two liquid phase biotransformation.



liquor and molasses. Fluorocatechol production from fluorobenzene by P.putida has been carried out in both defined media and in complex media containing molasses (Schofield,1988). Large scale industrial production of the biocatalyst may involve the cheaper complex carbon source but again it is important to consider the effect of these upon the downstream processing. Changes in growth conditions will not only effect the yield of biocatalyst but also the biocatalytic activity and solvent tolerance.

#### 1.3.1.1 - The effect of growth conditions on enzyme activity

Growth of Pseudomonas in a shake flask culture under different conditions had resulted in a number of changes in yield of catechol from benzene (Shirai,1987). Optimum medium conditions for catechol production were at a phosphate and ammonium chloride concentration of 100mM and 5g<sup>l</sup><sup>-1</sup>. Higher yield of catechol<sup>was</sup> obtained with resting cells rather than growing cells. Movement of pH from the optimum around to 8.5 and moving temperature from the optimum between 25-30°C to 15 or 40°C resulted in complete cessation of activity.

The toluene hydroxylation activity of P.putida UV4 had remained constant under full pH control but without this control the activity was variable during the fed batch stage (Hack,1992). Optimal activity had been obtained while the DOT was maintained at between 50-60 % aqueous saturation a decrease in DOT to 10 % aqueous saturation had resulted in lower activities.

Control of DOT may be essential for maintenance of biocatalytic activity, highest activities for catechol 1,2 dioxygenase in P.putida had been obtained at high DOT levels (Viliesid & Lilly,1992). Optimum DOT for induction of the 11  $\alpha$ -hydroxylase activity in Rhizopus nigricans occurred at low level of 10 % aqueous saturation (Hanisch et al,1980). DOT will affect each biocatalyst in a different way, a detailed review of the literature on the effect of DOT on enzyme

synthesis, metabolite synthesis and antibiotic production can be obtained in Suphantharika (1992).

The growth of Streptococcus faecalis was not greatly affected by pH (5.5 to 7.0) but the tyrosine decarboxylase activity had a pH optimum of 6.0 (Anderson et al,1987). At pH 7 no activity was observed and at a pH of 5.5 the activity was approximately 40 % of optimum. Decreasing the temperature from 37 to 25°C had yielded cells with 30 % of the optimum activity. These optima for pH and temperature are not only dependent on the organism but also on the enzyme since the optima for glucose oxidase and catalase of Alternaria alternata were significantly different (Caridis et al,1991). Glucose oxidase had an optimum of pH 7.9 at 32.4°C where as catalase was at pH 8.5 and 23.1°C. Optimisation of the growth conditions for biocatalytic activity must therefore be enzyme specific.

The rate of growth rate as well as the growth conditions will affect optimum expression of an enzyme since optimal  $\beta$ -D-galactosidase expression in continuous culture occurred at a dilution rate of 0.15 hr<sup>-1</sup> (Gomez & Castillo,1983). The profile of dilution rate against expression of superoxide dismutase by Saccharomyces cerevisiae was completely different to that of catalase (Hansson & Haggstrom,1983). Growth conditions will control the expression of each enzyme of a particular microorganism in <sup>a different way,</sup> catalase expression dropped to zero when the dilution rate increased from 0.05 to 0.15 hr<sup>-1</sup> whereas superoxide dismutase activity decreased by 50% upon moving to 0.05hr<sup>-1</sup> from the optimum dilution rate of 0.21 hr<sup>-1</sup>.

### 1.3.1.2 - The effect of growth conditions upon solvent tolerance

It has been shown that Gram + and Gram - bacteria <sup>demonstrate</sup> significant variations in their solvent tolerance which was thought to be due to differences in the composition of the outer membrane (Harrop et al,1989).

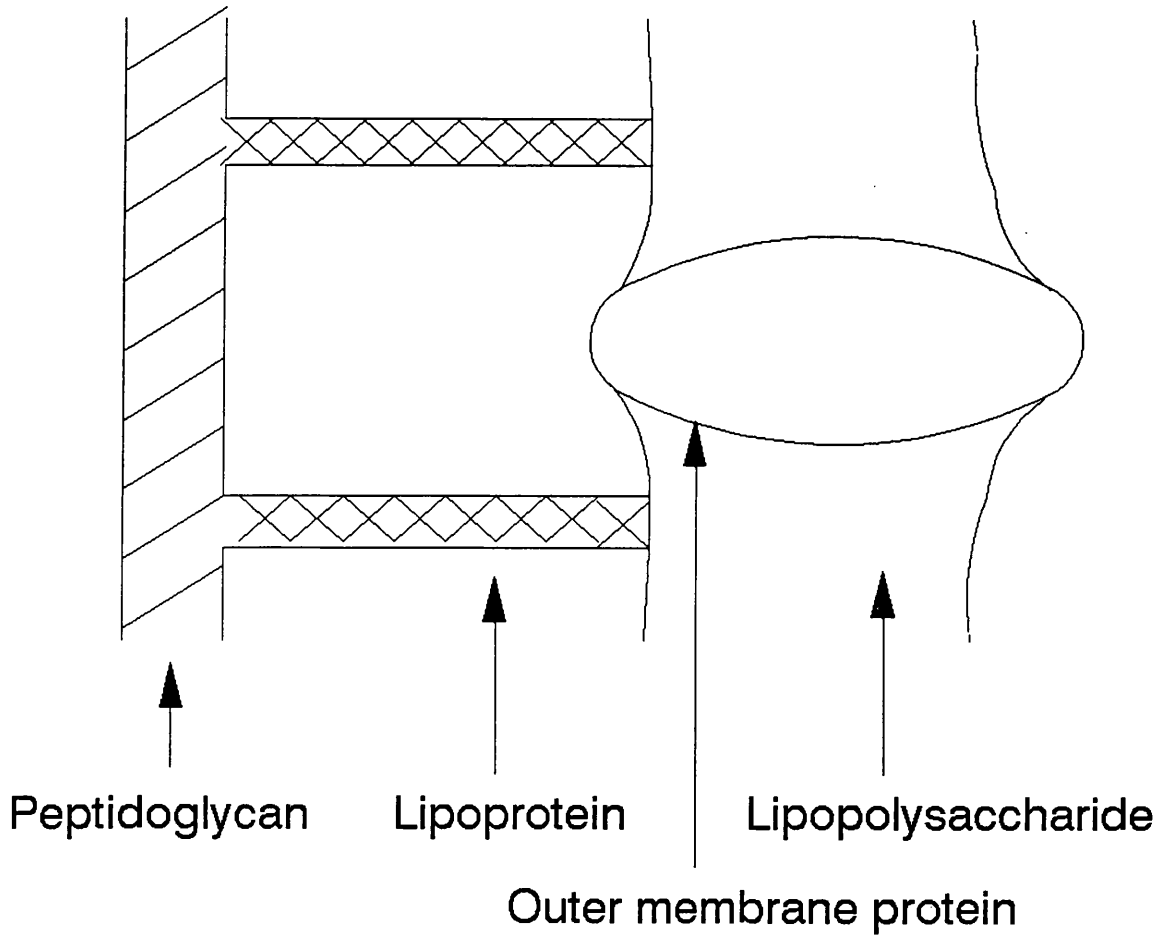
The structure of the outer membrane and lipopolysaccharide of a Gram - bacterium is shown in Figure 1.3 and Figure 1.4. Detailed analysis of the outer membrane has shown that a number of the structures have constant composition where as others are variable. The constant structures includes the lipoprotein (Vlasuk et al,1985) and the core region of the lipopolysaccharide molecule (Luderitz et al,1982). Changes in membrane permeability will be dependent on changes in the variable portion of the outer membrane. Solvent tolerance is dependent on membrane permeability.

Gross changes in the composition of the outer membrane due to changes in growth conditions include the amount of protein and carbohydrate (Ellwood & Tempest,1972). They had shown that when Aerobacter aerogenes had been grown in glycerol limited continuous culture the protein content was higher than <sup>in</sup> phosphate limited cultures. A significant drop in protein content (from 90 to 69 % of dried cell wall) in the glycerol limited cells had resulted when the dilution rate was increased from 0.1 to 0.7 hr<sup>-1</sup> whereas the phosphate limited cells did not changed significantly. Variations in the carbohydrate and <sup>2-keto-3-deoxyoctonic acid (KDO)</sup> composition had also been observed as a result of variations in the growth conditions.

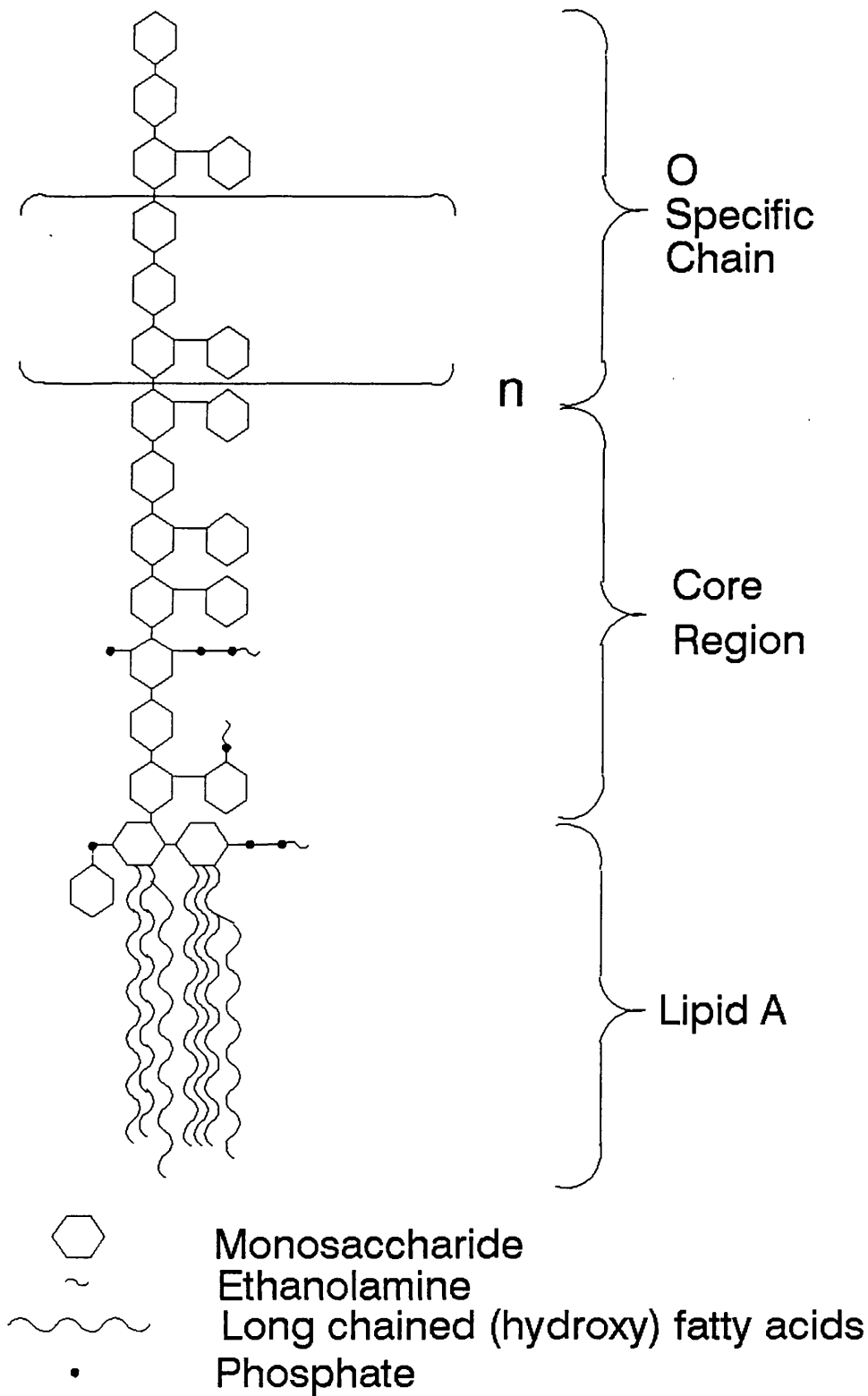
P.aeruginosa grown in magnesium deficient medium had resulted in the cells becoming more tolerant to EDTA and had 13.3 % more KDO and 18 % less phosphorus (Gilleland et al,1974). It was later shown that another P.aeruginosa strain grown in magnesium deficient medium had replaced the Mg<sup>2+</sup> with protein H1 (Nicas & Hancock,1980). Further



Figure 1.3 - The structure of the outer membrane of a Gram - bacterium .



**Figure 1.4** - The chemical structure of the lipopolysaccharide from the outer membrane of a Gram - bacterium (Luderitz et al,1982).



variations in the lipopolysaccharide<sup>(LPS)</sup> molecule were observed when E.coli had been grown at different growth rates (Dodds et al,1987). They had shown that at lower growth rates the ratio of high molecular weight LPS to low molecular weight LPS was higher than at high growth rates.

Not only is the composition of the LPS molecule dependent on growth conditions but also the lipid composition of the membrane. Minnikin et al (1972) had shown that B.subtilis grown under magnesium limited cultures had a higher phosphatidylglycerol concentration than diglucosyldiglyceride where as under phosphate limited growth the opposite had occurred. A higher growth temperature had resulted in E.coli having a greater proportion of saturated fatty acids which was thought to result in an increase in fluidity of the membrane (Sinensky,1971). It is clear that changes in growth conditions will result in variations in the bacterial membranes.

The effectiveness of the chemical antimicrobial agent chlorhexidine was shown to be dependent upon growth conditions in that at higher growth rates E.coli was shown to be more tolerant when grown under magnesium and phosphate limitation rather than nitrogen or carbon limitation (Wright & Gilbert,1987)b. E.coli had also been shown to alter its fatty acid composition when it is growing in the presence of alcohols so that it can increase its tolerance to them (Ingram,1976). It has been suggested that the alcohol induced changes in lipid composition was similar to the changes observed due to adaption to temperature (Berger et al,1980). The sensitivity of P.aeruginosa to chlorophenol was shown to be related to the lipopolysaccharide content of the cells (Gilbert & Brown,1978). Changes in the lipopolysaccharide content was brought about by changes in the growth rate and the nutrient limitation.

n-alkyltrimethylammonium bromide of varying Log P values

were analysed to determine their antimicrobial activity against E.coli grown under different conditions and growth rates (Wright & Gilbert,1987)<sup>a</sup>. Changes in the growth conditions had resulted in different compounds having optimal antimicrobial activity. This had suggested that the overall membrane lipophylicity was different under different growth conditions leading to different optimal value of Log P for compounds to traverse it. It is clear that changing growth conditions will lead to changes in solvent tolerance.

#### 1.4 - Experimental model system

The biocatalytic system chosen for investigation was the production of fluorocatechol from fluorobenzene by P.putida ML2 via the benzene degradation pathway. Aspects of the reaction makes it a suitable model system and these are described below.

##### 1.4.1 - Commercial interest

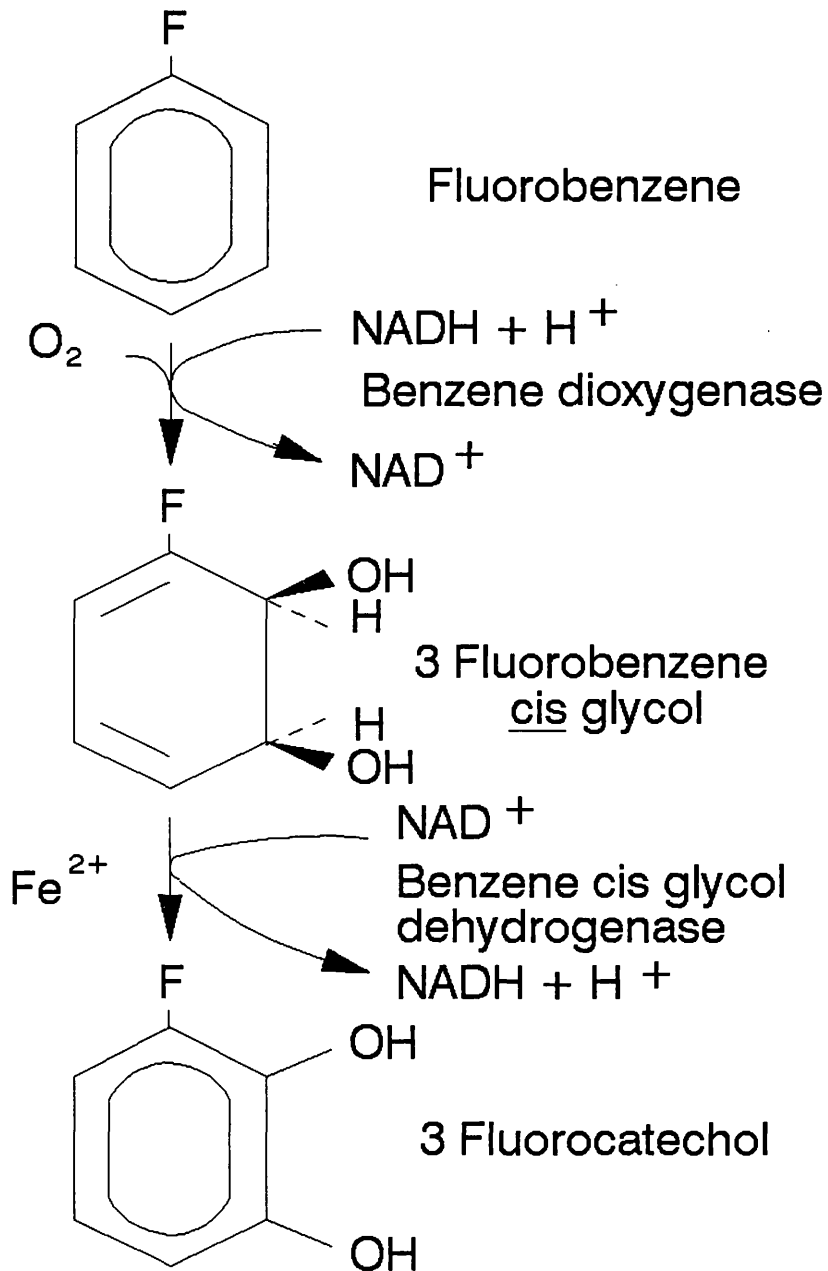
A simple cost-benefit analysis showed that the production of fluoroveratrole via the fluorocatechol with microbial oxidation is cheaper than conventional chemical synthesis (Johnston et al, 1987). Fluoroveratrole, which is chemically synthesised from fluorocatechol, is an intermediate in the production of fluorodimethoxyphenylethylamine a pharmacological active compound (Ladd & Weinstock,1981). Glaxo have submitted a number of patents on the production of pharmacologically active heterocyclic compounds with fluorocatechol as one of the starting materials (Cherry et al,1985; Hayman et al,1987).

It is important to note that complex disubstituted catechols can also be produced by P.putida (Johnston & Renganathan,1987) therefore allowing a range of novel products to be produced. Both Shell and I.C.I. have submitted patents on the production of fluorocatechol and various cis glycols accumulated by mutants lacking an active cis glycol dehydrogenase (Schofield et al,1987; Taylor,1987; Schofield, 1987). There is an obvious commercial interest in such systems once the developed pharmaceuticals have met the necessary regulatory requirements.

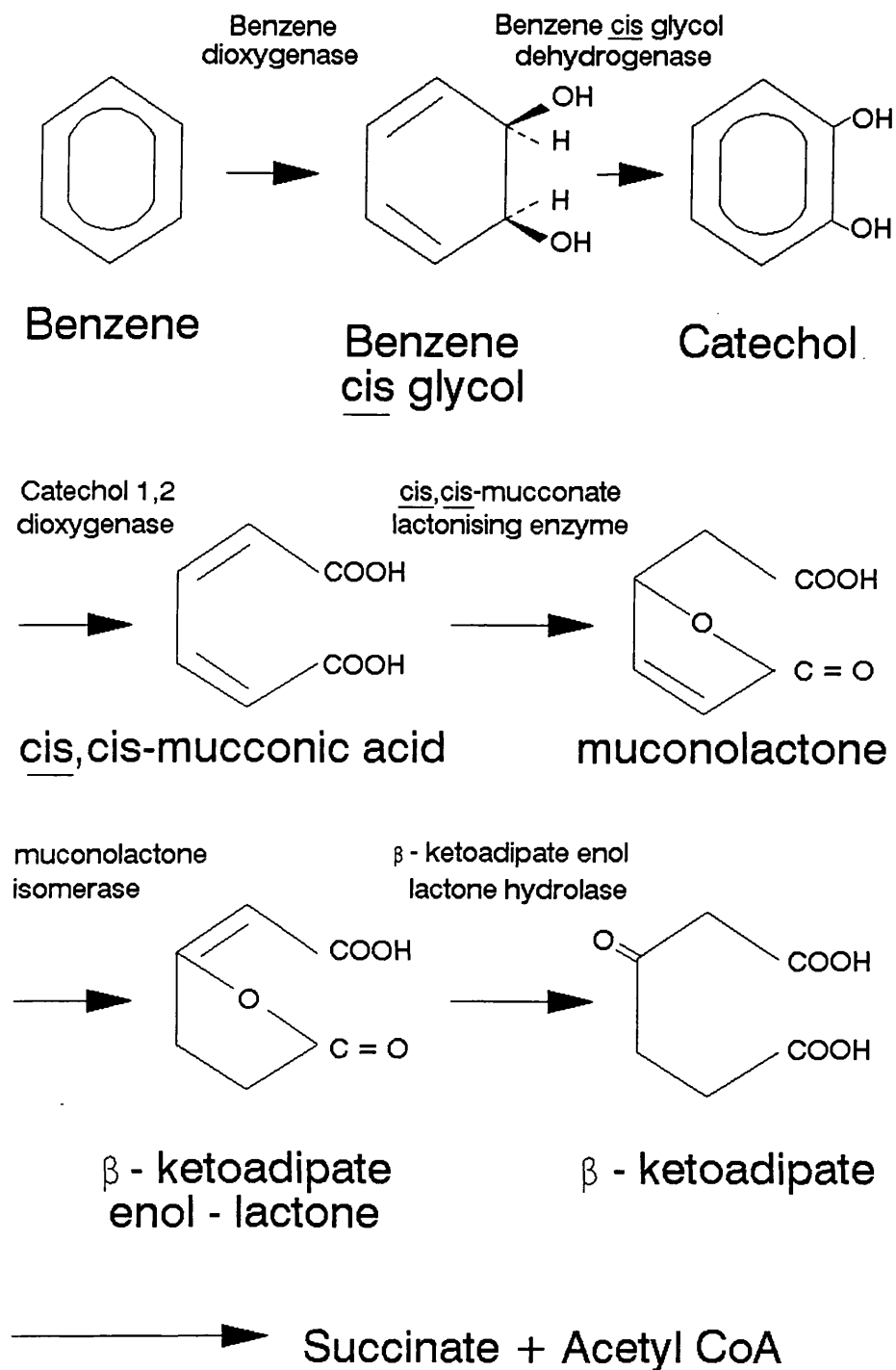
##### 1.4.2 - Enzyme system

The biotransformation pathway involves two enzymes and requires molecular oxygen and NADH as a cofactor which is regenerated by the second enzyme (Figure 1.5). These enzymes are part of the benzene degradation pathway (Figure 1.6) in which catechol 1,2 dioxygenase metabolises catechol but is unable to break down any substituted catechols (Dagley, 1986).

Figure 1.5 - The biotransformation of fluorobenzene to fluorocatechol by *P.putida* ML2.



**Figure 1.6** - The benzene degradation pathway of *P.putida* ML2  
 (Axcell & Geary, 1973; Ornston & Stanier, 1966).



Benzene minimal medium has been recognised as suitable selection medium for these enzymes.

#### 1.4.2.1 - Microbial dioxygenases

The benzene dioxygenase of P.putida ML2 is expressed from a 112 kb plasmid (Tan & Mason, 1990), its origin is likely to be from Acinetobacter rather than Pseudomonas (Tan et al, 1993). It is a relatively large enzyme comprising of three proteins of Mw 81,000, 12,300 and 215,000 each containing a iron sulphur group in the active site (Axcell & Geary,1975; Crutcher & Geary,1979; Geary et al,1984). The proteins are constitutively expressed therefore retention of biocatalytic activity will be dependent on plasmid copy number and hence stability.

Comparison of benzene, toluene and naphthalene dioxygenase in cell free extracts of P.putida ML2, NCIB 11767 and NCIB 9816 indicated a large variation in the rates at which benzene, toluene and naphthalene were metabolised (Zamanian,1989). Each strain metabolised a different substrate at maximum rate indicating large differences in substrate specificity. Dioxygenases initiate the degradation of a range of substrates (e.g. toluene, naphthalene, anthracene) with cis glycols as the products (Dagley,1986). A number of aromatic degradation pathways are encoded from a plasmid, including toluene, naphthalene, salicylate and chlorobenzoic acid (Frantz & Chakrabarty,1986).

The dioxygenase of Pseudomonas T12 has a broad substrate range including monosubstitution of the benzene ring with an alkyl side chain, halogen, nitrile or methoxyl group (Johnston & Renganathan,1987). The dioxygenases have also been demonstrated to act upon compounds which are not natural substrates including polychlorinated biphenols (Hofer et al,1993), azaarenes (Boyd et al,1993) and carbazole (Resnick et al,1993). Single ring substrates also include bromostyrene and norbornadiene which are converted into their equivalent dihydro diols (Konigsberger & Hudlicky,1993; Geary et al,1990). The large range of possible



products increases the potential commercial value of the bacterium.

#### 1.4.2.2 - Benzene cis glycol dehydrogenase

Benzene cis glycol dehydrogenase contains four subunits each of Mw 110,000 (Axcell & Geary,1973) which are constitutively expressed from a gene positioned close to the benzene dioxygenase gene on the P.putida ML2 plasmid (Tan & Fong, 1993).

#### 1.4.3 - Fermentation conditions

The study of bacterial physiology was an important part of this work, so that development of a suitable medium was essential. A minimal medium consisting of mineral salts and a single carbon source was preferable for the detection of any changes in carbon metabolism. The relative yield and catechol production of P.putida ML2 in a minimal salts medium with ten different carbon sources is shown in Table 1.3.

#### 1.4.4 - Substrate and product toxicity

Toluene (methyl substituted benzene) has been shown to have a toxic effect upon E.coli (DeSmet et al,1978) as well as inhibiting biocatalytic activity of P.putida UV4 at concentrations above 40 % aqueous saturation (Brazier, 1989). Likewise fluorobenzene (fluoro substituted benzene) is potentially toxic and has low solubility in water ( $1.54 \text{ g l}^{-1}$ , Riddick et al,1986). However it could be added in a water immiscible solvent to control the aqueous concentration below inhibitory levels.

The production of methyl catechol by P.putida 2312 was improved by removal of the product on to activated charcoal so reducing product inhibition and product denaturation (Robinson et al,1992). The potential use of an immiscible solvent as an extractant for inhibiting catechol or their derivatives is limited due to unfavourable partitioning from the aqueous phase of the water soluble product.

Table 1.3 - The effect of different carbon sources upon the yield of biomass and catechol production (Schofield et al, 1987).

Carbon Source	Concentration / $\text{gl}^{-1}$	Cell Density	Catechol Production
Succinate	10	+++	++
Fructose	10	++	++(+)
Sucrose	4	+/-	+/-
Galactose	10	+	+(+)
Lactose	10	+/-	+
Fumarate	10	++	+(+)
Formate	10	+/-	+/-
Citrate	10	++	+
Ethanol	10	+/-	+/-
Glycerol	10	+(+)	+

NB - + is an indicator of turbidity / intensity



### 1.5 - Objectives

The development of a successful two liquid phase biotransformation is dependent upon obtaining a biocatalyst which has high biocatalytic activity and solvent tolerance. Changes in the growth conditions of the bacterium will result in variations in the biocatalytic activity, solvent tolerance and yield of biomass.

The initial stage of this study will involve the development of a fermentation which can be characterised so that the growth of the bacterium can be modelled. The fermentation can then be compared on the basis of changes in growth rate and yield of biomass upon carbon source. This would allow changes in biocatalytic activity or solvent tolerance to be related either directly to the changes in growth conditions or to the changes in the rate of growth.

The aromatic oxidation activity will be characterised so that optimal substrate and cell concentration can be selected. Substrate and product toxicity will then be considered since these will influence the stability of the biocatalytic activity. Changes in the aromatic oxidation activity will then be induced by changing the DOT at which the bacteria are grown.

The initial analysis of solvent tolerance will be based upon obtaining a mathematical expression for the rate of inactivation of the biocatalyst so that changes could be quantified. A suitable solvent will be selected and be compared to a number of others available. Changes in solvent tolerance will be induced by changes in growth temperature.

The yield of biomass, aromatic oxidation activity and solvent tolerance of the bacterial<sup>culture</sup> of each fermentation will be discussed so allowing the advantages and disadvantages of each fermentation conditions to be compared.

## 2 - MATERIALS, EQUIPMENT AND METHODS

### 2.1 - Materials

#### 2.1.1 - Microorganism

Pseudomonas putida ML2 (NC1B 12190) was supplied by Shell Research Ltd, Sittingbourne, Kent. It had been isolated for its ability to grow on benzene from a soil sample taken from ground within the Shell Refinery in Pernis, Rotterdam (Schofield et al, 1987). The two enzymes involved in the biotransformation <sup>for this study did</sup> not require induction and are described in Section 1.4.2.

#### 2.1.2 - Chemicals

Aldrich Chemical Company Ltd, Gillingham, Dorset, UK.

Chemicals - Chloroxylenol  
Crystal violet  
 $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$   
 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$   
Fluorocatechol  
Fructose  
Glucose  
Indole  
 $\text{K}_2\text{EDTA}$   
 $(\text{NH}_4)_2\text{SO}_4$   
Succinate

Solvent - Benzene  
Decanol  
Ethyl acetate  
Fluorobenzene  
Heptanol  
Propanol

BDH Laboratory Supplies, Poole, UK

Chemicals - 2.5 M  $\text{H}_2\text{SO}_4$   
pH Indicator '678'  
Polypropylene glycol (antifoam)

Solvents - Dichloroethane  
Ethanol  
Hexane  
Pentanol  
Pentyl acetate

FSA Laboratory Supplies, Bishops Meadow Rd,  
Loughborough, UK

Chemicals -  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$   
 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$   
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$   
 $\text{H}_3\text{BO}_3$   
KCl  
 $\text{KH}_2\text{PO}_4$   
 $\text{K}_2\text{HPO}_4$   
KOH  
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   
 $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$   
 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$

Solvents - Acetonitrile  
Octanol

Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK

Chemicals - Agar Technical  
Nutrient agar  
Yeast extract

Sigma, Fancy Rd, Poole, Dorset, UK

Solvents - Butanol  
Butyl acetate  
Nonanol

### 2.1.3 - Growth Medium Composition

#### 2.1.3.1 - Solid growth medium

Two solid growth media were used which were nutrient agar and indole agar. Nutrient agar was used for subculturing the bacterium and indole agar had been used to detect the presence of the benzene dioxygenase activity. Both media were sterilised at 121°C for 20 mins.

The composition of indole agar was as follows:-

KH <sub>2</sub> PO <sub>4</sub>	1.33 gl <sup>-1</sup>
K <sub>2</sub> HPO <sub>4</sub>	2.65 gl <sup>-1</sup>
Yeast extract	3.00 gl <sup>-1</sup>
MgSO <sub>4</sub>	0.40 gl <sup>-1</sup>
Peptone	0.04 gl <sup>-1</sup>
Indole	0.10 gl <sup>-1</sup>
Agar technical	15.0 gl <sup>-1</sup>

#### 2.1.3.2 - Shake flask culture media

The minimal medium used to grow the bacterium in a shake flask was different to the one used in the fermenter. The composition of the shake flask medium is given below with succinate or benzene added as the carbon source. The medium was sterilised by autoclaving at 121°C for 20 mins.

Na <sub>2</sub> HPO <sub>4</sub>	3.00 gl <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	3.00 gl <sup>-1</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	1.00 gl <sup>-1</sup>
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.20 gl <sup>-1</sup>
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.015 gl <sup>-1</sup>
FeCl <sub>2</sub> .4H <sub>2</sub> O	0.016 gl <sup>-1</sup>
Trace element solution	2 ml l <sup>-1</sup>

The trace element solution contained the following salts:-

CaCl <sub>2</sub> .2H <sub>2</sub> O	0.66 gl <sup>-1</sup>
ZnSO <sub>4</sub> .H <sub>2</sub> O	0.18 gl <sup>-1</sup>
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.15 gl <sup>-1</sup>
MnSO <sub>4</sub> .4H <sub>2</sub> O	0.15 gl <sup>-1</sup>
CoCl <sub>2</sub> .6H <sub>2</sub> O	0.18 gl <sup>-1</sup>
H <sub>3</sub> BO <sub>3</sub>	0.10 gl <sup>-1</sup>
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.30 gl <sup>-1</sup>

Benzene minimal medium acted as a selective medium for the enzymes involved in the biotransformation. Benzene was assumed to be sterile and was added at a concentration of 0.2 ml l<sup>-1</sup> to the sterile medium described above. The flasks were sealed with 'subaseal' stoppers to prevent loss of benzene to the external environment Succinate was added at 10 gl<sup>-1</sup> to non-sterile medium which was then neutralised with 10 gl<sup>-1</sup> KOH before the whole medium was sterilised. The minimal medium occupied 20 % of the culture flask volume.

#### 2.1.3.3 - Fermentation media

The bulk of the fermentation medium (5 l) contained the following components:-

Succinate	22.0 gl <sup>-1</sup>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	6.5 gl <sup>-1</sup>
KH <sub>2</sub> PO <sub>4</sub>	1.06 gl <sup>-1</sup>
K <sub>2</sub> HPO <sub>4</sub>	2.12 gl <sup>-1</sup>
Polypropylene glycol	0.40 ml l <sup>-1</sup>

and neutralised with KOH to pH 6.9. The medium and fermenter were sterilised at 121°C for 20 mins.

The following components were added as a sterile concentrated solution by filtering through a Acrodisc, Gelman Sciences filter of pore size 2 µm (concentrations given are of the final fermentation broth concentration):-

MgSO <sub>4</sub> .7H <sub>2</sub> O	0.60 gl <sup>-1</sup>
FeSO <sub>4</sub> .7H <sub>2</sub> O	0.02 gl <sup>-1</sup>
Trace element solution	1.0 ml l <sup>-1</sup>

The carbon limited fermentation and the initial fermentation which had glucose and fructose as the carbon source were set up differently and are described in Section 2.3.1.4.

## 2.2 - Equipment

### 2.2.1 - Fermentation Equipment

#### 2.2.1.1 - Fermenter

A Chemap 7 litre fermenter was used which had two four bladed disc turbines which were motor driven from the bottom. The fermenter and medium was steam sterilised. The Ingold pH probe was calibrated at pH 4 and 7 at room temperature before sterilisation. The probe was recalibrated once it had been sterilised and cooled to 30°C to the pH of the medium. The Ingold DOT probe was calibrated at 0 and 100 % aqueous saturation after it had been sterilised and the medium temperature was 30°C. The zero point was calibrated by nitrogen sparging the medium free of oxygen. The probe was then calibrated at 100 % DOT once the medium had reached a steady oxygen concentration at a stirrer speed of 600 rpm and an airflow rate of 4 l min<sup>-1</sup>. The temperature probe required recalibration once or twice a year but was checked frequently against other thermometers.

#### 2.2.1.2 - Fermenter Control

The fermentation parameters were monitored and controlled by the Turnbull Control Systems Ltd controller. DOT was controlled by changes in stirrer speed and airflow rate. Temperature was controlled by passing water through



coils in the fermenter, an electrical heater controlled the temperature of this water. pH was controlled by the addition of sterile 2.5 M  $H_2SO_4$  to the fermenter by a Watson Marlow peristaltic pump.

#### 2.2.1.3 - Exit gas analysis

The exit gas from the fermenter was passed to the VG Gas Analysis Ltd MMG-80 Mass spectrophotometer so that the carbon dioxide and oxygen concentrations could be measured. It was calibrated with 1 % argon, 3 % carbon dioxide and 20 % oxygen gas. Helium gas was passed through the mass spectrophotometer so that the zero point could be calibrated.

#### 2.2.1.4 - Fermentation data retrieval

Data monitored by the Turnbull Control Systems Ltd controller and the VG Gas Analysis Ltd MMG-80 mass spectrophotometer was recorded every 3 mins by the BioI, Biotechnology Computer Systems package. It could also calculate the CER, OUR and RQ values. The data was then converted into a IBM compatible textfile by the 'Reflections' (version 4) computer package.

#### 2.2.1.5 - Automatic sampling of fermentation broth

The MX3 Bio Sampler, New Brunswick had been used to automatically sample some of the fermentations overnight at 2 hr intervals. The apparatus was set up and run as described in the operation manual, and included the input of cooling water. Samples were taken by the 'Bio Sampler' which overlapped the manual sampling so that the two sets of data could be compared.

#### 2.2.1.6 - Loadcell measurement of pH reservoir

The weight of the pH reservoir was measured with a loadcell (Defiant Weighing) so that the number of moles of

acid added to the fermenter could be calculated. The data obtained was recorded online by the BioI, Biotechnology Computer Systems package. The density of 2.5 M H<sub>2</sub>SO<sub>4</sub> was calculated to be 1145 gl<sup>-1</sup>.

#### 2.2.2 - Biomass concentration

The biomass concentration was determined from the A<sub>670</sub> value of the bacterial culture which was measured in the Pye Unicam PU 8600 UV/VIS spectrophotometer. In all cases the samples were diluted tenfold and a hundredfold so that A<sub>670</sub> was below 1.

The standard curve of the A<sub>670</sub> value of the bacterial culture (grown upon succinate minimal medium in a shake flask) against its dry cell weight is given in Figure 2.1. In all calculations the following equation was used :-

$$\text{Cell dry weight (gl}^{-1}\text{)} = 0.457 A_{670}$$

#### 2.2.3 - Carbon analysis of fermentation broth

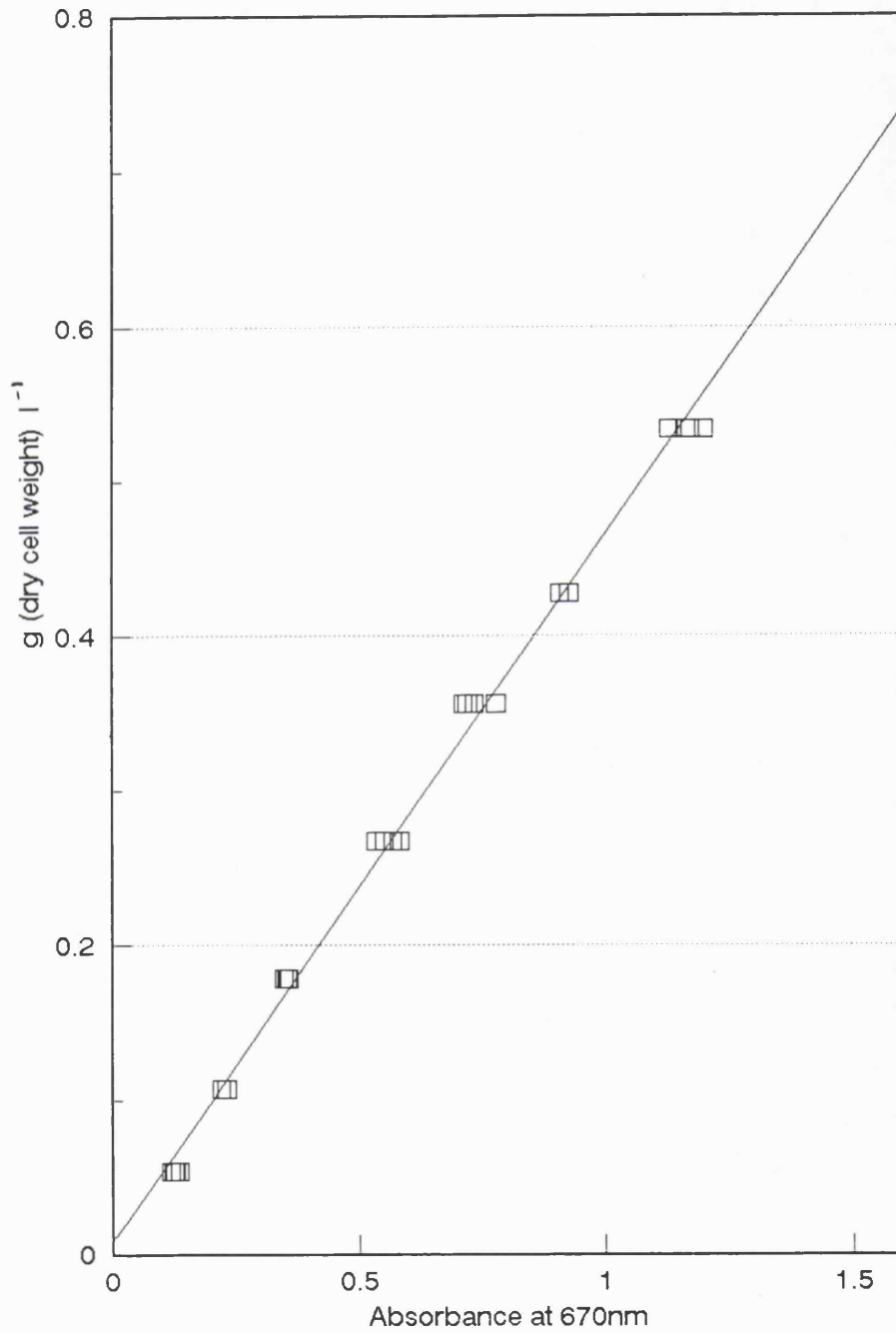
The total carbon content of the fermentation broth was measured by the Total Organic Carbon Analyser, Model TOC-5050, Shimidzu Corporation. The procedure was as described in the manual with standards at 100, 500 and 1000 ppm. Samples were diluted with water to a carbon content concentration below 1000ppm.

The samples taken from the fermentation were stored at -20°C before analysis.

#### 2.2.4 - Clark type oxygen electrode

A number of assays had been developed which were dependent upon the analysis of the bacterial culture OUR by using the Clark type oxygen electrode obtained from Rank Brothers.

Figure 2.1 - Standard curve of the  $A_{670}$  of the bacterial culture against dry cell weight.



A maximum of three electrodes was used at any one time. Solvents could damage the perspex oxygen electrode therefore the glass electrode was used for the solvent tolerance assay. The setting up of the oxygen electrode had been modified slightly from the operations manual. A 1 cm<sup>2</sup> piece of lens tissue (no hole in the centre) was placed on the platinum electrode and was then flooded with saturated KCl solution, covered with a 1 cm<sup>2</sup> piece of teflon membrane and sealed with an O ring. The incubation chamber was then clamped/screwed onto the baseplate making sure no air bubbles were present underneath the membrane. With the glass oxygen electrode another O ring was placed around the bottom of the incubation chamber portion of the electrode and was in contact with the baseplate to prevent loss of water by evaporation from the electrolyte. With both the glass and perspex electrodes they were set up the day before to allow equilibration and were stable for 2 to 3 days. Between each run the oxygen electrode was washed with water.

The temperature of the incubation chamber was maintained at 30°C by the use of a water bath (Grant Instruments Ltd) to pump water through the heating jacket. A 0.6 V polarising voltage was provided across the electrode and the reaction mixture in the incubation chamber was stirred by a magnetic stirrer (in house constructed apparatus - Electronic Workshop, Department Chemical Biochemical Engineering and the Electronic Workshop, Department Biochemistry).

The readings taken from the oxygen electrode were recorded on a potentiometric chart recorder (10mV full scale), chartspeed 10 mm min<sup>-1</sup>. The zero DOT calibration was obtained by sparging with nitrogen gas or complete exhaustion of oxygen in the reaction chamber by bacterial consumption. The data from the solvent tolerance assay (Section 2.3.4) was recorded by a BBC Microcomputer (Programme written by M.Vale, Electronic Workshop, Department Chemical Biochemical Engineering) and was then converted into a IBM compatible

textfile by the 'Kermit' programme.

Atkinson & Mavituna (1983) quoted a value of 1.16 mmol l<sup>-1</sup> for the aqueous saturated oxygen concentration at 30°C, this value was used in all OUR calculations.

#### 2.2.5 - Computer analysis

Fermentation data was transferred to an IBM compatible textfile which was then analysed on the Microsoft Excel spreadsheet (version 4). Error signals and negative values were deleted and the elapsed time was converted to hr after inoculation. It was also possible to calculate the total carbon dioxide evolved (TCER) and the total oxygen consumed (TOUR) using the spreadsheet and the equations:-

$$TCER_{t_1} = ((t_1 - t_0) \cdot (CER_{t_1} + CER_{t_0}) / 2) + TCER_{t_0}$$

$$TCER_{t_n} = ((t_n - t_{n-1}) \cdot (CER_{t_n} + CER_{t_{n-1}}) / 2) + TCER_{t_{n-1}}$$

TCER<sub>t<sub>0</sub></sub> is always set to zero. Total oxygen consumed was calculated in a similar manner. The files were then converted into a worksheet format so that the data could be imported into the 'Freelance', Lotus 1,2,3 graphics package.

The data from the solvent tolerance assay which had been converted into an IBM textfile was modified in the Microsoft Excel Spreadsheet (version 4). Times were converted into hr after chloroxylenol/solvent addition and the drop in DOT after its addition was also calculated. The data was then converted into a worksheet format and imported into the Microsoft 'Origin' programme so that the equation:-

$$(DOT_0 - DOT_t) = ((OUR_0 - OUR_f) / C) \cdot (1 - \exp^{-ct}) + (OUR_f \cdot t)$$

could be fitted to the data available. The data could also be imported into the 'Freelance', Lotus 1,2,3 graphics package.

## 2.3 - Methods

### 2.3.1 - Culturing methods

#### 2.3.1.1 - Storage and subculturing

Bacterial<sup>culture</sup> were subcultured every fortnight, a colony was inoculated into benzene minimal medium and cultured overnight at 30°C, 220 rpm in a New Brunswick orbital shaker. The bacterial culture was streaked onto nutrient agar plates and cultured overnight at 30°C before storing at 4°C.

#### 2.3.1.2 - Shake flask culture

The composition of the benzene and succinate minimal medium is given in Section 2.1.3.2.

In all experiments the culture was initially grown overnight in benzene minimal medium before 1 ml was transferred to either 50 or 100 ml of fresh medium. Samples were taken from benzene minimal medium without removing the subbaseal by using a sterile microlance and 'plastipak' syringes (Becton Dickinson) to sample through the subbaseal with its surface sterilised with ethanol. When larger volumes of bacterial culture were required (e.g. 400 ml) a larger inoculum (50 ml) grown on benzene minimal medium was added.

In the analysis of the toxicity of fluorobenzene upon growth of the bacteria in succinate minimal medium, the fluorobenzene was assumed to be sterile and added directly to the medium. It was allowed it to dissolve in the medium before the experiment was started.

In all experiments involving fluorocatechol the medium was prepared as normally with the fluorocatechol added by sterile filtration through a 2 µm 'Acrodisc' filter (Gelman Sciences).

#### 2.3.1.3 - Fermentation at 30°C and 20 % DOT

This was the main fermentation to which the others were compared. The composition of the medium is described in Section 2.1.3.3. The equipment are described in Section 2.2.1. pH was controlled at 6.8 by the addition of 2.5 M H<sub>2</sub>SO<sub>4</sub> and the temperature was at 30°C. DOT was controlled at 20 % aqueous saturation by automatic changes in stirrer speed and airflow rate, minimum stirrer speed was 250 rpm.

The inoculum development was as follows:-

- a) a single colony taken off a nutrient agar plate and transferred to benzene minimal medium and was grown overnight at 30°C, 220 rpm in a New Brunswick orbital shaker.
- b) 1 ml of the benzene grown bacterial culture was transferred to 50 ml of benzene minimal medium and grown for 7hr at 30°C and 220 rpm.
- c) fermenter was then inoculated with the 50 ml benzene grown culture.

#### 2.3.1.4 - Alternative fermentation conditions

The composition of the initial fermentation medium provided by Shell Research Ltd, Sittingbourne, Kent was similar to that described in Section 2.1.3.3. The difference was that the carbon source was glucose (11 gl<sup>-1</sup>) and fructose (11 gl<sup>-1</sup>) together instead of succinate (22 gl<sup>-1</sup>). The medium did not require neutralising with KOH. Glucose and fructose were sterilised separately from the rest of the medium. Stirrer speed was 600 rpm and the airflow rate was 4 l min<sup>-1</sup>. The inoculum volume was 200 ml and had been grown overnight. The development of this fermentation to the one described in Section 2.3.1.3 is described in Section 3.1.1 and Section 3.1.2.

The medium for the carbon limited fermentation was the same as for the fermentation described in Section 2.3.1.3 but

with the succinate (neutralised with KOH) added at a rate of  $1.53 \text{ g hr}^{-1}$  and not batched in at the start. The inoculation regime was the same as described in Section 2.3.1.3.

In some of the fermentations they had become iron limited therefore additional iron sulphate was added by filter sterilising through a  $2 \mu\text{m}$  'Acrodisc' (Gelman Sciences).

In all other fermentations the medium was identical to that described in Section 2.1.3.3 and the inoculum development the same as described in Section 2.3.1.3. *P.syringae* which has a similar optimum growth temperature to *P.putida* had been shown to have a growth rate of 67 % of optimum at  $33.5^\circ\text{C}$  and 27 % of optimum at  $34.9^\circ\text{C}$  (Young et al, 1977). It was decided to grow the bacteria at  $34^\circ\text{C}$  which would give a growth rate of approximately 50 % of optimum. The lower temperature was  $26^\circ\text{C}$  (i.e.  $4^\circ\text{C}$  lower than  $30^\circ\text{C}$  instead of  $4^\circ\text{C}$  higher) and did not require further adjustments in the temperature control to obtain a better cooling of the fermenter. Selection of 5 % DOT was based on lowering the oxygen concentration so that the bacterial growth was affected but allowed a margin for error involved with the automatic DOT control to prevent it reaching zero. The reasons and the methods for culturing the bacteria at zero DOT and low  $K_L a$  are given in Section 3.1.4.2 and Section 3.1.4.3.

### 2.3.2 - Cell harvesting and preparation

The bacterial <sup>culture</sup> required washing to remove the media surrounding the <sub>cells</sub> so that the endogenous OUR was as low as possible which made the aromatic oxidation activity assay easier to perform.

The bacteria were harvested from either fermentation samples or from shake flask cultures. There were two



centrifugation stages to give a washed cell suspension in 50 mM phosphate buffer pH 6.8 lower than 17  $\text{gl}^{-1}$  but <sup>which</sup> was generally around 10  $\text{gl}^{-1}$ . Cells harvested from shake flask cultures were initially <sup>centrifuged</sup> at 6000 rpm, 4°C for 30 mins in the MSE 18 Highspeed centrifuge. The pellet surface was washed with phosphate buffer and then resuspended in buffer. The second centrifugation stage was in a MSE Micro Centaur at high speed for 3 mins. The pellets surface were washed with phosphate buffer, <sup>pellets were</sup> then resuspended in buffer.

The first washing stage for the bacteria from the fermentation samples was carried out in the MSE Micro Centaur at high speed for 3 mins since this was more rapid and the fermentation broth had a higher cell concentration. The second stage was as described above for the shake flask culture.

In all cases the samples and the resuspended and washed cells were kept on ice as much as possible to maintain aromatic oxidation activity.

### 2.3.3 - Aromatic oxidation activity

The development of this assay is described in Section 3.2.1. The setting up of the Clark type oxygen electrode is described in Section 2.2.4 and the harvesting and washing stage is described in Section 2.3.2. The total volume which was the initial volume of 50 mM phosphate buffer pH 6.8 and the cell suspension added once the assay had started was 3 ml. Before each assay run the electrode would be allowed to reach saturation which was indicated by a steady reading on the chart recorder.

The cell concentration in the oxygen electrode was dependent on the endogenous OUR but was always below 0.5  $\text{gl}^{-1}$  and would give an activity around 2 % DOT  $\text{min}^{-1}$ . If two oxygen electrodes were available the samples were analysed at

two different cell concentrations. The cell suspension would be added to the incubation chamber using a Hamilton syringe and allowed to reach a steady OUR which would take 10 to 15 mins. 1  $\mu$ l of fluorobenzene was then added with a Hamilton syringe to the chamber making sure the droplet had left the end of the syringe and dispersed throughout the chamber. The OUR was allowed to reach linearity before the run was stopped.

The aromatic oxidation activity was calculated by subtracting the initial endogenous OUR from the final OUR after addition of fluorobenzene.

#### 2.3.4 - Solvent tolerance assay

Chloroxylenol (3, chloro 4,5 dimethyl phenol) was selected as the toxic hydrophobic compound to determine variations in cell susceptibility; the development of this assay is described in Section 3.3.1. Chloroxylenol toxicity was determined by the rate of decrease of OUR which was measured in a Clark type oxygen electrode. The setting up of the apparatus was described in Section 2.2.4, cell harvesting and washing was described in Section 2.3.2 and the computer analysis of the data was described in Section 2.2.5.

Total volume in the oxygen electrode was 3 ml. The 50 mM phosphate buffer pH 6.8 in the incubation chamber was allowed to saturate (indicated by a steady reading on the chart recorder) before cell suspension and 10  $\mu$ l of ethanol was added. The cell concentration in the oxygen electrode would be dependent on the specific OUR of the bacteria but was below 0.5  $\text{gl}^{-1}$  and would ideally give a OUR between 5 and 10 % DOT  $\text{min}^{-1}$ . Once the DOT had reached 60 % aqueous saturation and the OUR had reached linearity, 10  $\mu$ l of 20  $\text{gl}^{-1}$  of chloroxylenol in ethanol was added to the chamber. The run was allowed to continue until the OUR had reached a steady value or the DOT had reached zero.

A number of solvents had been analysed to determine whether the equation describing the loss of OUR due to chloroxylenol toxicity could also be fitted to other compounds. The assay was performed as described above but with solvents added in ethanol and not chloroxylenol. Since most of the solvents had densities lower than one they could not be added as a second phase since they would float to the top of the chamber and remain there unmixed. After completion of the assay the incubation chamber was initially washed with ethanol to remove residual solvent before being washed with water.

The effect of  $MgSO_4$  and  $K_2EDTA$  upon the solvent tolerance of the bacteria had also been analysed. These compounds were added to the electrode before each experiment was started.  $K_2EDTA$  was selected rather than EDTA since it had higher solubility.

#### 2.3.5 - Fluorocatechol inhibition of OUR and aromatic oxidation activity

These experiments were performed in the Clark type oxygen electrode, the setting up of which is described in Section 2.2.4.

The inhibition of endogenous OUR was followed by adding the cell suspension to the oxygen electrode and allowing a steady OUR value to be reached before the fluorocatechol in 50 mM phosphate buffer pH 6.8 was added. The assay was stopped once the OUR had again reached a steady value.

In the analysis of the effect of fluorocatechol upon aromatic oxidation activity, the fluorocatechol was added to the incubation chamber before the experiment was started. The assay was then run the same way as described in Section 2.3.3.

#### 2.3.6 - Plasmid stability

The bacteria were initially grown overnight in benzene minimal medium at 30°C and 220 rpm in a New Brunswick orbital shaker before 1 ml was inoculated into succinate minimal medium. The  $A_{670}$  of the inoculum and the culture after it had been inoculated was measured. Bacteria were repeatedly subcultured into succinate minimal medium until the cells had lost the benzene dioxygenase activity. Bacteria containing the plasmid and the ones not containing the plasmid were differentiated by plating on indole agar. The bacteria with benzene dioxygenase activity produced the blue pigment indigo which turned the yellow colonies green.

### 3 - RESULTS

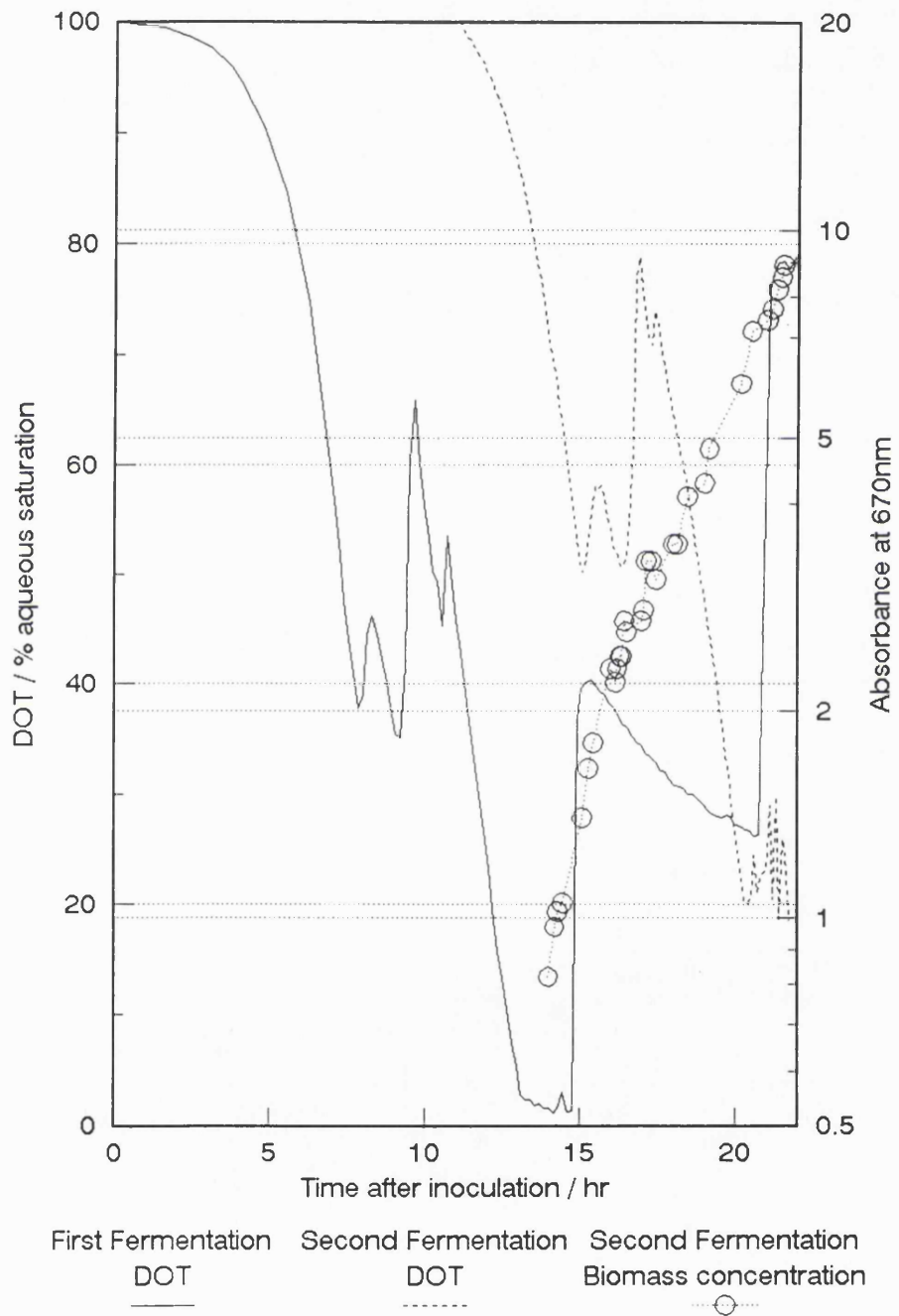
#### 3.1 - Fermentation

The fermentation conditions provided by Shell Research Ltd were described in Section 2.3.1.3 and 2.3.1.4 with a 50% glucose and 50% fructose mix as the carbon source and the DOT not controlled. The stirrer speed and airflow rate were constant at 600 rpm and 4 l min<sup>-1</sup>. The first fermentation is shown in Figure 3.1. The profile was complex indicating that a number of byproducts were produced which were later metabolised. In subsequent fermentations the inoculum volume to the fermenter was reduced from 200 to 25 ml so that the fermentation could proceed overnight allowing samples to be taken at the point where the metabolism seemed to be varying dramatically. The cells did not become oxygen limited, fermentation time had lengthened considerably. The biomass profile for the fermentation with 25 ml inoculum indicated a change in the specific growth rates of the bacteria 17 hr after inoculation at which time the DOT rose. It is not clear from the profile whether all the changes seen in the DOT profile were reflected in the biomass profile. It was decided that the metabolism was complex and difficult to interpret and therefore an alternative carbon source was selected so that a simple profile could be obtained.

##### 3.1.1 - Carbon source selection

The selection of the carbon source was based on the production of cells with high aromatic oxidation activity. Ten carbon sources had been analysed (Schofield et al, 1987) to determine their effect upon the aromatic oxidation activity and yield of cells. Both fructose and succinate gave high aromatic oxidation activity and good yield of cells as compared to the other eight. Succinate was initially selected since fructose in combination with glucose had given a complex profile. The yield of biomass upon succinate was similar to the yield on glucose (Zamanian, 1989), succinate

Figure 3.1 - DOT and biomass profile of the fermentations with cells grown on glucose / fructose as carbon source.



was therefore added at  $22 \text{ g l}^{-1}$ , the same concentration as glucose and fructose together in the previous fermentations. The pH was moved to neutrality by addition of potassium hydroxide.

The DOT profile obtained from the growth of the bacteria on succinate is shown in Figure 3.2. The rapid growth resulted in the DOT falling below 10 % aqueous saturation but even so the profile was much simpler than with the previous fermentations on glucose/fructose. In subsequent fermentations the  $K_L a$  was increased with the stirrer speed increased from 600 to 750 rpm and the airflow rate from 1.0 to  $2.5 \text{ l min}^{-1}$  to remove oxygen limitation. The fermentation finished earlier because at no point was growth oxygen limited.

Succinate was therefore selected as the carbon source and the effects of inoculum and oxygen transfer were then analysed.

### 3.1.2 - Fermentation development

A simple fermentation profile had therefore been obtained for cells grown upon succinate as <sup>the</sup> carbon source with high oxygen transfer rate to prevent oxygen limited growth. The effect of changing the inoculum was examined (Figure 3.3) using three different inoculation regimes. In all four fermentations the cells were not oxygen limited. One had high but constant  $K_L a$  with DOT kept above 40 % aqueous saturation, where as the remaining three were controlled to 20 % DOT. Maintaining a strict inoculation regime resulted in a similar profile of the OUR as demonstrated by fermentations controlled at 20% DOT with 50ml inoculum which had been cultured for 7 hr. Doubling the inoculum resulted in a faster growth of the bacterium which was undesirable for the following two reasons:-

Figure 3.2 - DOT profile of two fermentations with succinate as carbon source at different  $K_La$  values.

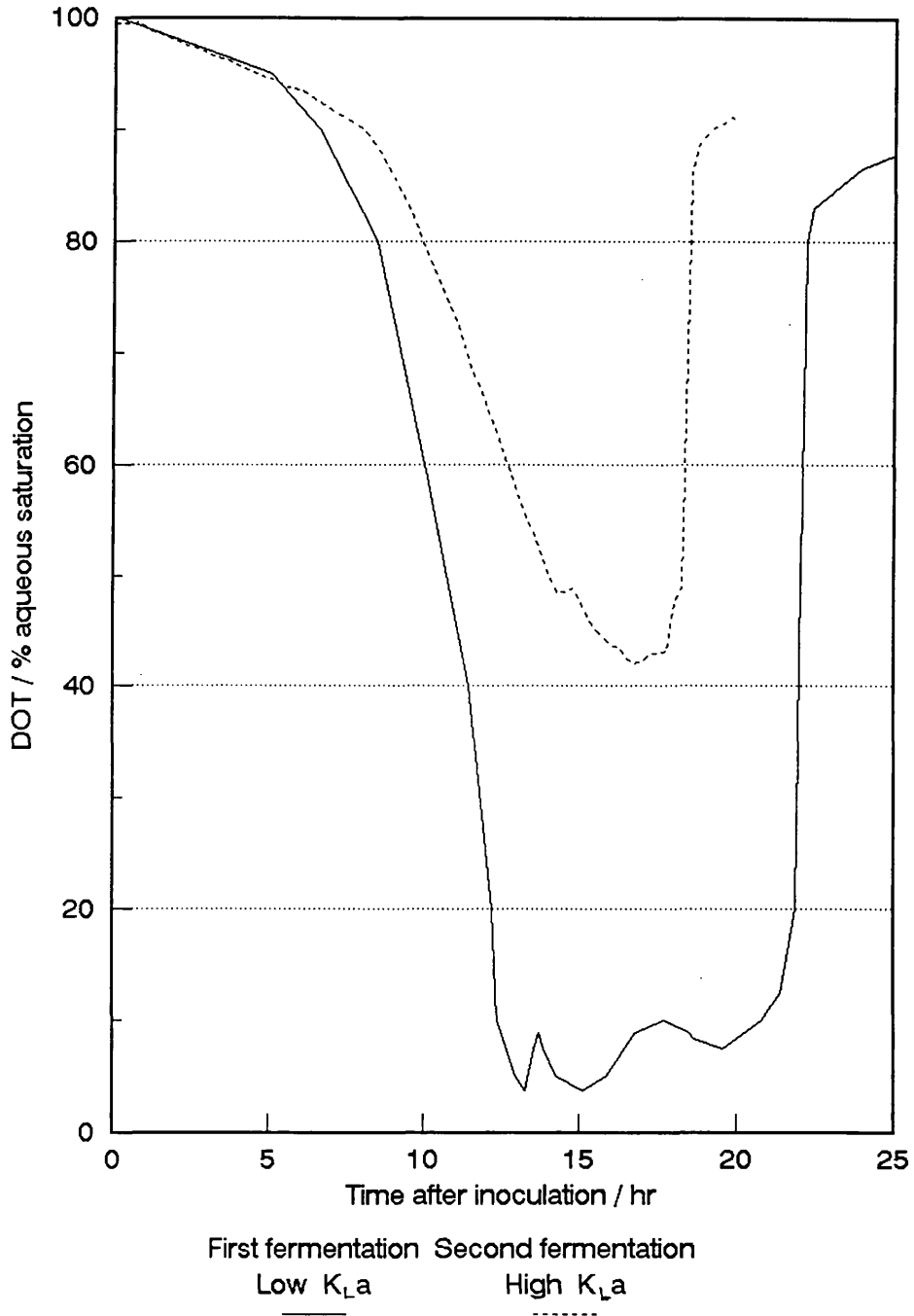
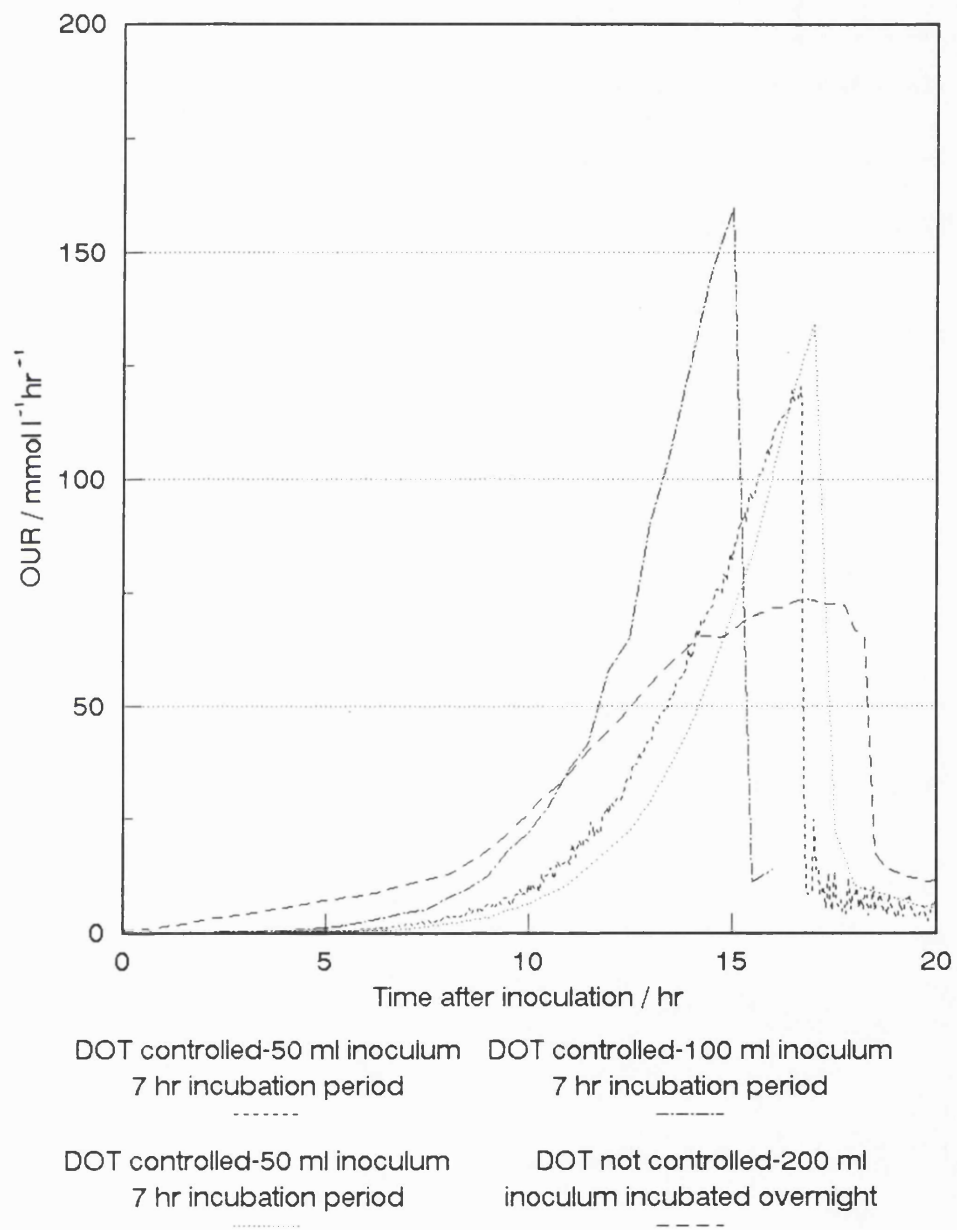




Figure 3.3 - The effect of changes in the inoculum upon the growth of *P.putida* ML2.



- a) The fermenter would initially have to be inoculated much later at night which would make it impracticable.
- b) The growth stage was actually shorter allowing less time for sample analysis.

However, it was recognised that a reduction in the inoculum volume would increase the possibility of contamination since the cell concentration would be at a lower value for a longer time. The time for culturing the inoculum prior to inoculation of the fermenter was maintained at 7 hr to make sure all the cells were in the growth phase.

The control of DOT was considered to be essential since changes in the growth conditions (e.g. growth temperature) could lead to a change in the OUR profile which would then result in variations to the DOT profile. It has been shown previously by Matyashova (1976) that when the cells go from high to low DOT levels the specific growth rate of the cells decrease. Good growth was observed when the DOT was controlled at 20 % aqueous saturation, a value chosen to be above the growth-limiting value. This DOT was selected as the standard value for subsequent experiments.

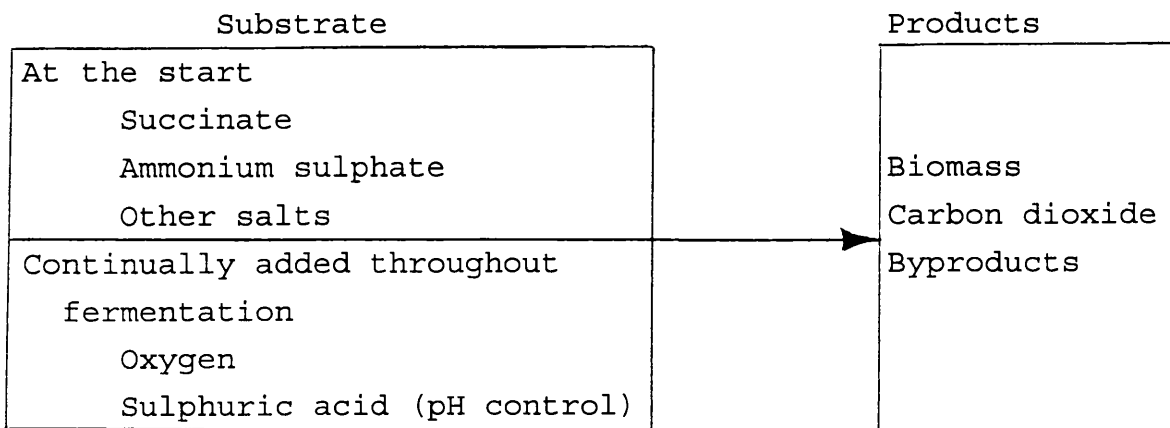
In the fermentations at a controlled DOT level it would be possible to distinguish the effect of oxygen concentration upon the cell growth and metabolism from the effect of other changes in the growth conditions.

3.1.3 - Characterisation of the fermentation controlled at 20% DOT and 30°C.

In this work the effect of growth conditions upon the aromatic oxidation activity and solvent tolerance of P.putida ML2 was analysed. The development of the fermentation which was controlled at 20% DOT with succinate as carbon source was described in Section 3.1.1. and 3.1.2. The fermentation conditions, medium composition and inoculum development are described in Section 2.3.1.3. These fermentation conditions were used as a standard against which other fermentations were compared. This fermentation was analysed in detail so that suitable growth parameters (e.g. growth rate) could be compared to other fermentations. It was essential that the reproducibility of fermentations was established so that the significance of any changes observed under different fermentation conditions could be determined.

A change in fermentation condition will result in changes in growth parameters e.g. growth rate. Changes in aromatic oxidation activity or solvent tolerance could be a result of the change of metabolism and independent of the change in growth conditions. Changes in aromatic oxidation activity and solvent tolerance should therefore be compared to the growth parameters as well as the changes in growth conditions.

During the fermentations a flow of chemicals will occur to produce biomass as shown below:-



The following five parameters were chosen for monitoring the fermentation:-

- Biomass concentration
- Carbon dioxide evolved
- Oxygen consumed
- Sulphuric acid input
- Succinate concentration

Biomass accumulation and succinate concentration were monitored off-line, the other three were monitored on-line.

The most sensitive indicator of changes in metabolic and growth rates is DOT. However, as indicated earlier, DOT was controlled at a constant value throughout the fermentations to ensure that it was not the cause of any changes observed.

Measurement of exit gas analysis to determine oxygen uptake rates (OUR) and carbon dioxide evolution rates (CER) is also a powerful on-line techniques and was considered first.

#### 3.1.3.1 - Exit gas analysis

Exit gas analysis, because it could be monitored online, allowed monitoring throughout the fermentation. Off-line data were restricted to the number of samples taken; sampling usually occurred once an hour over a twelve hour period. Accurate values for growth parameters can be difficult to obtain from offline data due to the limited amount of data available. Online data can be taken throughout the fermentation where as in these fermentations samples were only taken when the cell concentration was high enough to analyse the aromatic oxidation activity and solvent tolerance of the culture.. It was also possible to estimate the biomass concentration at the start of the fermentation from the OUR and CER values.

The reproducibility was examined by carrying out three fermentations under identical conditions. The OUR values (Figure 3.4), CER and RQ values (Figure 3.5) were almost identical. The initial RQ values for the fermentations were unsteady due to the low values for CER and OUR. At the end of the growth phase both the CER and OUR values fell towards zero very suddenly indicating that after the cells had completely utilised the succinate there was no further metabolism of any products which might have been formed during the growth phase.

The profile of RQ against time indicated three portions to the growth phase which were:-

- 1) from 3 to 7 hr after inoculation a RQ value below one was obtained.
- 2) an increase in RQ is seen from 7 hr to 12 hr after inoculation.
- 3) a fairly steady RQ value of approximately 1.4 from 12 hr after inoculation to end of growth phase.

This strongly suggest that the metabolism was changing dramatically with the specific oxygen uptake rate or the specific carbon dioxide evolution rate changing during the growth phase. The initial lower RQ values suggest that the organic carbon was being fixed rather than metabolised for energy. The other fermentation data were analysed to determine reasons for these changes in RQ values.

Variations in the RQ value during the growth phase were observed and therefore the CER and OUR values were analysed in more detail. These were plotted on a logarithmic scale to determine which activities were increasing exponentially as would be expected with cells growing under non-limiting growth conditions. OUR and CER values from the Fermentation 2 were plotted on a logarithmic scale in Figure 3.6. The OUR values up to 3 hr after inoculation were unstable resulting in unstable values for RQ. The OUR increased exponentially

Figure 3.4 - OUR profiles of three fermentations controlled at 30°C with DOT controlled at 20 % aqueous saturation.

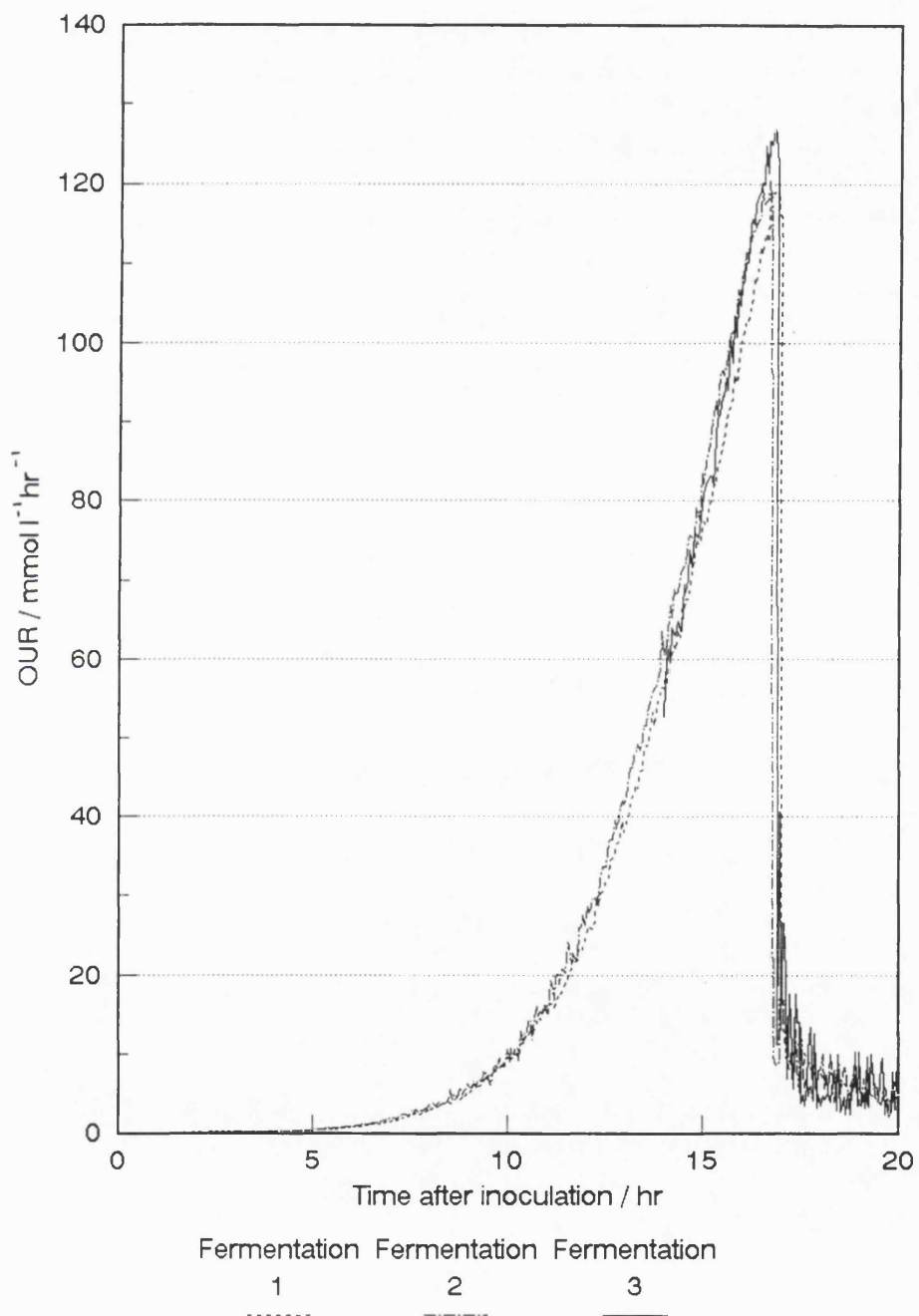
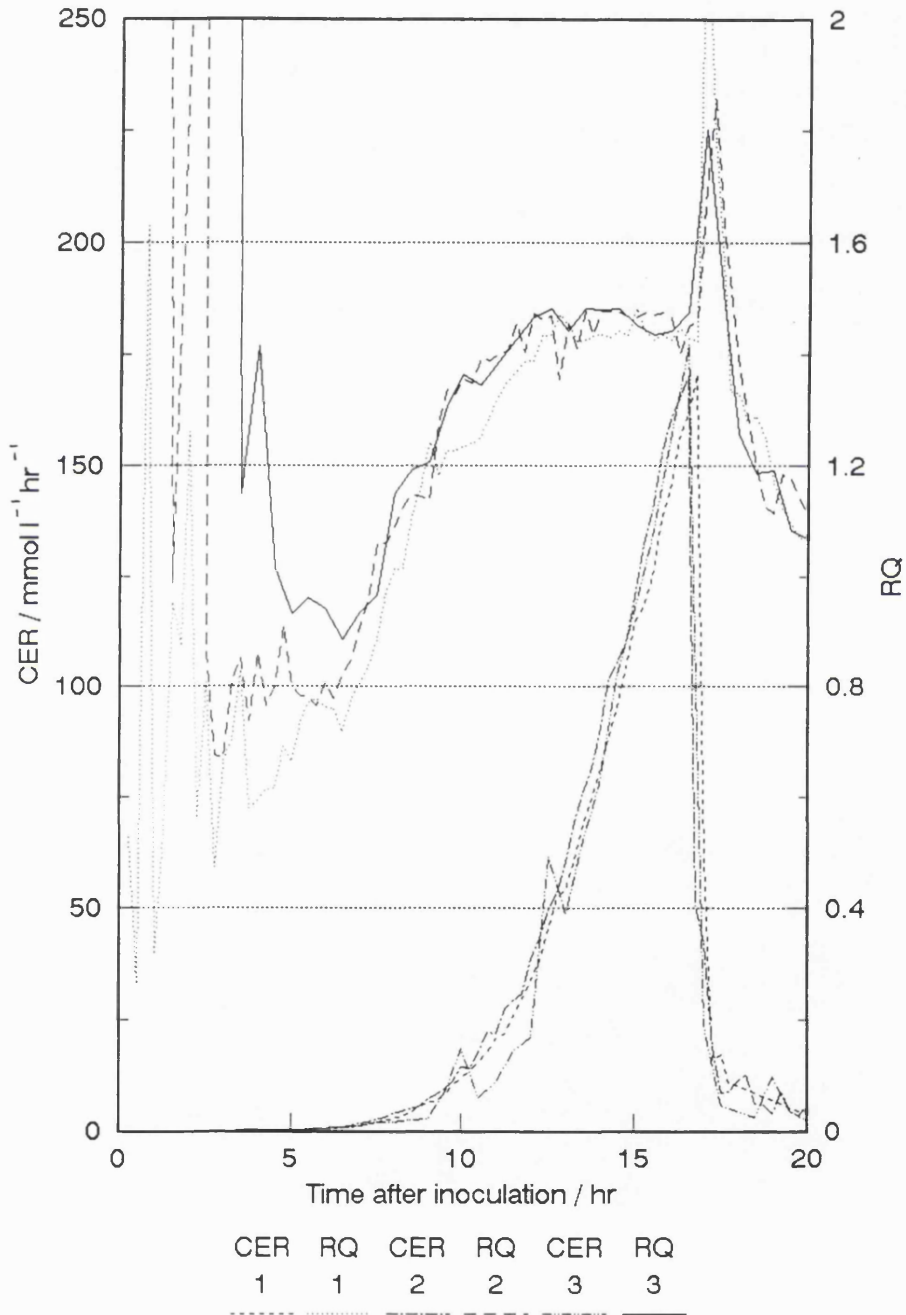


Figure 3.5 - CER and RQ profiles of three fermentations controlled at 30°C with DOT controlled at 20% aqueous saturation.



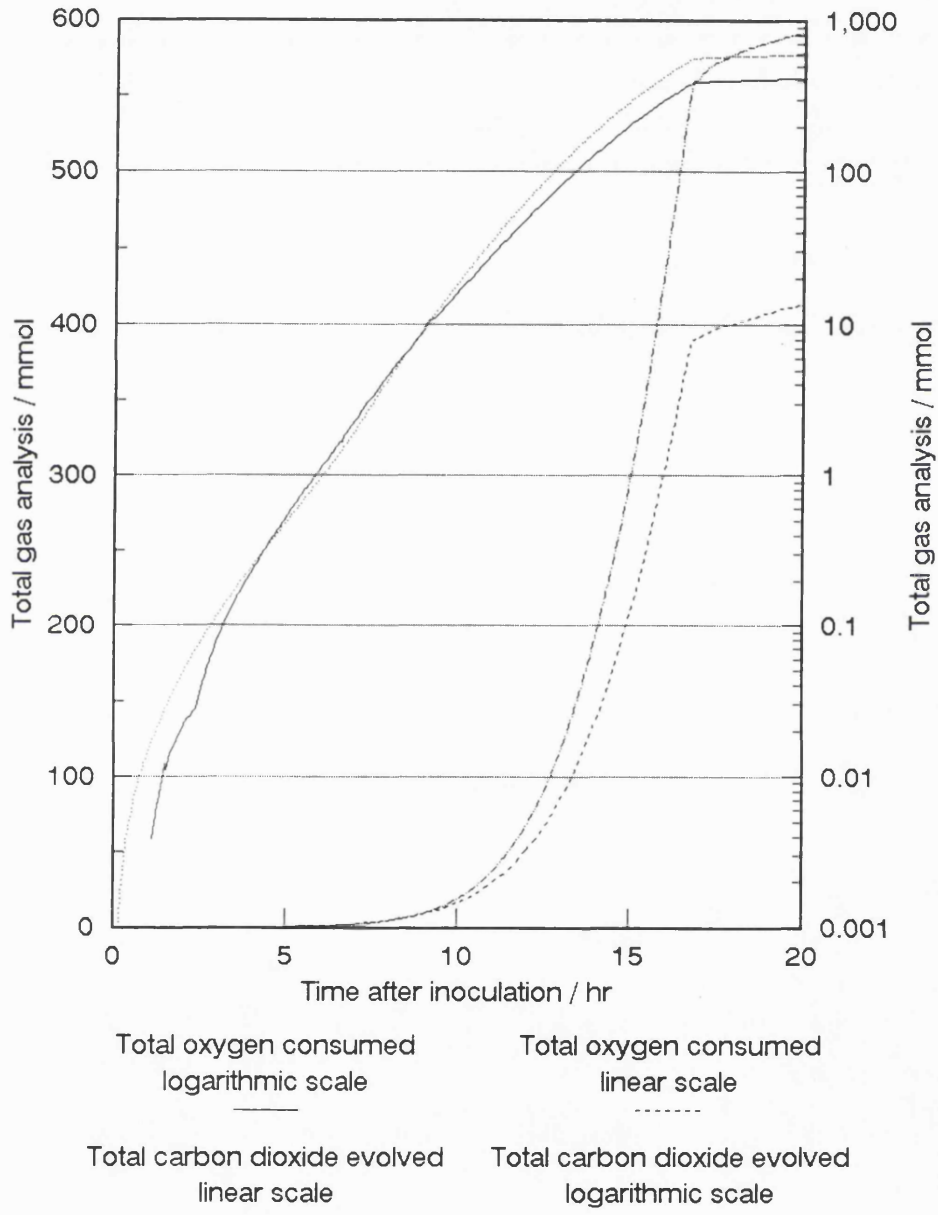




from 3 to 13 hr after inoculation but 8 hr after inoculation the exponential rate of increase of OUR decreased from  $0.70 \text{ hr}^{-1}$  to  $0.52 \text{ hr}^{-1}$ . The CER values increased exponentially ( $0.72 \text{ hr}^{-1}$ ) up to 10 hr after inoculation and were also stable below 3 hr. The CER and OUR values did not increase exponentially at the end of the growth phase. The data were analysed to see if they rose linearly at this point in the fermentation but this was not observed. The stable RQ value suggests that the carbon metabolism was not changing dramatically and therefore another metabolite could be limiting growth.

The OUR and CER values of Fermentation 2 were totalised so that the quantity of carbon evolved as carbon dioxide and the quantity of oxygen consumed were obtained. Total oxygen consumption has been shown to be directly related to the biomass concentration achieved in the fermenter (Buckland et al, 1985). In this work the pH was controlled, it was therefore possible to totalise the CER values. Figure 3.7 gives the totalised values for the gases, in the first 12 hours only approximately 15% of the total organic carbon was evolved. The changes in the RQ values from 7 to 12 hr after inoculation were therefore unlikely to be due to carbon limitation. The three fermentations were compared for total carbon evolved from the fermenter, 17 hr after inoculation. The fermentations were similar with 6.66, 6.70 and 6.70  $\text{gl}^{-1}$  of carbon evolved as carbon dioxide. The totalised values of Fermentation 2 are also shown on a logarithmic scale, exponential increase in total carbon dioxide evolved is seen from 4 to 11 hrs after inoculation. The variations in the RQ values and the changes seen in the exponential increase in CER and OUR could be due to the accumulation of byproducts which would then be metabolised at the end of the growth phase. The changes in RQ were observed 11 hr after inoculation, at this stage only approximately 10 % of the total carbon dioxide has been evolved. The conversion of all the succinate to a byproduct is therefore unlikely.

Figure 3.7 - Totalised values for carbon dioxide evolved and oxygen consumed for the fermentation controlled at 30°C with DOT at 20% aqueous saturation. Data expressed on both a linear and logarithmic scale.



### 3.1.3.2 - Biomass

The results in the previous section suggested that inhibition of growth had occurred since both the oxygen uptake and the rate of carbon evolution did not increase exponentially at the end of the fermentation. Maximum biomass concentration was reached after a longer time in Fermentation 1 although the OUR and CER profiles did not reflect this. Logarithmic plots of the biomass data in Figure 3.8 show that the growth of the bacteria was exponential to the end of the growth phase. Growth rates are similar at 0.40, 0.41 and 0.41  $\text{hr}^{-1}$ . Thus the changes in the OUR and CER profiles were not reflected in the biomass data. This demonstrates the value of exit gas analysis to detect metabolic changes. Specific OUR and specific CER of Fermentation 2 decreased linearly by more than 30% in the last 3 hr of the growth phase.

Figure 3.9 shows that there was not a linear relationship for the three fermentations between the yield of biomass, the total oxygen consumed, and total carbon evolved. Extrapolation of a curve through the data points would intersect the cell concentration axis at an  $A_{670}$  of 2. The  $A_{670}$  of the culture at the start of the fermentation (i.e. before 1 hr after inoculation) was below 0.1. The curve was therefore expected to go through the  $A_{670}$  axis near this value. The curve appears to have a sigmoidal shape.

### 3.1.3.3 - Acid control

The cells metabolised succinate as the carbon source and this resulted in an increase in the alkalinity of the culture medium. The pH was controlled by the addition of sulphuric acid. The metabolism of succinate could therefore be indirectly followed by analysing the addition of sulphuric acid to the fermenter.

The acid input to the fermenter is plotted in Figure

Figure 3.8 - Logarithmic plot of the biomass data to observe exponential increase in cell concentration.

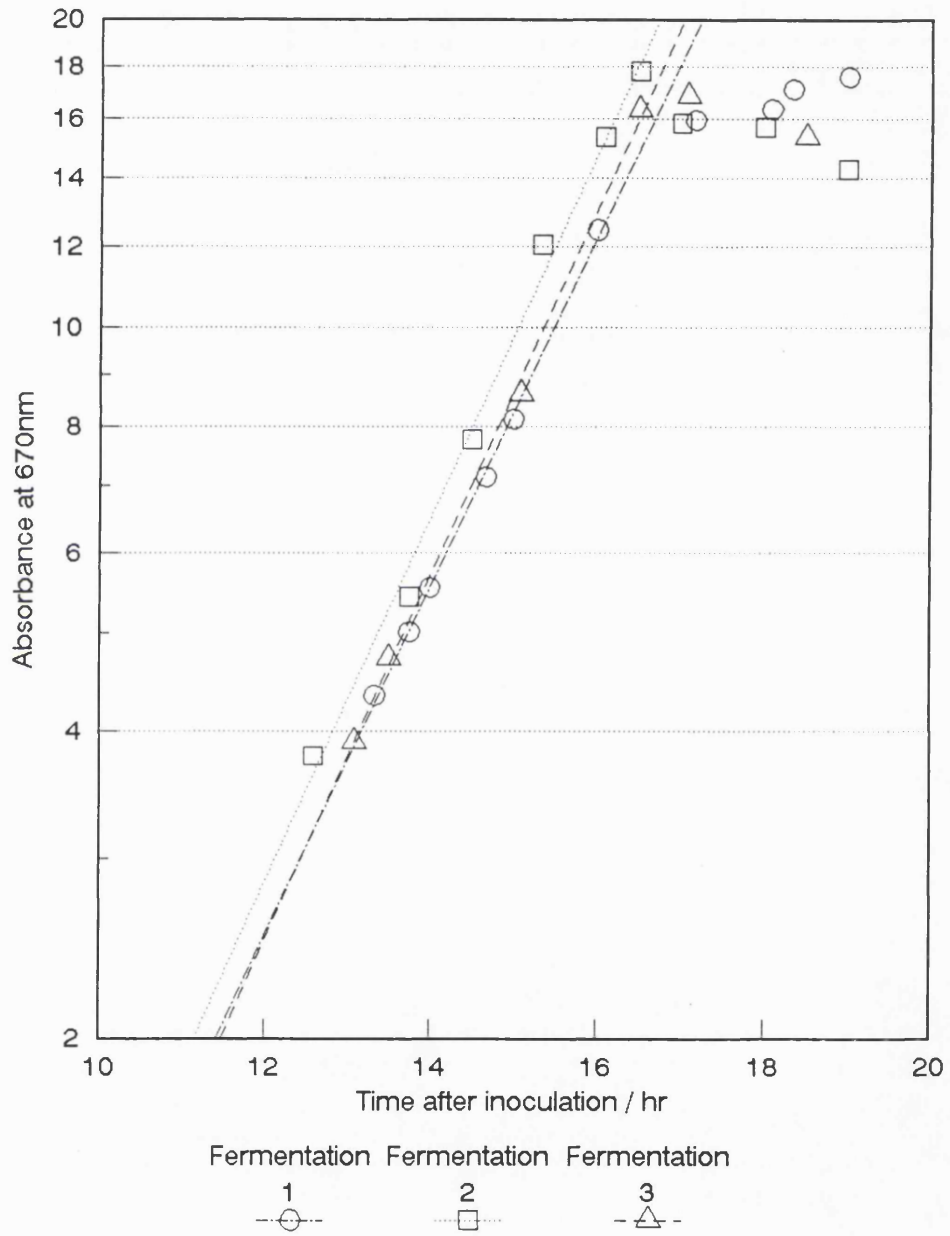


Figure 3.9 - Relationship between the total carbon evolved as carbon dioxide, total oxygen consumed and the cell concentration of the fermentation controlled at 30°C and 20 % DOT.

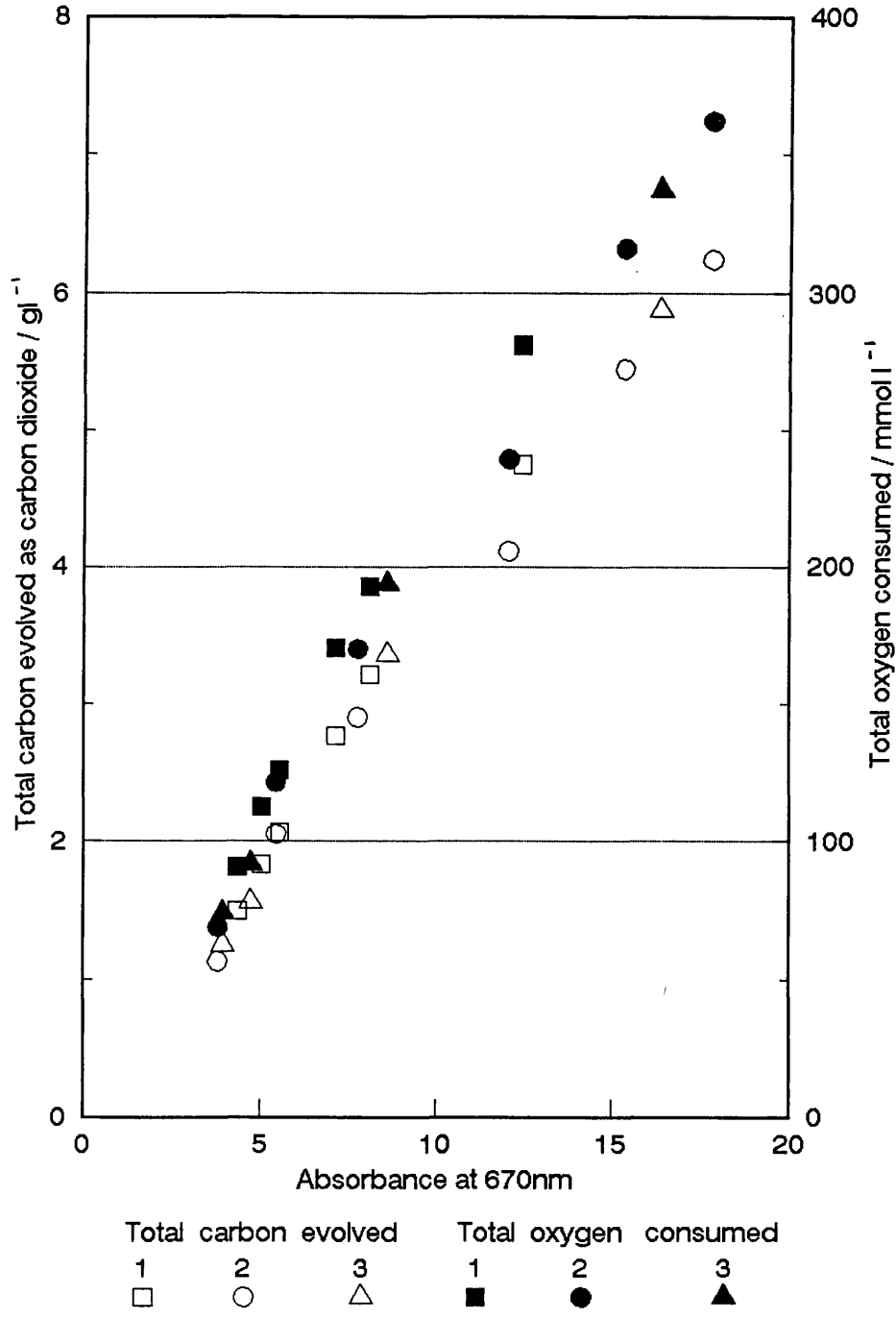
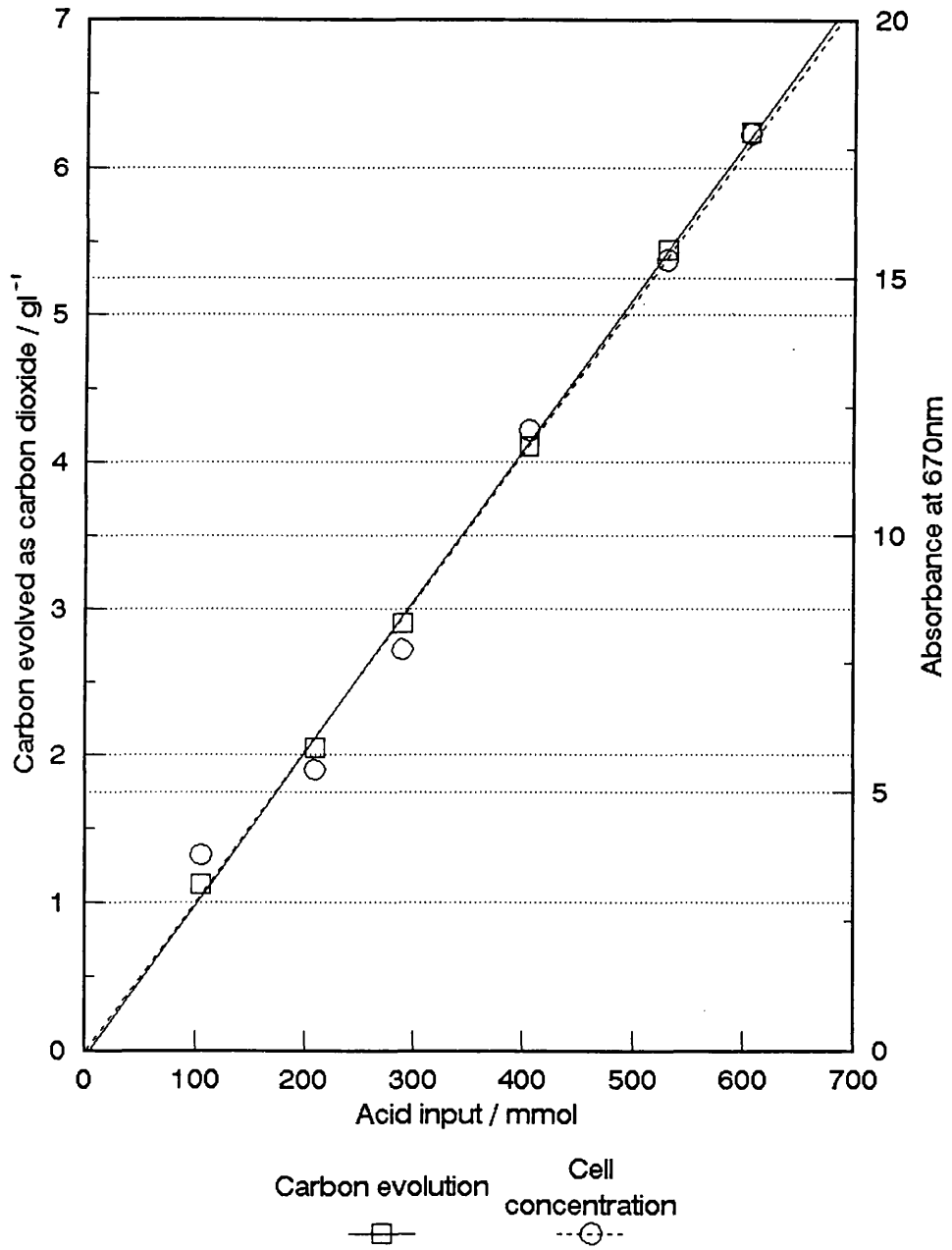


Figure 3.10 - Relationship between the cell concentration and carbon evolved from the fermenter as carbon dioxide to the acid added to control the pH.



3.10 against organic carbon evolved and the absorbance at 670nm of the cell culture. A linear fit was obtained for both; a closer fit was obtained for the carbon out data. This could be due to the carbon data being more accurate or that it was a closer measure of carbon metabolism than the cell concentration. This again leads to the idea that the carbon metabolism is more complex than was first envisaged and cannot be directly related to biomass concentration. For every mole added to the fermenter, 51.7 g of carbon was evolved as carbon dioxide and 13.33 g of biomass was produced.

The readings for acid input were prone to errors since the markings on the acid reservoir were every 5 ml which was equivalent to 12.5 mmol. The good correlations obtained, however indicate the usefulness of monitoring acid addition. The analysis of acid input was improved in the later fermentations by using a loadcell which measured the weight of the acid reservoir and the data was obtained online.

#### 3.1.3.4 - Carbon data analysis

Fermentation 2 was carbon balanced so that the uptake of succinate could be analysed.

Cell concentration (dry cell weight) was calculated from the  $A_{670}$  values, carbon content was obtained by assuming 50% of dry cell weight as carbon. Total carbon out of the fermenter as carbon dioxide was calculated by totaling the CER values. Carbon content of the fermentation culture and supernatant was measured using the total organic carbon analyser as described in Section 2.2.3. All samples had been stored at -20°C prior to analysis. Total carbon balance was obtained by adding the carbon content of the fermentation broth to the total carbon out of the fermenter as carbon dioxide.

Figure 3.11 shows the carbon data profiles from the fermentation. The CER and OUR profiles in Section 3.1.3.1 had indicated that the cells had metabolised all of the succinate at the end of the growth phase. The profiles for cell concentration and fermentation culture come together in Figure 3.11 which also indicate that the carbon source was exhausted and that the standard curve of  $A_{670}$  against dry cell weight (Figure 2.1) is also correct for the samples at the end of the fermentation. The total carbon balance did not remain at  $8.95 \text{ gl}^{-1}$  which is the carbon content of the culture medium but increased with time. This indicated an error with the measurements. The error in the measurements could either be due to the total carbon analysis of the fermentation culture or the calculation of the total carbon evolved as carbon dioxide. The error is unlikely to be due to the total carbon analysis of the fermentation culture for the following reasons:-

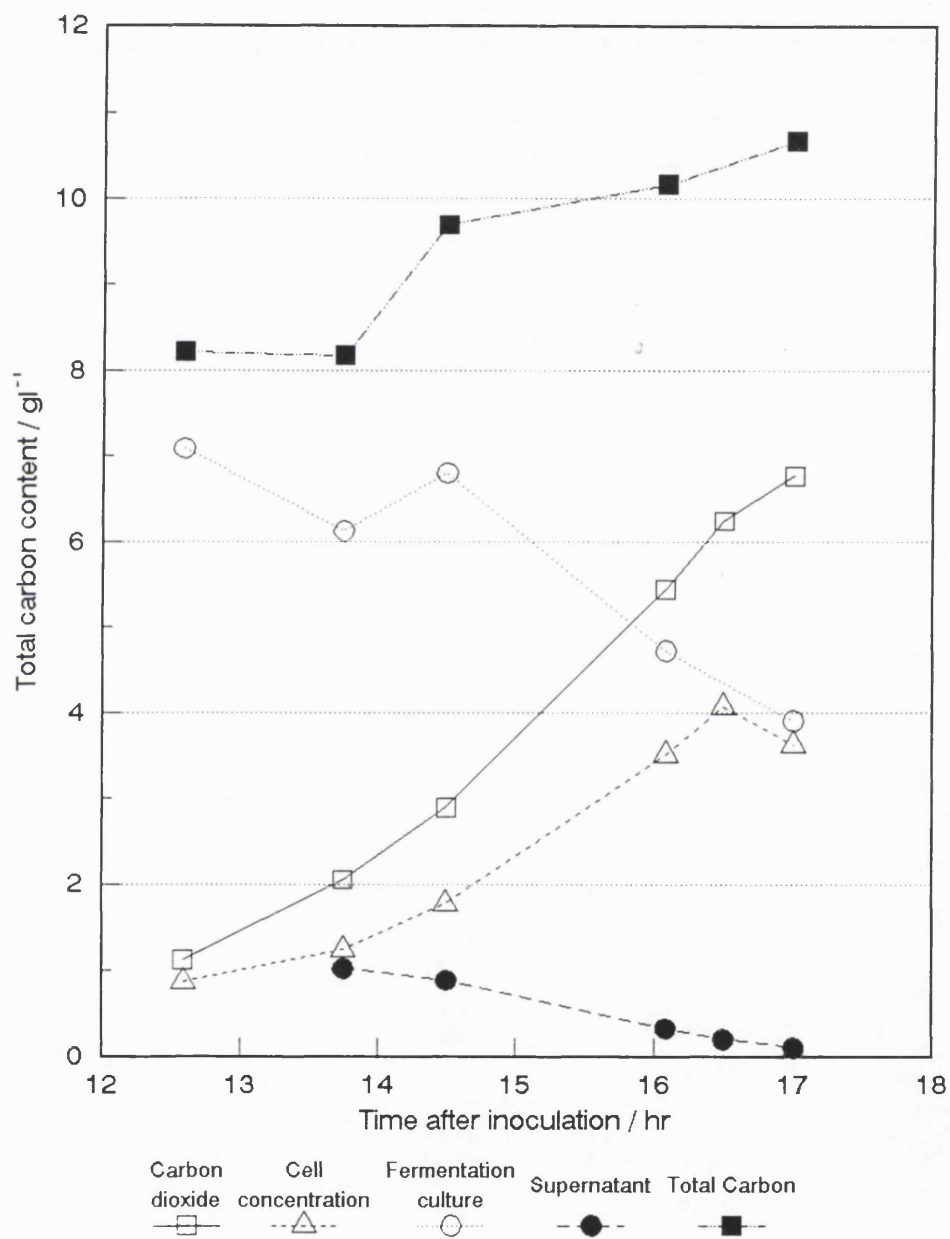
- a) the error at the start of the fermentation is small in comparison to the error at the end. The carbon content of the fermentation culture at the start is greater than at the finish. In general the error on a measurement is likely to be proportional to the measured amount and not inversely proportional.
- b) the calculated carbon content of the cells alone obtained from the  $A_{670}$  value of the sample at the end of the growth phase was equal to the carbon content of the fermentation culture.
- c) the error with carbon evolved data would be cumulative in that an error at the start would be counted in all the data-points. The error observed does increase with time.

The error due to the measurement of the total carbon evolved as carbon dioxide could either be the CER readings or/and the calculation of the total carbon dioxide evolved.

The CER readings are dependent on the volume of the



Figure 3.11 - Carbon balance and profile of the fermentation controlled at 30°C and 20% DOT.



culture but in these fermentations changes in volume were not taken into consideration when calculating the total carbon data. The volume of the fermentation culture will change in three ways:-

- a) by the addition of acid to control pH.
- b) a reduction in volume due to culture sampling
- c) loss of water in the exit gas due to evaporation.

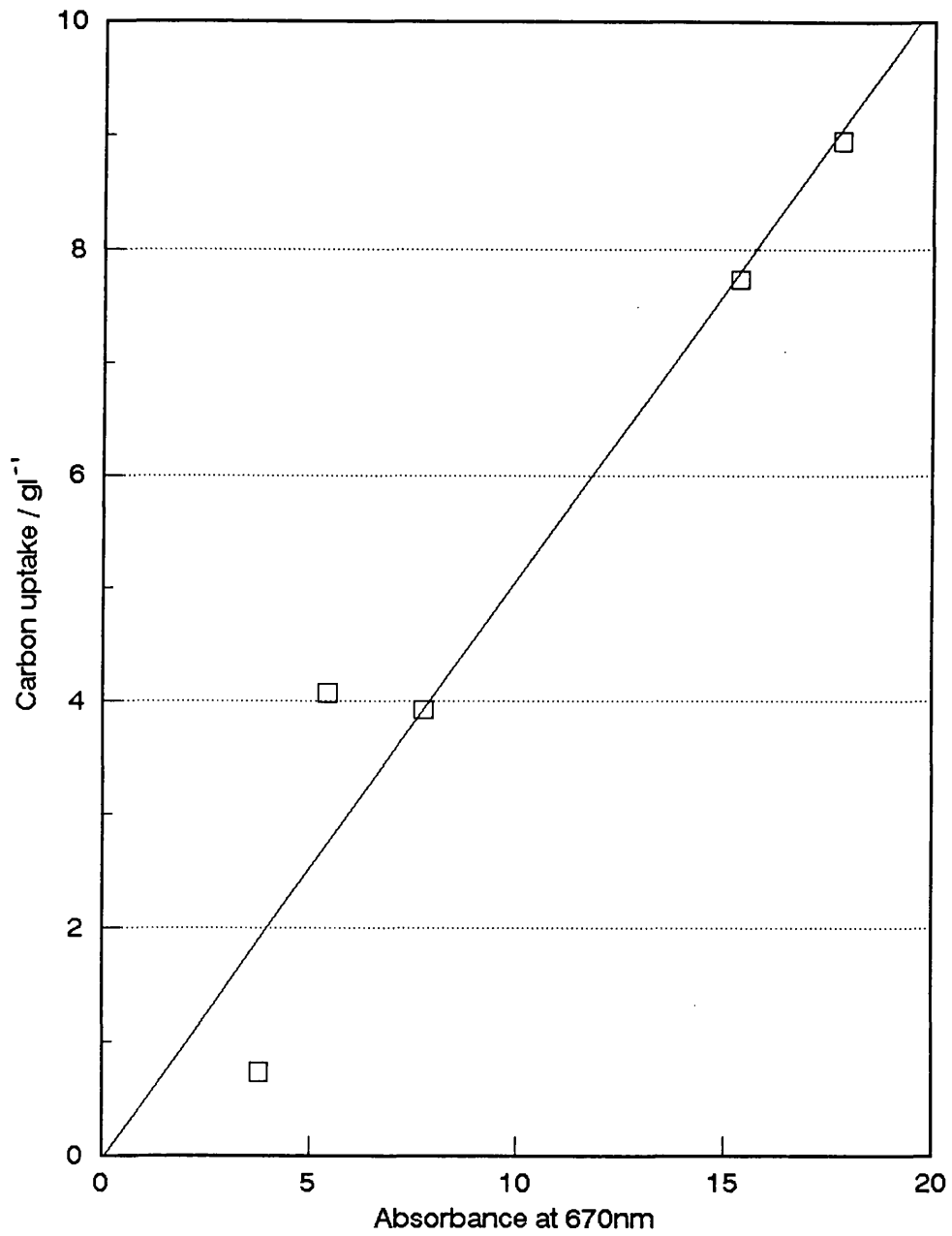
In this fermentation 242 ml of acid was added, 6 samples were taken of total volume of approximately 240 ml (each sample approximately 40 ml = 25 ml sample volume + 15 ml initially discarded so that the culture in the sample tube was not analysed). The loss of water due to evaporation is likely to be low. Initially the airflow rate was low which would allow a longer contact time between exit gas and condenser. At the end of the fermentation which was during the daytime, the humidity of the inlet gas was likely to be high since the steam supplies in the fermenter room were operational. The difference in humidity of the inlet and outlet gases was likely to be low. Large volume changes are unlikely to occur.

The error could also be due to the calculation of the total carbon evolved. The CER values were obtained every 3 min and values obtained after totalising were based on the CER increasing linearly between each point where as in reality a curve would be obtained between these point. The calculated carbon evolved as carbon dioxide at the end of the growth phase from 16.40 to 16.45 hr after inoculation was 102 mg l<sup>-1</sup>. Only 1.6 mg l<sup>-1</sup> was evolved due to the increase in the CER value from 168 to 173 mmol l<sup>-1</sup> hr<sup>-1</sup> (16.40 to 16.45 hr after inoculation), this is only 1.6 % of the total amount and therefore the actual error between the curve and straight line joining the data points will be much lower. The error on the totalised CER values could be due to the calibration of the airflow rate into the fermenter or the calibration of the mass spectrophotometer.

The calculated supernatant carbon content obtained by subtracting cell concentration from the fermentation broth was significantly larger than the experimental values. Precipitate was seen in the defrosted supernatant samples which may account for this discrepancy. The carbon concentration of the supernatant tends towards zero which indicates that the carbon is exhausted at the end of the growth phase.

The carbon uptake by the cells was calculated by subtracting the calculated supernatant concentration from  $8.95 \text{ g l}^{-1}$ , the carbon concentration of the medium. Figure 3.12 relates the cell concentration to the carbon uptake by the cells. Maximum cell concentration had been achieved at the end of the growth phase where carbon concentration was assumed to be zero. A linear fit was obtained and cell yield was calculated to be  $0.90 \text{ g dry cell mass (g carbon source)}^{-1}$ . This is equivalent to  $0.36 \text{ g dry cell mass (g succinate)}^{-1}$ .

Figure 3.12 - Relationship between cell concentration and concentration of carbon consumed.



### 3.1.4 - Fermentation under different DOT controlled conditions

Section 3.2.2.1 <sup>demonstrate</sup> that bacteria grown in a shake flask maintained <sup>high aromatic oxidation</sup> activity for 7 days after harvesting <sup>due to oxygen</sup> possibly <sup>limited</sup> growth since cells grown at 20 % DOT in the fermenter lost activity within hours of harvesting. The bacteria were therefore grown at different oxygen concentrations to determine its effect upon the aromatic oxidation activity.

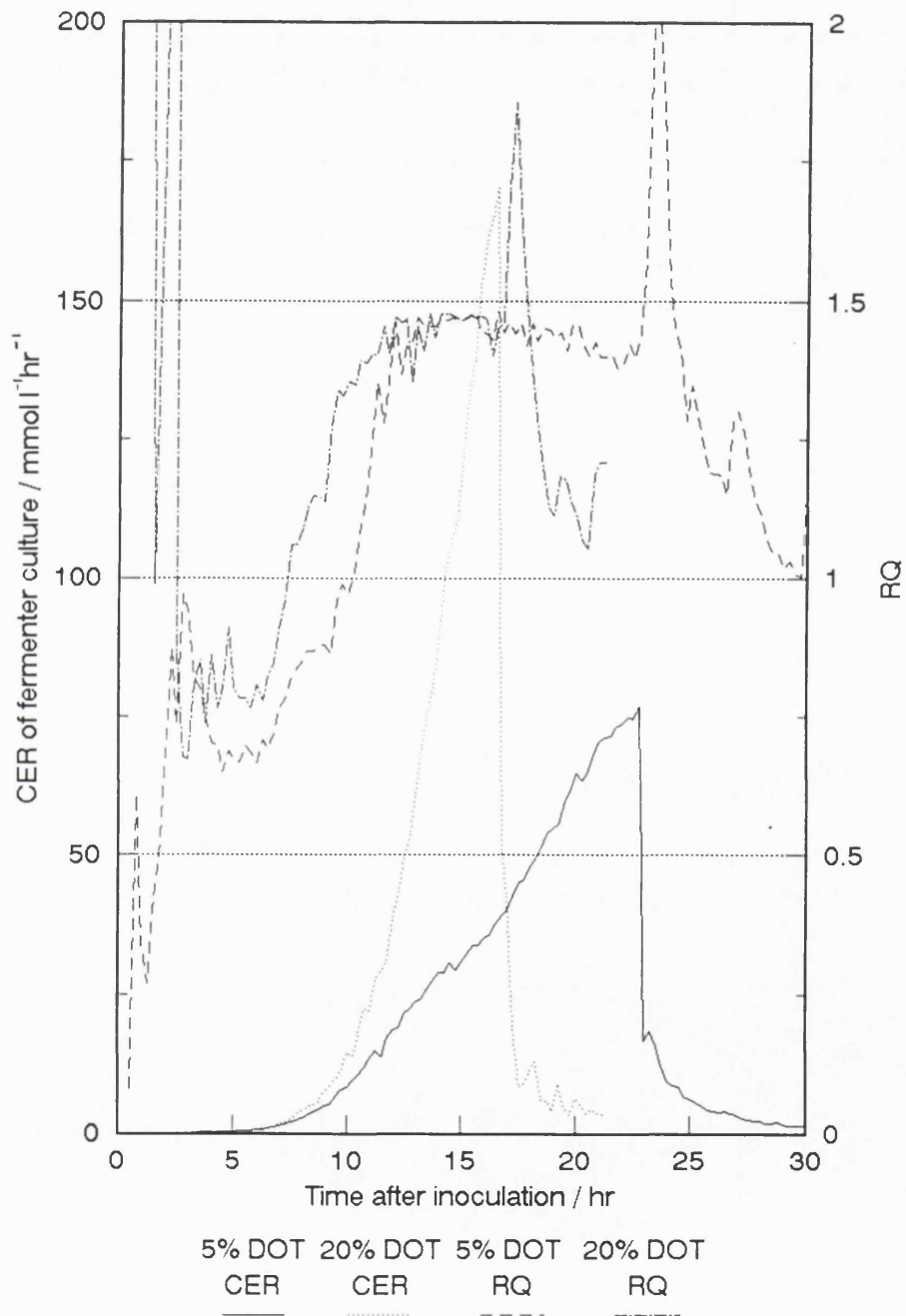
The fermentations were characterised so that changes in the growth could be detected. Changes observed in the aromatic oxidation activity could then be correlated to changes in the growth physiology.

#### 3.1.4.1 - Fermentation controlled at 5% DOT

The cells were initially grown at the lower oxygen concentration of 5% DOT. This oxygen concentration would allow a margin of error for the automatic control of the DOT so preventing it reaching zero.

The CER and RQ profile of the cells grown at 5% DOT was compared to the cells grown at 20% DOT. Figure 3.13 shows that the maximum CER value reached was 78 mmol l<sup>-1</sup> hr<sup>-1</sup> as compared to 176 mmol l<sup>-1</sup> hr<sup>-1</sup> for the cells grown at 20% DOT. The fermentation finished 22.8 hr after inoculation rather than 17 hr. The RQ values were compared to determine if obvious variations in metabolism could be detected. The RQ values are similar at 1.4 at the end of the growth phase. Major variations in the carbon metabolism are not indicated. Biomass concentration had reached a maximum A<sub>670</sub> value of 15.6 which was equivalent to 7.13 gl<sup>-1</sup>, the yield of cells upon succinate was therefore 0.324 g dry cell (g succinate)<sup>-1</sup>. This absorbance value at 670nm was similar to that obtained, (between 15 and 17), for cells grown at 20 % DOT. When CER and biomass profiles were plotted on a logarithmic scale

Figure 3.13 - CER and RQ profiles of the fermentations at 30°C and DOT controlled at 20 and 5% aqueous saturation.



(Figure 3.14), exponential increases in biomass and CER were detected throughout the growth phase. Both the exponential rate of increase of CER and biomass decrease midway through the growth phase clearly indicating that there were two phases to the growth of the bacterium. The growth rate of the bacteria decreased by 40%, 15 hr after inoculation and the exponential rate of increase of the CER decreased by 81%, 11 hr after inoculation. There are definite differences between the growth of the bacteria and the carbon metabolism.

The cell concentration, total carbon dioxide evolved from the fermenter and the acid added to control pH were compared to determine any correlations. A linear fit was obtained when the total carbon evolved from the fermenter as carbon dioxide was related to the cell concentration as seen in Figure 3.15a. The line curves from the origin to a linear fit with 1.089 g dry cell weight produced per g of carbon evolved. Two linear fits were obtained when the total carbon evolved from the fermenter as carbon dioxide was compared to the acid input to control pH as shown in Figure 3.15b. This would indicate two phases to the growth of the bacterium with 46.5 and 52.1 g carbon evolved per mole of acid added. Cell concentration was compared to acid input and a single linear fit was obtained with 10.7  $\text{gl}^{-1}$  of cells produced per mole of acid added.

A carbon balance was formed to determine the carbon consumption by the cells. This was calculated in the same way as for the fermentation at 20 % DOT and 30°C (Section 3.1.3.4). Figure 3.16 indicates the flow of carbon from the medium to biomass and carbon dioxide. The calculated value for carbon remaining in the supernatant and the carbon consumed are also shown. Similarities were observed between these curves and the ones obtained for the fermentation at 20 % DOT and 30°C (Figure 3.11). These include the carbon content of the fermentation culture at the end of the growth phase is similar to the calculated value for cell

Figure 3.14 - Logarithmic profile of the CER and biomass data of the fermentation controlled at 30°C and 5 % DOT.

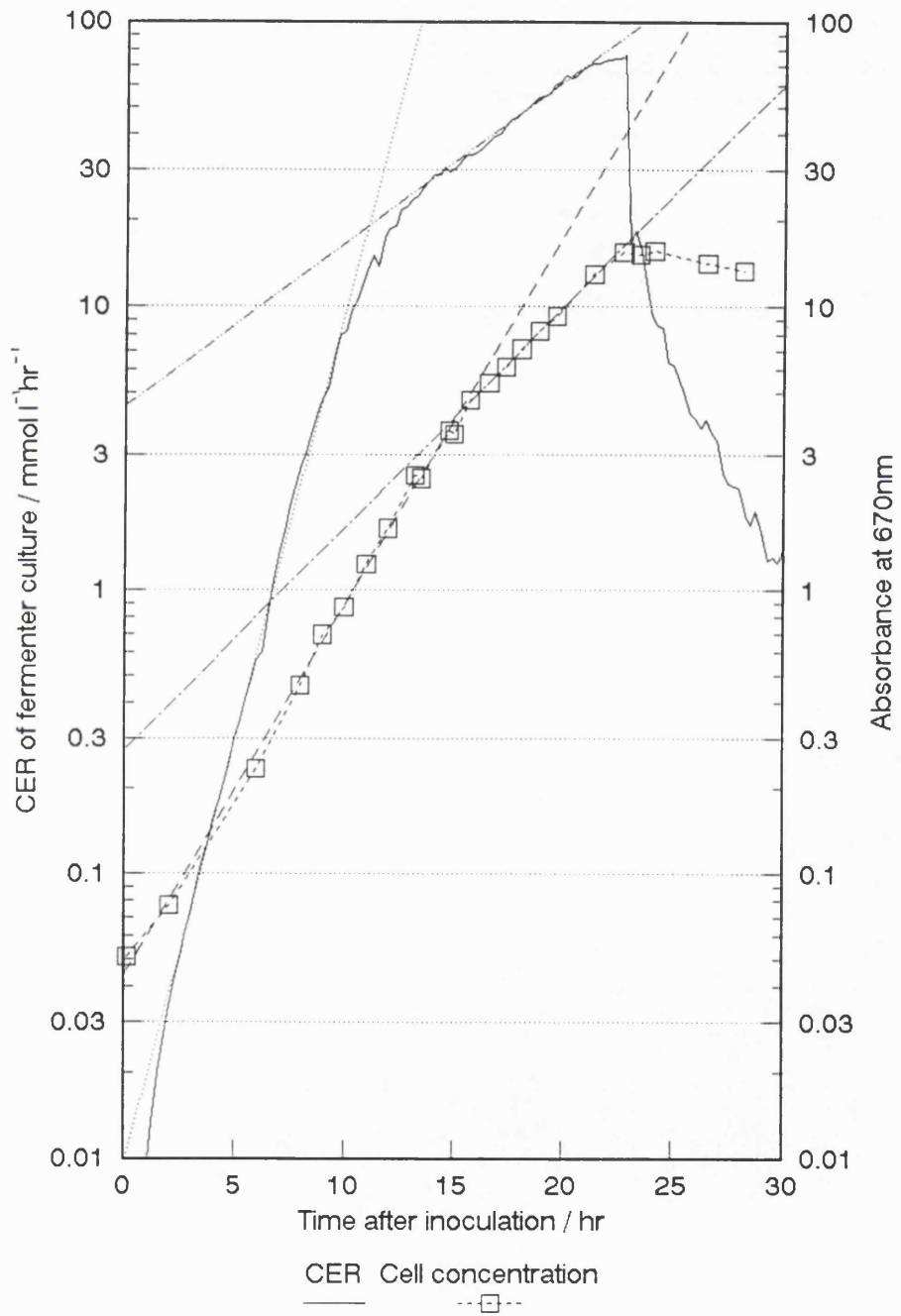
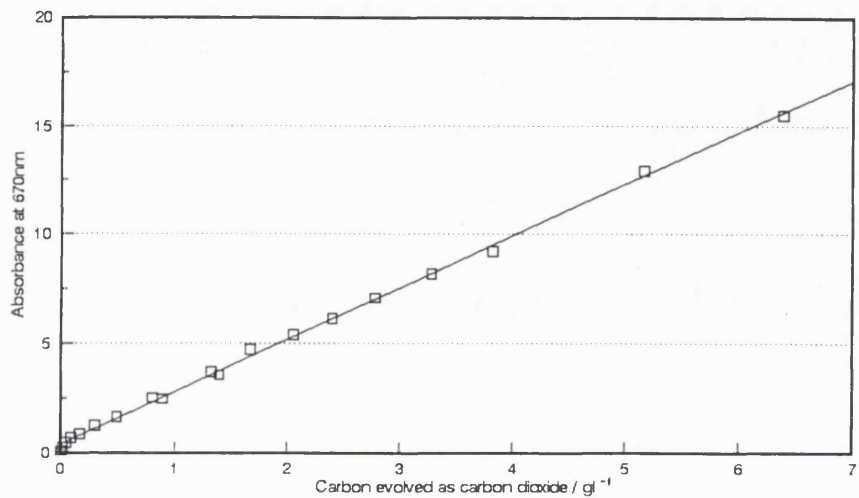


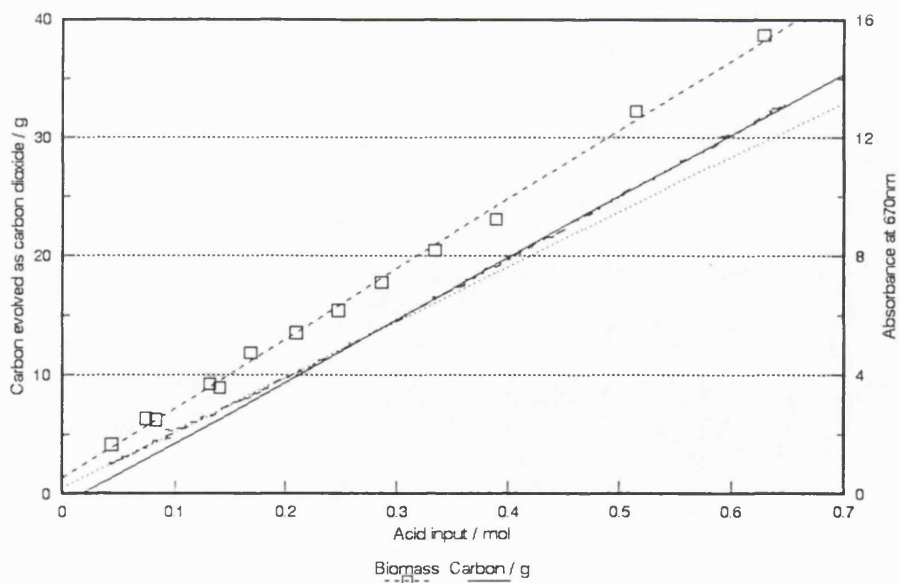


Figure 3.15 - Correlation between biomass concentration, carbon dioxide evolved and acid input to control pH.

a) Correlation between biomass and carbon dioxide evolved.

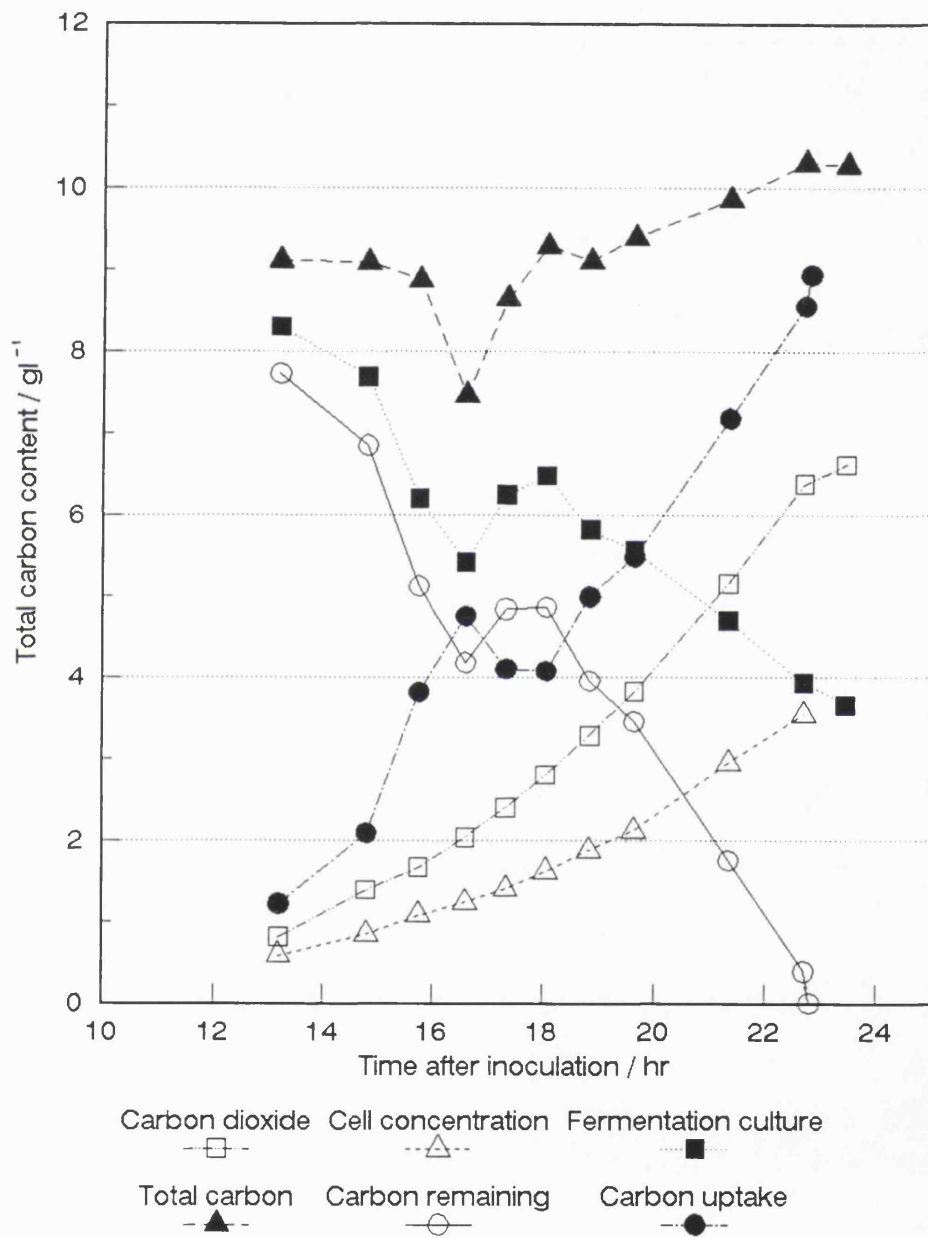


b) Correlation between the acid input to control pH and the biomass concentration and total carbon evolved from the fermenter.



12 - 23 hr after inoculation

Figure 3.16 - Carbon balance of the fermentation controlled at 30°C and 5 % DOT. The calculated carbon content of the supernatant and the carbon uptake by the cells are also shown.



concentration measured by its absorbance at 670nm. The total carbon content increases with time indicating that the calculated value of the carbon out as carbon dioxide was greater than the actual value. A number of samples between 15 hr and 18 hr do not fit the profiles for carbon remaining and taken up which is due to the low readings obtained from the total organic carbon analyser. Figure 3.17 demonstrate a linear relationship between the carbon uptake and cell concentration if the three samples obtained between 15 to 18 hr after inoculation were ignored. The yield of cells was 0.33 g dry cell weight (g succinate)<sup>-1</sup>.

The resuspended and washed cells were analysed for endogenous OUR and OUR due to ethanol consumption. The profiles of these activities are shown in Figure 3.18. Endogenous OUR increased during the growth phase where as the ethanol activity decreased.

#### 3.1.4.2 - Fermentation with transition of DOT from 20 to 0 % aqueous saturation

High aromatic oxidation activity and stability were achieved with cells grown in a shake flask. It was thought that this was due to oxygen limitation since a linear growth curve had been observed. A fermentation was set up to look at the effect of 0 DOT upon the aromatic oxidation activity of the cells. A high biomass concentration was initially achieved by growing at 20 % DOT and then the DOT was brought down to zero to increase the aromatic oxidation activity.

The CER profile obtained had indicated a slower growth than the fermentations described in Section 3.1.3. This was due to a lower inoculum concentration and therefore the CER profile is shown with a 3 hr lag time subtracted and is compared to the fermentation controlled at 20 % DOT (Figure 3.19). The DOT was reduced 18.67 hr after inoculation, a

Figure 3.17 - Correlation between total carbon uptake and cell concentration.

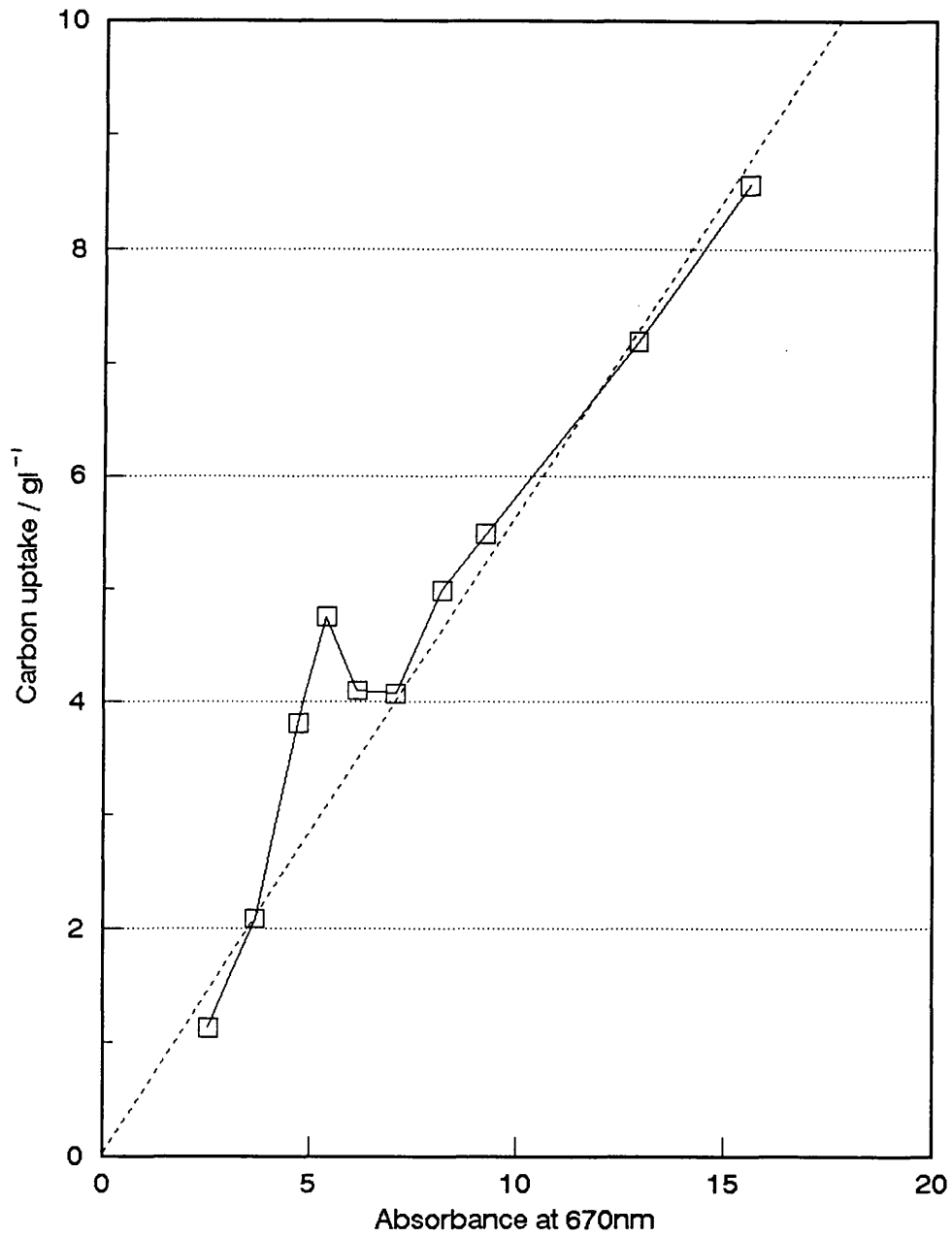


Figure 3.18 - Endogenous OUR, OUR due to ethanol consumption of the resuspended and washed samples of the fermentation at 30°C and 5 % DOT with the fermentation CER profile shown.

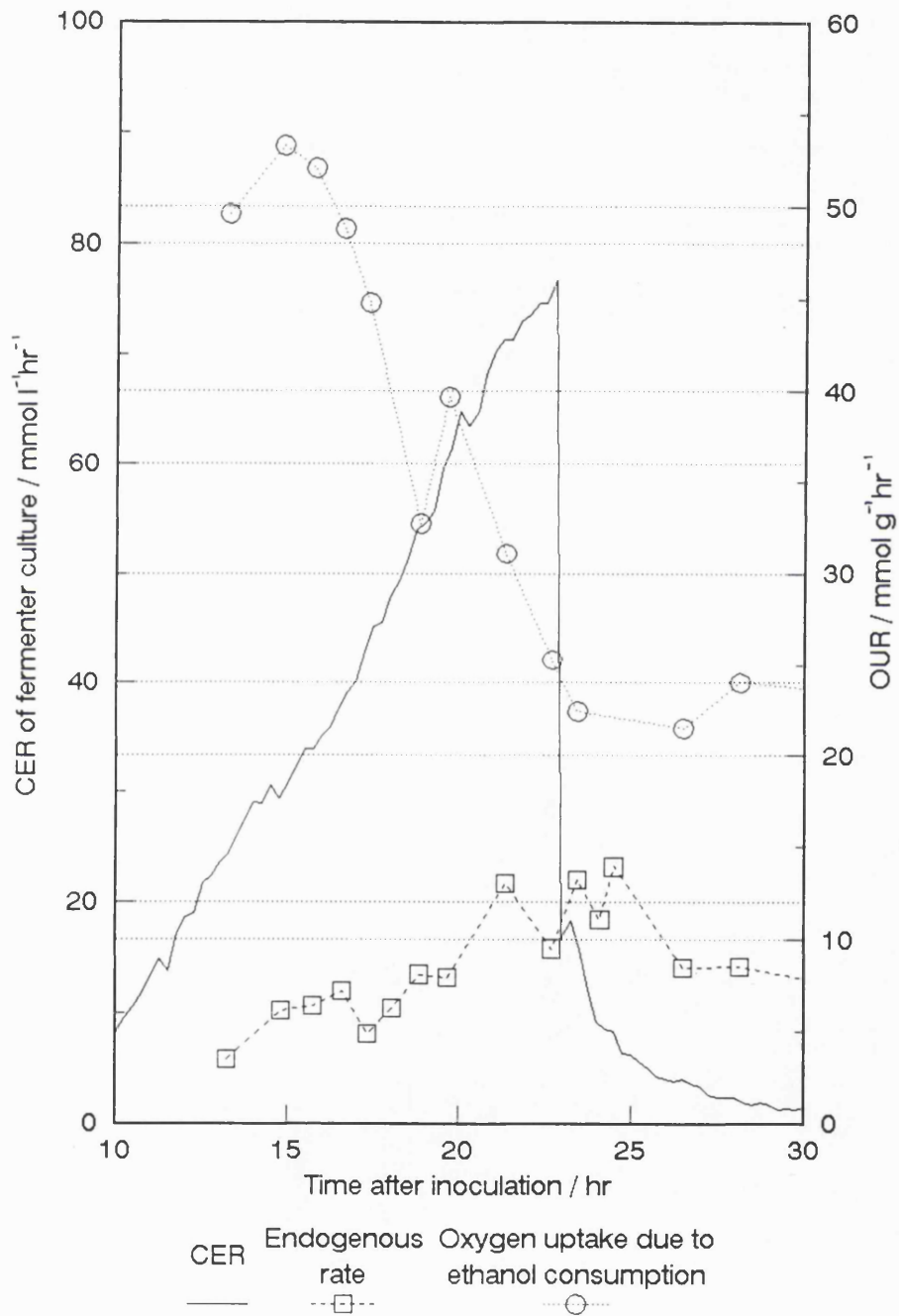
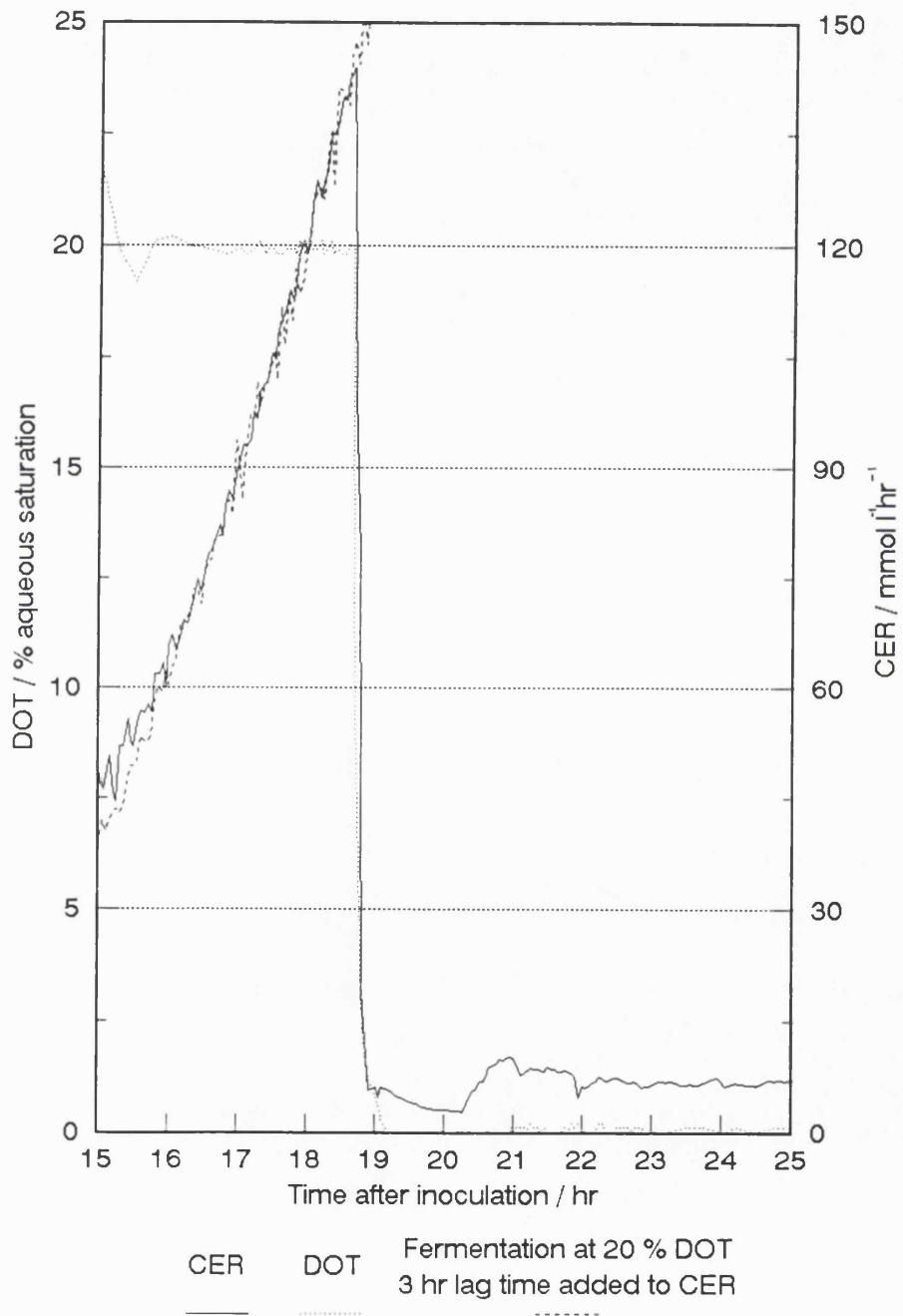


Figure 3.19 - DOT and CER profiles from 15 to 25 hr after inoculation to show the effect upon the CER of decreasing the DOT to 0. The CER profile is compared to the fermentation controlled at 20 % DOT throughout but with a 3 hr lag time subtracted.



decrease in cell concentration had been observed. Figure 3.19 demonstrate the effect of changing the DOT level upon the CER profile of the bacterial culture. Once the DOT setpoint had been moved to zero the oxygen transfer rate had decreased. The oxygen transfer rate decreased further even after the DOT had reached zero since negative readings could not be taken. The setpoint was then moved to 0.1 % aqueous saturation. The oxygen transfer rate then increased with a resulting increase in the fermentation culture CER value. The DOT was controlled between 0 and 0.2 % aqueous saturation and the CER then increased towards 8 mmol l<sup>-1</sup> hr<sup>-1</sup>.

The specific OUR due to ethanol consumption of the samples taken from the fermentation had increased during the growth phase to a maximum value of 24 mmol g<sup>-1</sup> hr<sup>-1</sup> which was lower than the value obtained for the fermentation at 5 % DOT and 30°C. Once the DOT had been moved towards zero the specific OUR had decreased to 4 mmol g<sup>-1</sup> hr<sup>-1</sup>.

In a second fermentation the reading obtained from the probe for the oxygen free culture medium obtained after sparging with nitrogen was set at 0.4 % DOT. The setpoint was moved from 20 % to 0.5 % and the CER had stabilised to a value between 2.3 and 2.9 mmol l<sup>-1</sup> hr<sup>-1</sup>.

In these experiments further growth of the cells was not seen once the DOT had been decreased. This is different from shake flask cultures where linear growth had occurred.

#### 3.1.4.3 - The effect upon the growth of cells of moving from 20 % DOT to a low $K_L a$ which results in oxygen limited growth

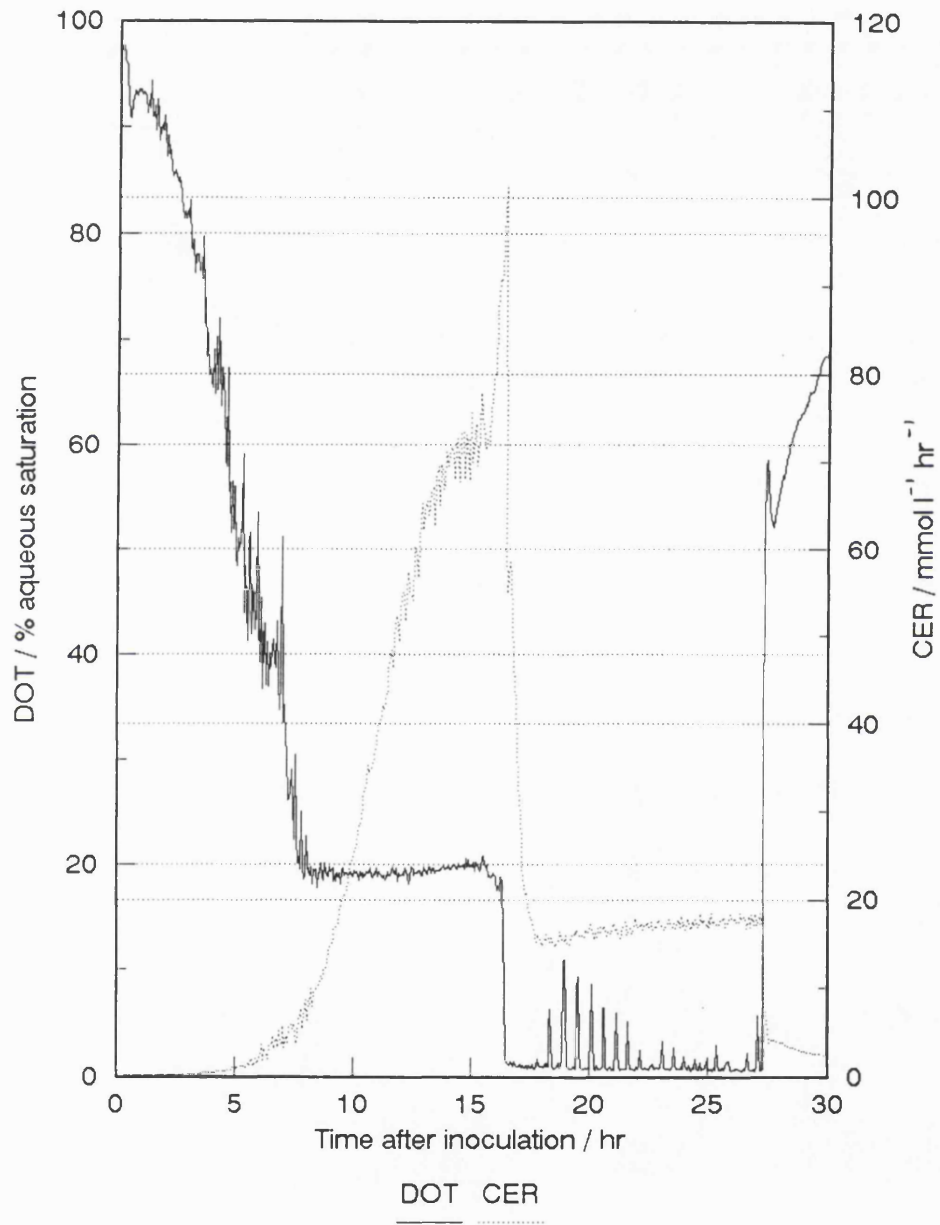
The aromatic oxidation activity of the bacterium obtained from a shake flask was stable for a week as compared to bacteria obtained from a fermentation at 20 % DOT which had lost their activity by 2 hr after sampling. The reason for this retention in activity was seen to be important in a biocatalytic process. In Section 3.1.4.2 the biocatalyst had been grown at 20 % DOT before reducing to 0 at the end of the growth phase. The cells did not continue to grow which was different from the oxygen limited shake flask cultures since the cells remained in a growth phase. A fermentation was carried out in which the cells were subjected to oxygen limitation but the oxygen transfer rate was high enough to allow the cells to grow.

The profile for the fermentation is shown in Figure 3.20 and was composed of two main sectors. In the first sector the cells are not oxygen limited with the DOT controlled at 20 % aqueous saturation from 7 to 16.25 hr after inoculation but had become nutrient limited. Iron sulphate (0.2 g) was added to the fermentation, 14.6 hr after inoculation which resulted in a rapid increase in CER.

In the second sector the cells then became oxygen limited by reducing the  $K_L a$  to a low but constant value. The stirrer speed was set at 350 rpm and the airflow rate at 2 l  $\text{min}^{-1}$ , 16.25 hr after inoculation until the end of the fermentation. There was a rapid decrease in the DOT which led to a rapid fall in metabolic activity as indicated by the CER profile. The spikes in the DOT indicate that the metabolic activity of the cells was changing rapidly and not maintained at one level. The acid input into the fermenter was reduced from 80.7  $\text{mmol hr}^{-1}$  during growth at 20 % DOT to 17.4  $\text{mmol hr}^{-1}$  when the bacteria were oxygen limited which would indicate that the succinate uptake has been reduced to



Figure 3.20 - CER and DOT profile of the fermentation controlled at 20 % DOT and 30°C but near the end of the growth phase the  $K_L a$  had been reduced.

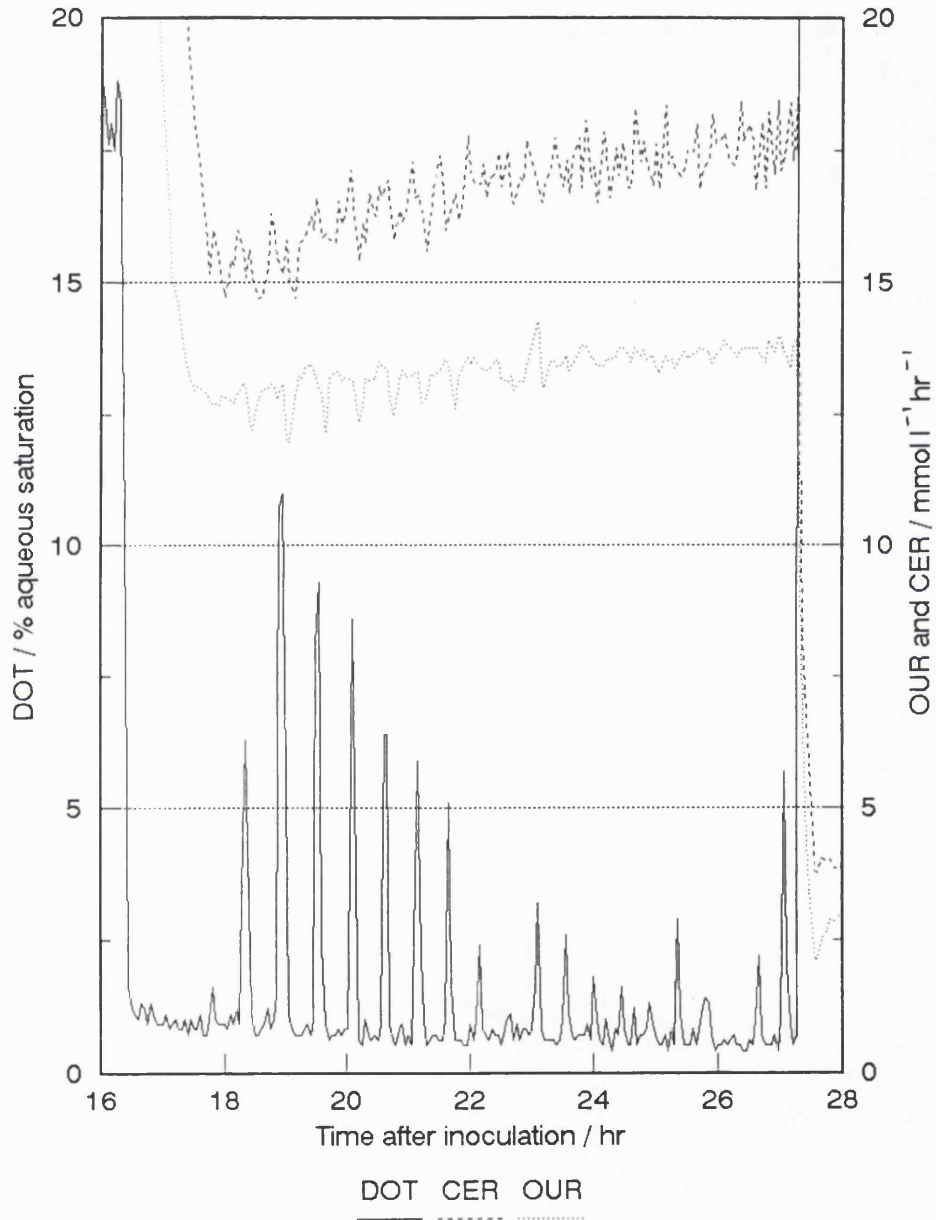


21.6 % of its value at 20 % DOT. Acid input then fell to zero, 27 hr after inoculation which corresponded to a rapid increase in DOT (Figure 3.20) which was likely to be due to starvation of the carbon source.

The biomass, carbon dioxide evolved from the fermenter and the oxygen consumed was correlated to the acid added to control the pH. The six biomass readings taken during the growth phase were correlated with the acid input with a calculated value of 8.59 g of dry weight cells per mol of acid added. The CER was  $0.861 \text{ mol (mol acid added)}^{-1}$  and OUR was  $0.663 \text{ mol (mol acid added)}^{-1}$  during the growth phase. Once the oxygen transfer rate had been decreased the CER and the OUR increased to 0.974 and  $0.766 \text{ mol (mol of acid added)}^{-1}$ .

The DOT, OUR and CER profiles at low  $K_L a$  value (i.e. 16 to 26 hr after inoculation) are shown in Figure 3.21. The CER value was above  $15 \text{ mmol l}^{-1} \text{ hr}^{-1}$  which was greater than for the cells where the DOT was controlled at 0.1 % aqueous saturation since the CER value had decreased to below  $8 \text{ mmol l}^{-1} \text{ hr}^{-1}$ . The DOT profile was complex with a number of spikes indicating that the cellular metabolism was changing rapidly. These changes in cellular metabolism were also demonstrated by decreases in the OUR readings at the same time. The idea that oxygen limited cells remain at one minimum DOT value is therefore untrue in this case.

Figure 3.21 - DOT, CER and OUR profile of the fermentation at low  $K_L a$  after high biomass had been achieved.



### 3.1.5 - Fermentations at different growth temperature

#### 3.1.5.1 - 26°C

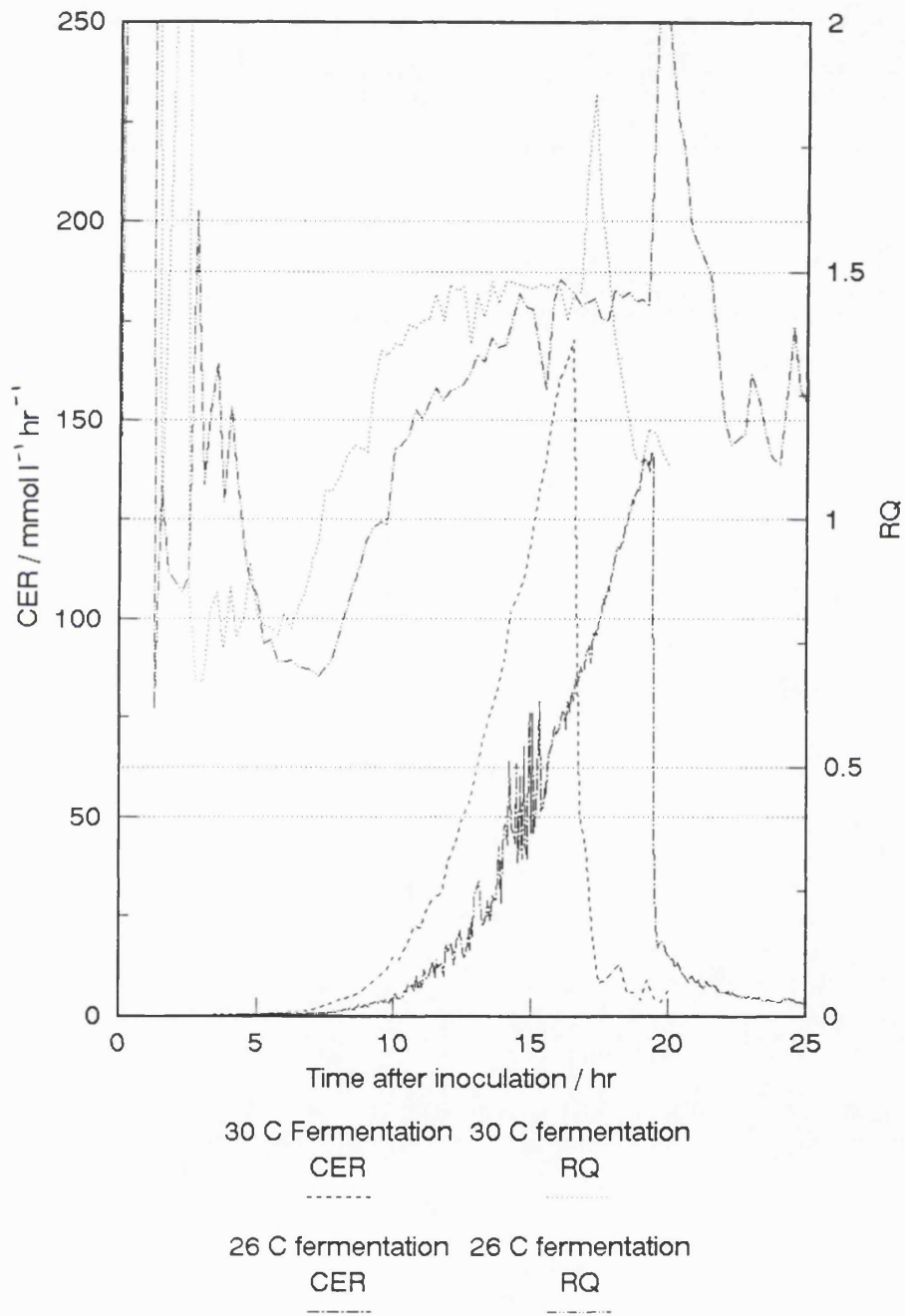
The CER and cell concentration profile were similar in shape to those obtained for cells grown at 30°C with the biomass following the CER profile until the end of the growth phase. The growth phase finished 19.4 hr after inoculation with the peak CER value reaching 142 mmol l<sup>-1</sup> hr<sup>-1</sup>. Cell concentration then decreased, indicating cell death and lysis, and CER also dropped to a low value. The yield of biomass was lower at 6.26 gl<sup>-1</sup> compared to 7.96 gl<sup>-1</sup> for cells grown at 30°C.

The profiles in Figure 3.22 indicate that the bacterium was growing at a slower rate. Both the RQ and CER profiles are similar in shape indicating that significant variations in metabolism were not observed. The RQ values increased during the growth phase reaching a steady value of 1.4 by the end of the growth phase. The specific growth rate of the bacteria at 26°C was 0.36 hr<sup>-1</sup> which was 44 % lower than the rate of increase of CER which had an exponential rate constant of 0.63 hr<sup>-1</sup>. Biomass did not increase exponentially at the end of the growth phase where as the CER increased at a rate of 0.22 hr<sup>-1</sup> from 15.6 to 19 hr after inoculation. This clearly indicates that the carbon dioxide evolved from the fermenter per g cells was changing during the course of the fermentation.

The total carbon data which was obtained by integrating the CER values was analysed on a logarithmic scale and showed that between 5 and 15 hr the total carbon evolved as carbon dioxide increased at an exponential rate of 0.638 hr<sup>-1</sup> but after 15 hr the rate declined.

The acid input into the fermenter which is an indirect measure of succinate uptake was compared to the cell

Figure 3.22 - Comparison of the CER and biomass profile of the cells grown at 30°C and 26°C.



concentration and carbon out as carbon dioxide. Acid input was analysed online with 220 readings obtained. Figure 3.23 demonstrates good correlation between acid input to control pH and the total amount of carbon dioxide evolved. 50.88 g of carbon was evolved from the fermenter for every mole of acid added. The fit between cell concentration and acid input was poorer with 8.68  $\text{gl}^{-1}$  of cells produced per mole of acid added.

A carbon balance was performed to analyse the uptake of the carbon source. The calculations were performed as described in Sections 3.1.3.4 and 3.1.4.1. Figure 3.24 shows the profile of the carbon content of the cells, fermentation broth and total carbon out of the fermenter as carbon dioxide. The calculated carbon source concentration in the medium and the carbon uptake are also shown. The three samples between 14 and 16 hr after inoculation had total carbon balance value around 9  $\text{gl}^{-1}$  which would indicate that the total organic carbon analysis of the fermentation broth was correct. Similarities between the profiles obtained here and for the other fermentations include the carbon content of the fermentation culture at the end of the growth phase having a similar value to the calculated value for cell concentration indicating starvation of the carbon source. The total carbon increased from 9  $\text{gl}^{-1}$  towards 11  $\text{gl}^{-1}$  from 16 hr after inoculation onwards indicating an error with the calculation of the total carbon dioxide evolved from the fermenter.

The carbon uptake data were plotted against cell concentration in Figure 3.25 to obtain a yield of cells upon the carbon source. The last datum point was obtained from a sample taken after the growth phase had finished and was therefore not included in the curve fitting since it demonstrated endogenous respiration where the cells are metabolising biomass to survive and not succinate. The yields of cells upon carbon source was 0.785 g dry cell

Figure 3.23 - Correlations between total carbon dioxide evolved from the fermenter, cell concentration and the acid added to the fermenter.

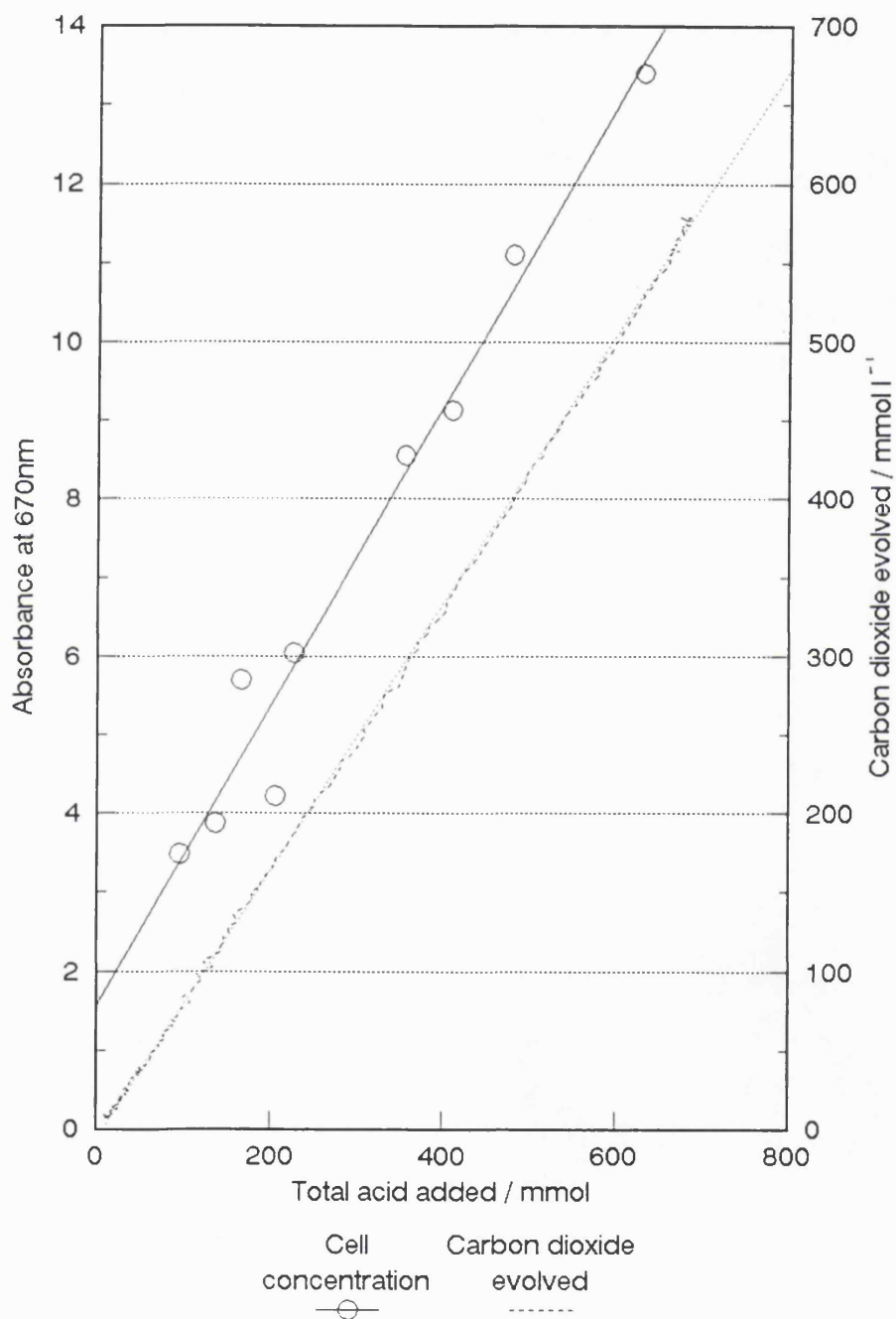


Figure 3.24 - Carbon balance of the fermentation at 26°C and controlled at 20 % DOT. The calculated carbon source concentration and uptake by the bacteria are also shown.

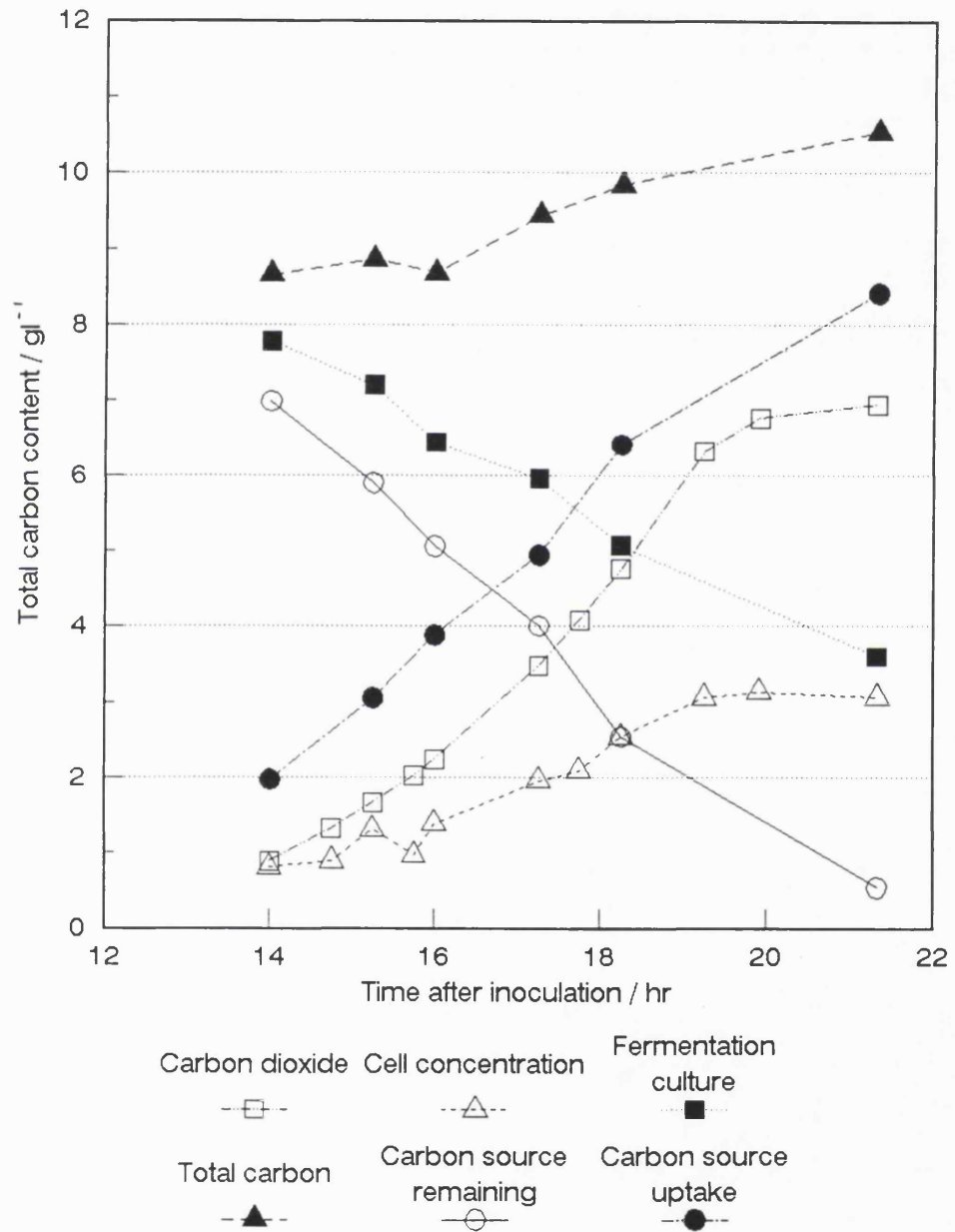
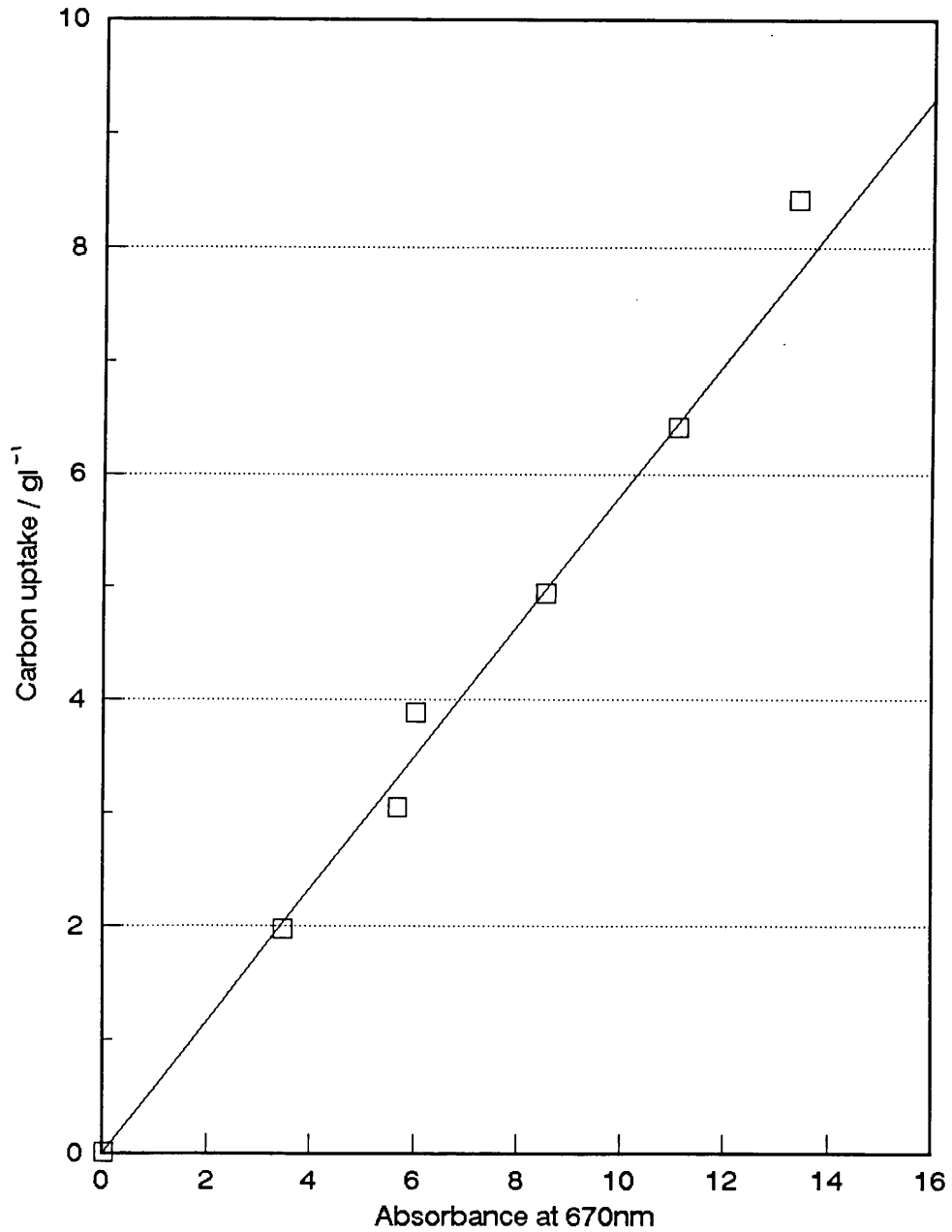




Figure 3.25 - Correlation between the calculated carbon source uptake and cell concentration of the cells grown at 26°C and DOT controlled at 20 % aqueous saturation.



weight / g of carbon which is equivalent to 0.319 g dry cell weight / g succinate.

The specific OUR due to ethanol consumption of the samples taken from the fermentation had increased from 18 mmol g<sup>-1</sup> hr<sup>-1</sup>, 14 hr after inoculation to a maximum of 26 mmol g<sup>-1</sup> hr<sup>-1</sup>, 17.25 hr after inoculation. This maximum value was similar to that obtained in samples taken from the fermentation at 30°C and 20 % DOT (Section 3.1.4.2) but lower than the values obtained for the fermentation at 30°C and 5 % DOT (Figure 3.18). The specific OUR had then decreased to 16 mmol g<sup>-1</sup> hr<sup>-1</sup> at the end of the growth phase.

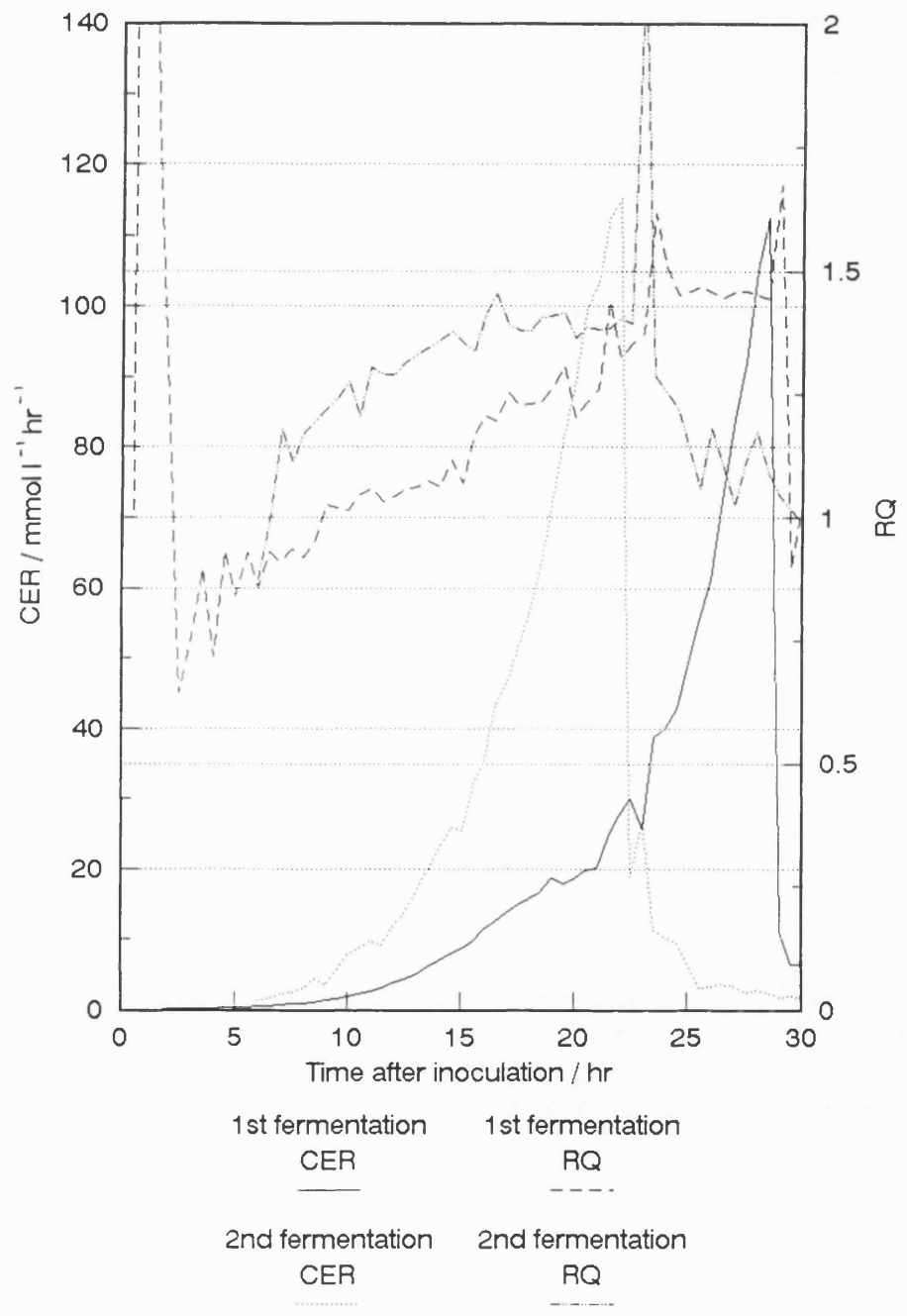
The biomass and CER data suggest that there are two phases to the growth of the bacteria but clear differences in the correlations between biomass, carbon dioxide evolved and acid input were not obtained. The growth of the bacteria at 26°C was similar to cells grown at 30°C but at a slower rate.

#### 3.1.5.2 - 34°C

Figure 3.26 shows the profile obtained for CER and RQ for two fermentations at 34°C and 20 % DOT. Variations in these profiles are due to differences in the inoculum level. The first fermentation had inoculum concentration of 0.226 gl<sup>-1</sup> (7 hr culture) resulting in the fermentation finishing 28.8 hr after inoculation. The second fermentation had an inoculum concentration of 0.457 gl<sup>-1</sup> (11 hr culture) with the fermentation finishing 22.3 hr after inoculation. The RQ values of these two fermentations are similar to each other and to the fermentation controlled at 30°C.

The carbon evolved as carbon dioxide was analysed to obtain an expression for the metabolic activity of the cells. When the CER values were plotted on a logarithmic scale convex curves were obtained for both curves unlike the fermentations at 26°C (Section 3.1.5.1) and 30°C (Figure 3.6).

Figure 3.26 - CER and RQ profiles of the two fermentations controlled at 34°C and 20% DOT



The profile for total carbon evolved as carbon dioxide for the first fermentation was plotted in Figure 3.27 to determine any metabolic relationships. It was seen that towards the end of the growth phase the increase in carbon out did seem to be exponential but in the early part no such relationship could be obtained. The carbon evolved from the fermenter was compared to that of the 30°C fermentation in Figure 3.27. The profile for the fermentation at 30°C was moved from 0 to 16.75 hr after inoculation to 12.05 to 28.8 hr so that the points at which the maximum organic carbon evolved from the fermenter is reached at the same time. This allowed an easier comparison of the data to be made. Two conclusions can be drawn:-

- a) the total carbon dioxide evolved was the same at 30°C and 34°C indicating that the proportion of the succinate metabolised to provide energy was unchanged.
- b) at 34°C the metabolic rate was lower.

Only a few samples were analysed for biomass accumulation in the two fermentations with the growth profile of the first fermentation shown in Figure 3.28. It is seen that there is an exponential fit to the available data but it is poor. It is seen from this data that the bacteria behave very differently at the higher temperature due to its damaging effect. The damaging effect is reflected by the major difference between the growth of the cells at 34°C as compared to 30°C.

#### 3.1.5.3 - Comparison of the initial growth of the bacterium at different temperatures

Temperature has clearly demonstrated an effect upon the growth of the bacterium (Section 3.1.5.1 and 3.1.5.2). The growth phase of the fermentations at 30°C finished before 17 hr after inoculation whereas the fermentation at 26°C took

Figure 3.27 - Comparison of the carbon out of the fermentation at 30°C and 34°C. Profile of 30°C fermentation which was from 0 to 16.75 hr after inoculation was moved to 12.05 to 28.8 hr after inoculation so that end of growth phase was at the same time as the fermentation at 34°C.

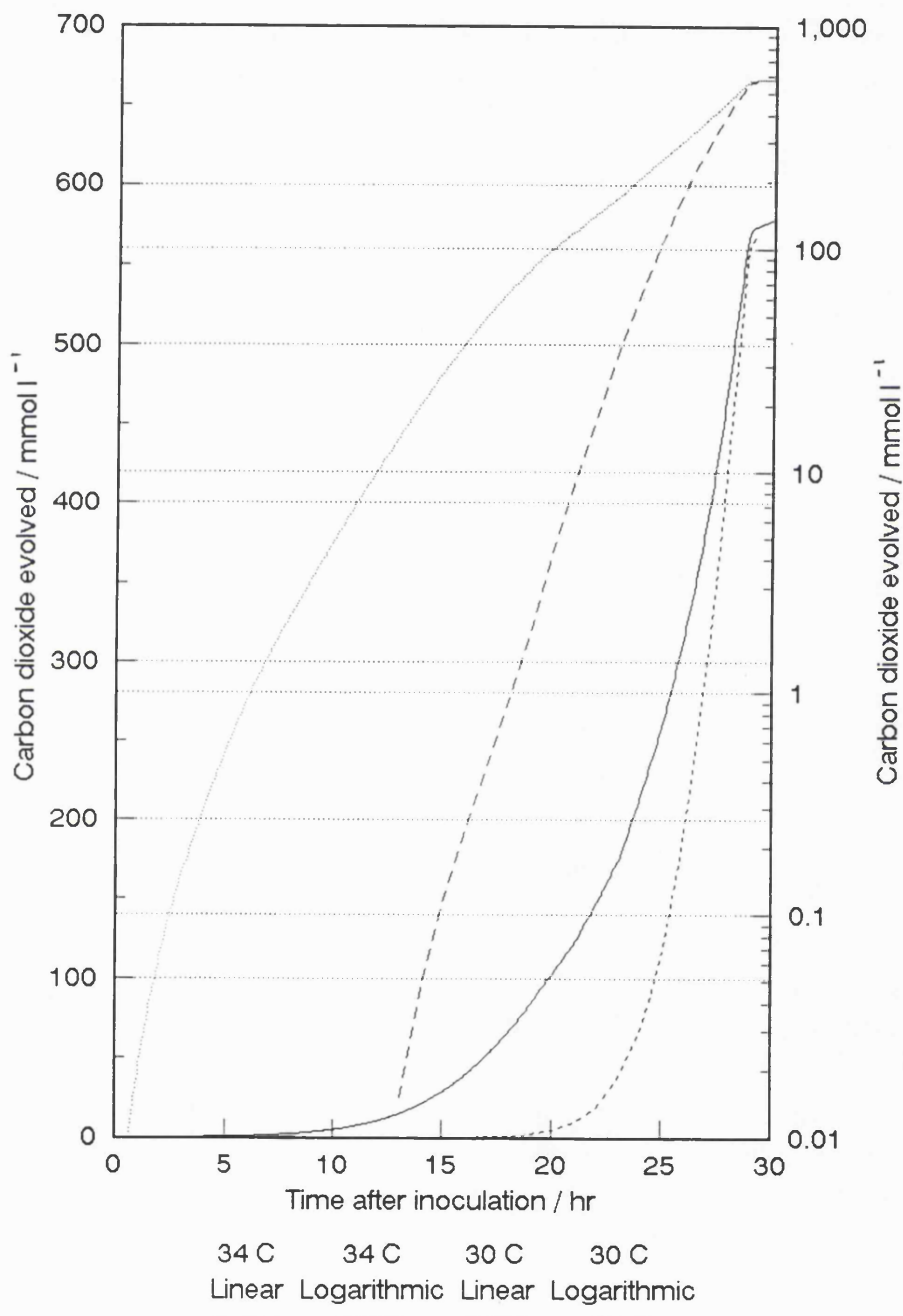
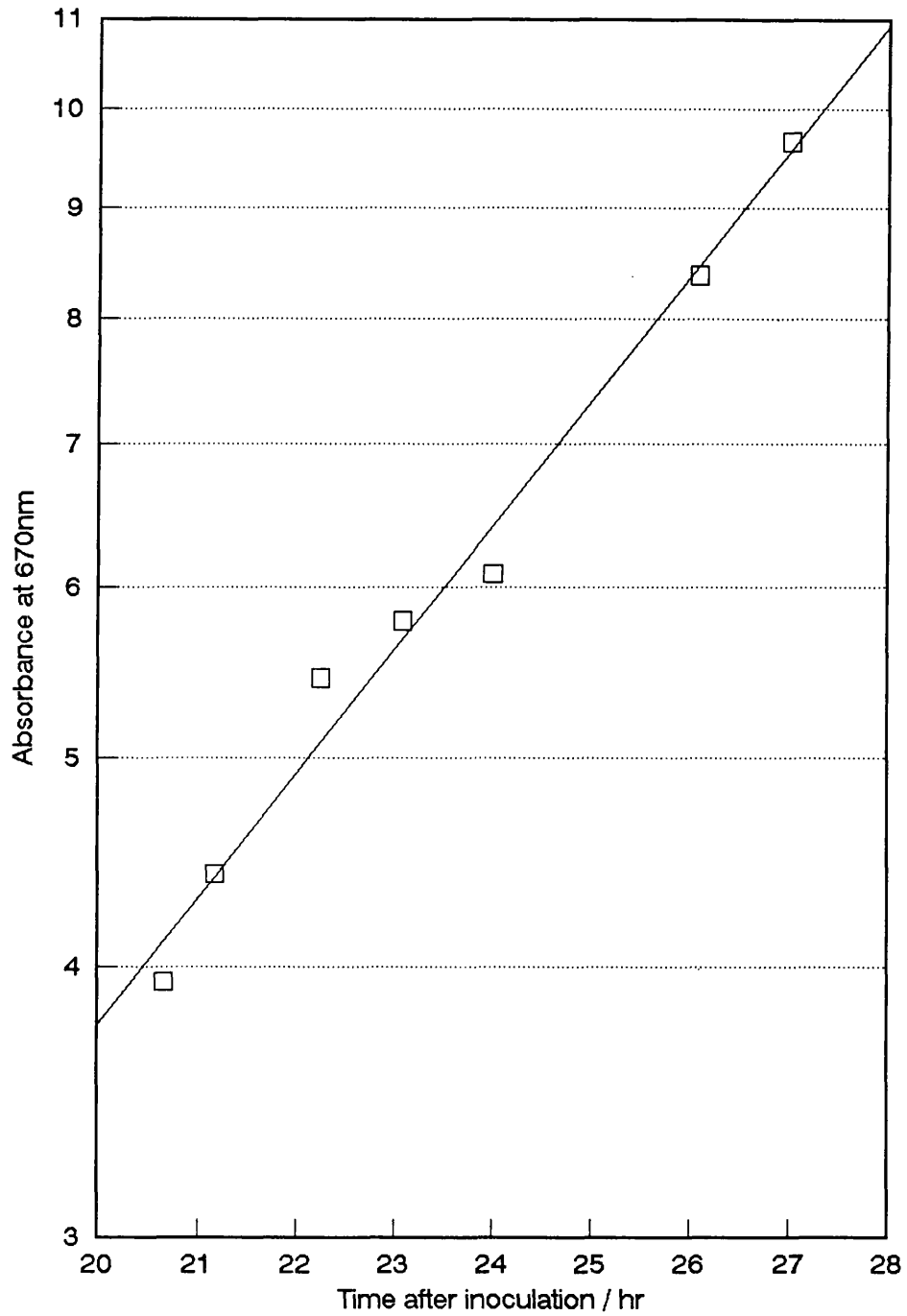


Figure 3.28 - Biomass profile of the growth phase of the first fermentation at 34°C and 20% DOT



19.4 hr and the two fermentations at 34°C took over 22.3 hr to finish. Variations in the exponential increase in CER and total carbon dioxide evolved had also been observed. At the start of the fermentation off-line data had not been obtained for the fermentations at 30 and 34°C therefore these were compared by analysing the CER data. Another method of analysing the start of the growth phase was sought. It would also allow the comparison of the fermentations under different growth conditions but at 30°C (e.g. 5 % DOT, Section 3.1.5.1).

The DOT profiles obtained at the start of the fermentations were compared since at this stage the stirrer speed and airflow rate were fixed at their minimum value until the DOT reached 20 % aqueous saturation.

The drop in DOT was due to an increase in the OUR as the biomass increased. The OUR readings obtained for the fermentation at 30°C and 20 % DOT has already been shown to increase exponentially from 3 to 8 hr after inoculation (Figure 3.9). If we take the OUR to be equal to the oxygen transfer the following equation is obtained:-

$$\text{Oxygen uptake rate} = \text{Oxygen transfer rate} = k_L a (C^* - C_L)$$

where  $C^*$  is the dissolved oxygen concentration that is in equilibrium with the gas phase and  $C_L$  is the concentration in the bulk liquid. The system reaches an equilibrium during the calibration of the DOT probe where  $C^*$  will be equivalent to the aqueous saturated concentration of oxygen at 30°C. The following equation can be applied if we assume in all our calculations that  $C^*$  is 100% :-

$$\text{OUR} = K_L a \cdot \frac{\text{Saturated oxygen concentration} \cdot (100 - \text{DOT \% aqueous saturation})}{100}$$

At the start of the fermentations the  $K_L a$  was constant therefore the expression can be simplified to:-

$$\text{OUR} = \text{constant} \cdot (100 - \text{DOT})$$

Since the OUR increased exponentially we should see the expression 100-DOT increasing exponentially at the start of the fermentation.

Profiles of (100-DOT) at the start of a number of fermentations were obtained with six of the profiles shown in Figure 3.29. The fermentation at 30°C and 20 % DOT described in Section 3.1.3 had a gradient of 0.56 hr<sup>-1</sup>. Four other fermentations at 30°C analysed were:-

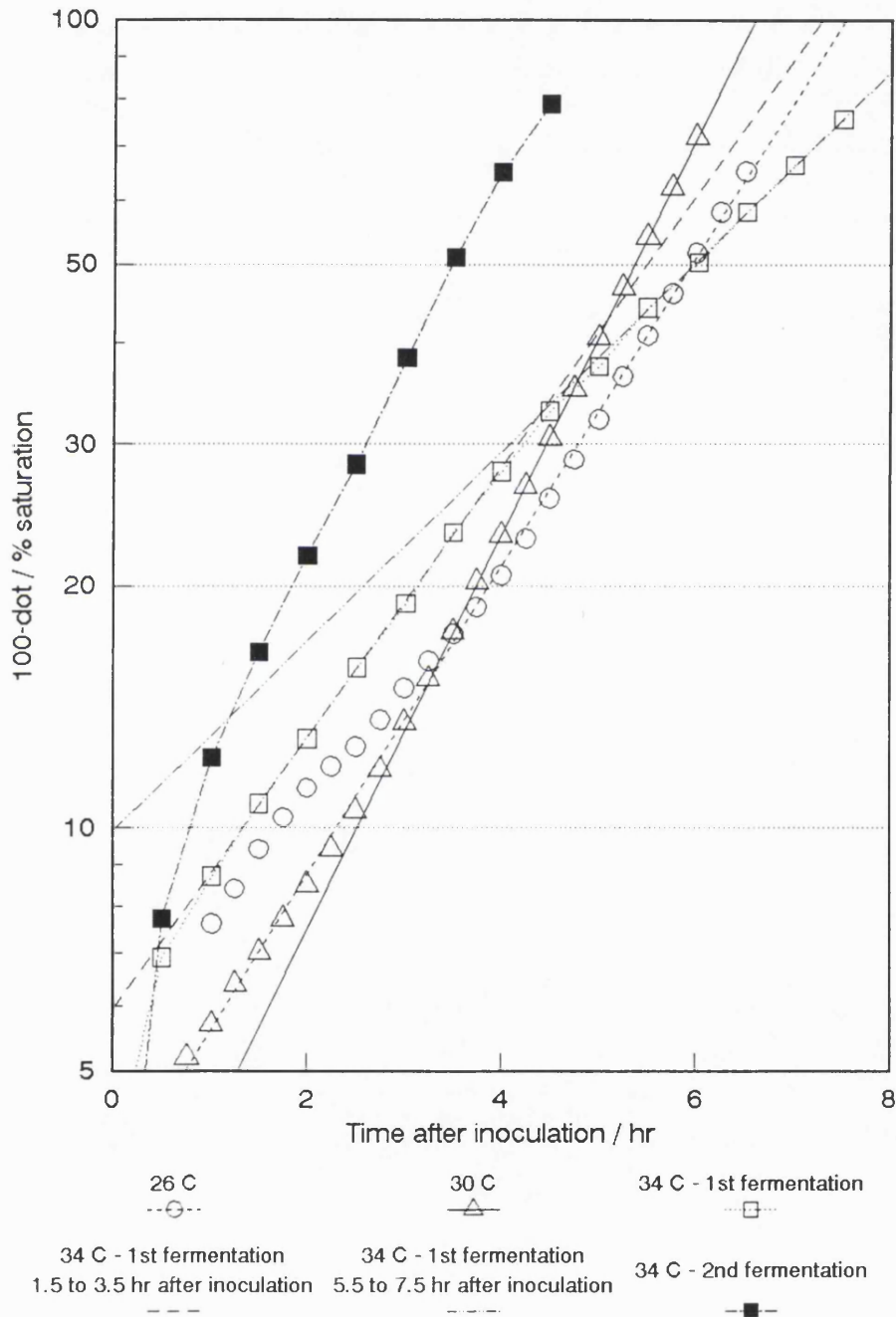
- a) two more fermentations described in Section 3.1.3 with 50 ml of inoculum.
- b) one fermentation with 100 ml described in Section 3.1.2 (Figure 3.6).
- c) the fermentation which was controlled at 5 % DOT and 30°C (Section 3.1.4.1).

The average value for the gradient was 0.59 ± 0.03 hr<sup>-1</sup>. The gradient obtained for the fermentation at 26°C was lower at 0.44 hr<sup>-1</sup> indicating the slower growth of the bacteria at this temperature (Section 3.1.5.1).

The convex curves obtained for the fermentations at 34°C were dependent on cell concentration. The first fermentation had similar inoculum to the fermentation at 26 and 30°C. The initial gradient (0.39 hr<sup>-1</sup>, 1.5 to 3.5 hr after inoculation) was similar to the gradient at 26°C but drops to a low level of 0.27 hr<sup>-1</sup> from 5.5 to 7.5 hr after inoculation. This indicates that the cells were experiencing the toxic effect of the higher temperature with a resulting decrease in the metabolic activity. The overall effect was to increase the length of the growth phase to 28.8 hr.



Figure 3.29 - Comparison of the initial activity of the cells in the fermentations at 26, 30 and 34°C with DOT at 20 % aqueous saturation. Activities were expressed as a profile of (100-DOT) against time



### 3.1.6 - Carbon limited

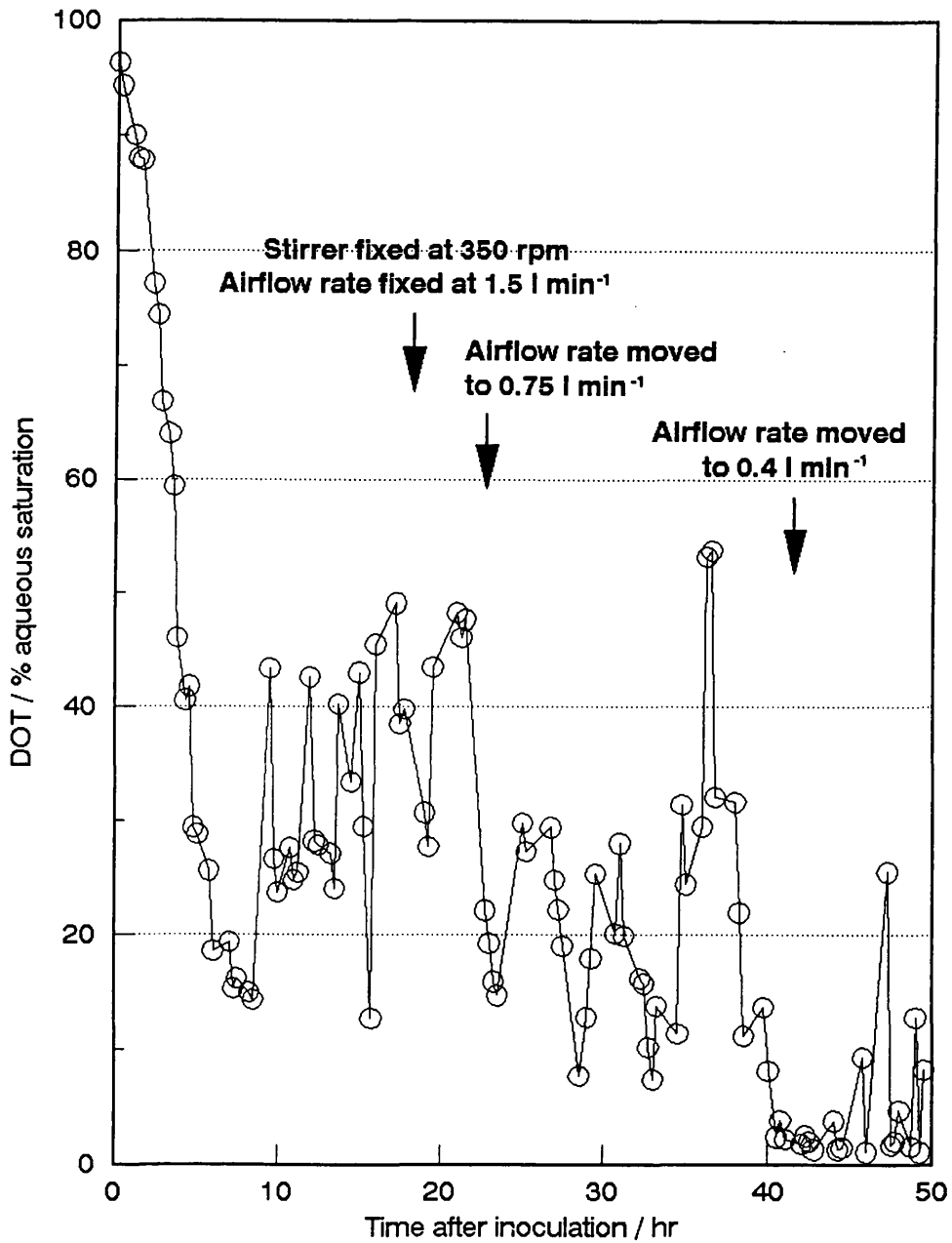
The effect of oxygen concentration upon the growth of P.putida ML2 has been analysed. In the previous fermentations the cells were allowed to grow at 5 or 20 % DOT until the carbon source had finished. A carbon limited fermentation was analysed since the growth rate could be independently controlled from the effect of the oxygen concentration. Succinate was added at a constant rate with DOT controlled at 20 % aqueous saturation. Succinate was added at a rate of  $1.53 \text{ g hr}^{-1}$  and biomass accumulated at a rate of  $0.25 \text{ g hr}^{-1}$ . The yield of cells upon succinate was calculated to be  $0.16 \text{ g dry cell weight (g succinate)}^{-1}$  which was 44 % of the yield for the fermentation at 20 % DOT,  $30^\circ\text{C}$  with succinate batched in at the start.

The DOT was highly unstable as shown in Figure 3.30 and could not be maintained at 20 % aqueous saturation. The  $K_L a$  was therefore set at one level, 18.25 hr after inoculation with the stirrer speed and airflow rate fixed at 350 rpm,  $1.5 \text{ l min}^{-1}$ .

The DOT level was still unstable therefore indicating that it was due to the feeding of succinate into the fermenter and not the automatic control of the stirrer speed and airflow rate. The succinate solution was pumped in at a steady flow rate but resulted in drops of the solution forming on the end of the inlet needle before falling into the fermentation culture. This pulsed input of succinate resulted in oscillations in the metabolic activity of the cells.

The effect of the oxygen transfer rate upon the metabolism of the cells was determined by reducing the airflow rate. The stirrer speed was not reduced since variations in the mixing time would result in the succinate concentration varying throughout the fermenter leading to further oscillations in the metabolic activity of the cells.

Figure 3.30 - The DOT profile of the carbon limited fermentations which had the oxygen transfer rates changed to determine its effect upon the growth of the cells.

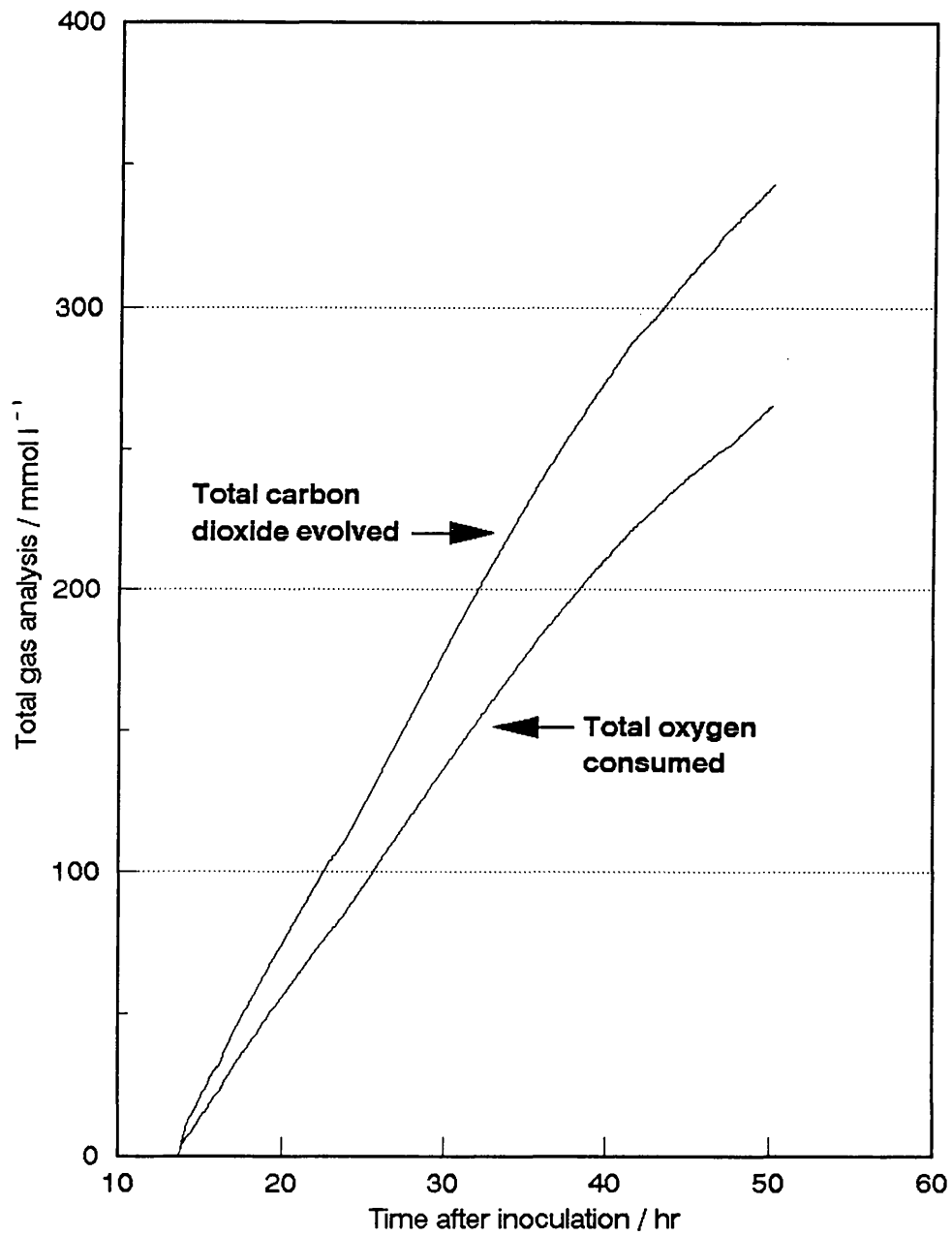


The cells appeared to become oxygen limited when the airflow rate was fixed at  $0.4 \text{ l min}^{-1}$  since the DOT had reached zero. The CER and OUR values were highly unstable prior to fixing the stirrer speed and airflow rate and were still unstable after fixing the oxygen transfer rate but appear to be decreasing with time.

The CER and OUR values were totaled to obtain smooth curves which are shown in Figure 3.31. These profiles were shown to be linear between 15 and 35 hr after inoculation with a CER and OUR value of  $10.45$  and  $8.15 \text{ mmol l}^{-1} \text{ hr}^{-1}$ . The RQ value was  $1.28$  which is lower than for previous fermentations in which a value of  $1.4$  was obtained for cells at the end of the growth phase. The gradient of the curves in Figure 3.31 decreased from 40 hr onwards when the cells had become oxygen limited. Acid was added to the fermenter at a rate of  $10.6 \text{ mmol hr}^{-1}$  from 20 to 40 hr after inoculation.

In this fermentation problems were encountered with oscillations in the metabolism of the cells due to the pulsing of succinate into the fermenter. Steady DOT values were not obtained. Linear correlations were obtained and oxygen limited growth was detected. The fermentation was not repeated due to the problems in preventing the pulsing of succinate into the fermenter and preventing large increases in broth volume. Similar problems were envisaged with other nutrient limited fermentations (e.g. magnesium, nitrogen) therefore these were not investigated.

Figure 3.31 - Total oxygen consumption and the total carbon dioxide evolved from the carbon limited fermentation.



## 3.2 - Fluorobenzene Oxidation activity

### 3.2.1 - Assay development and characterisation of the enzyme activity.

The preferred method for measuring enzyme activity was to follow the accumulation of the product during a biotransformation. It would also be useful to follow the substrate uptake by the cells and loss from the system through the airstream due to its volatility. The biotransformation assay needed to be carried out on a small scale either in a shake flask or in a stirred reactor of 60ml working volume. If larger volumes were required this would lead to problems arising from large changes in fermentation volume due to the necessary removal of sufficient quantity of biocatalyst. The first step in the biotransformation assay development was the selection of an analytical method to detect the product and substrate.

Fluorocatechol was first analysed by Normal Phase HPLC as described by Harrop (1990). Fluorocatechol was loaded in dichloroethane, as the loading solvent, and a standard curve obtained at a concentration of  $2.0 \text{ gl}^{-1}$  was ten times greater than the maximum achievable concentration for the following reasons. First the initial concentration of fluorobenzene would be lower than aqueous saturation ( $1.54 \text{ gl}^{-1}$ ; Riddick et al, 1986) since substituted benzenes (e.g. toluene) have been shown to be toxic to the cells (Jackson & DeMoss, 1965). Brazier (1989) had found that toluene was toxic above 40 % aqueous saturation therefore in this assay the fluorobenzene concentration would have to start below a concentration of 50 % aqueous saturation. A portion of this (e.g. 10%) would also be lost in the airstream due to its volatility. Thus the maximum achievable amount of fluorocatechol in the aqueous sample was  $0.9 \text{ gl}^{-1}$ . The HPLC analysis of naphthalene developed by Harrop (1990) resulted in a 25 fold dilution of the naphthalene concentration after the sample had been added to dichloroethane which was the loading solvent. This was

undesirable in this analysis since a low concentration of fluorocatechol would be obtained, the ratio of the sample volume to loading solvent volume was therefore decreased. This would still result in the concentration of fluorocatechol in dichloroethane being approximately a quarter of the concentration of the initial sample. This would lead to a maximum achievable concentration of approximately  $0.2\text{gl}^{-1}$  in the dichloroethane. Since fluorocatechol has a higher solubility than naphthalene cis dihydrodiol it is more likely to remain in the aqueous phase rather than be extracted to the dichloroethane phase. The sensitivity of the detector was increased so that a lower concentration of fluorocatechol of approximately  $0.01\text{gl}^{-1}$  could be detected. When analysing the standard solutions at a higher sensitivity a large amount of electrical noise was observed. Further problems would also be observed when the samples from the biotransformation were analysed since these would also contain impurities. Fluorocatechol was therefore reanalysed using Reverse Phase HPLC as described by Brazier (1989) so that a aqueous sample could be loaded. Similar problems with the sensitivity of the detector were again observed.

The HPLC method was therefore found to be unsuitable. An alternative Gas Chromatographic method of analysis was developed by Mr R. Lynch for the study of this biotransformation system. Problems were encountered with the development of this assay and therefore a simpler assay based on the OUR rather than product accumulation was instigated. The assay is based on the fact that one mole of oxygen is required to convert one mole of fluorobenzene to fluorocatechol.

The Clark type oxygen electrode has previously been used to determine the aromatic oxidation activity of Pseudomonas putida ML2 (Zamanian, 1989). The substrate was added in aqueous solutions which was vigorously mixed prior to

addition to the electrode. The activity of the cells which were grown in benzene shaken flasks were initially determined by this method. Preliminary experiments showed that aromatic oxidation activity was observed when 1.0 ml of buffer saturated with fluorobenzene was added to 3 ml of a cell suspension. Toluene hydroxylation had been shown to be substrate (toluene) limited at low levels, initially the fluorobenzene was added at a high concentration (but below 40 % aqueous saturation which could be toxic) so that the reaction was not substrate limited. It was demonstrated that the addition of the substrate brought about an increase in oxygen uptake and therefore could be used as the assay for the aromatic oxidation activity. The assay was therefore selected. Additional advantages included:-

- a) Simplicity - water bath + oxygen electrode.  
Equipment problems less likely to arise.
- b) The assay took less than an hour to complete and did not involve a large number of manual operations. This allowed other assays to be performed at the same time.
- c) It would be reasonably easy to increase the number of electrodes for the assay allowing greater number of runs on each sample.

The buffer saturated with fluorobenzene was at a higher oxygen concentration than the culture in the chamber. The addition of the buffer saturated with fluorobenzene resulted in an increase in the average DOT value which was indicated by a large shift in the DOT immediately after addition of the buffer. This as well as the problem of diluting the cell concentration in the oxygen electrode complicated the calculation of the aromatic oxidation activity. Therefore the fluorobenzene was added in a water-soluble alcohol to minimise the increase in volume.



### 3.2.1.1 - Substrate Concentration

The first priority was to determine which alcohol should be used to supply the fluorobenzene to the electrode and at what concentration. Four water soluble alcohols were considered - ethanol, butanol, propanol and pentanol. The profiles of the specific oxygen uptake rate due to ethanol and propanol are shown in Figure 3.32. Ethanol was unsuitable as the loading solvent due to the high oxygen consumption which resulted even when added at low concentration. Butanol also had a high oxygen uptake requirement and pentanol was toxic to the cells. Propanol was therefore selected as the alcohol in which to add the fluorobenzene since it had a low oxygen requirement and could be used at higher volumes.

Fluorobenzene was dissolved in propanol and added in 10  $\mu$ l volume. The aromatic oxidation activity of the cells at various fluorobenzene concentrations was determined. Figure 3.33 shows the effect of fluorobenzene concentration upon the OUR of the cells. The fluorobenzene concentration is quoted as percentage of the aqueous saturation where the saturated concentration is 1.54  $\text{g l}^{-1}$  (Riddick et al, 1986). At fluorobenzene concentrations of 60% or higher the OUR was lower than 4  $\text{mmol g}^{-1} \text{hr}^{-1}$  due to the toxicity of fluorobenzene. At 5% saturated concentration the reaction is substrate (fluorobenzene) limited since the oxygen uptake due to fluorobenzene alone is approximately a third of that obtained at 10% saturation concentration. The activity of the cells were therefore analysed at concentrations of fluorobenzene from 0 to 40% aqueous saturation. Figure 3.34 shows the runs with the fluorobenzene in propanol added to 5, 10 and 20% aqueous saturation in the electrode. When the profiles were superimposed it was clear that the OUR increased as the fluorobenzene concentration increased. The OUR was linear for at least five minutes before and after the addition of fluorobenzene. If fluorocatechol was toxic or inhibitory it would be expected that its accumulation in the

Figure 3.32 - The effect of ethanol and propanol concentration upon the specific OUR of the cells.

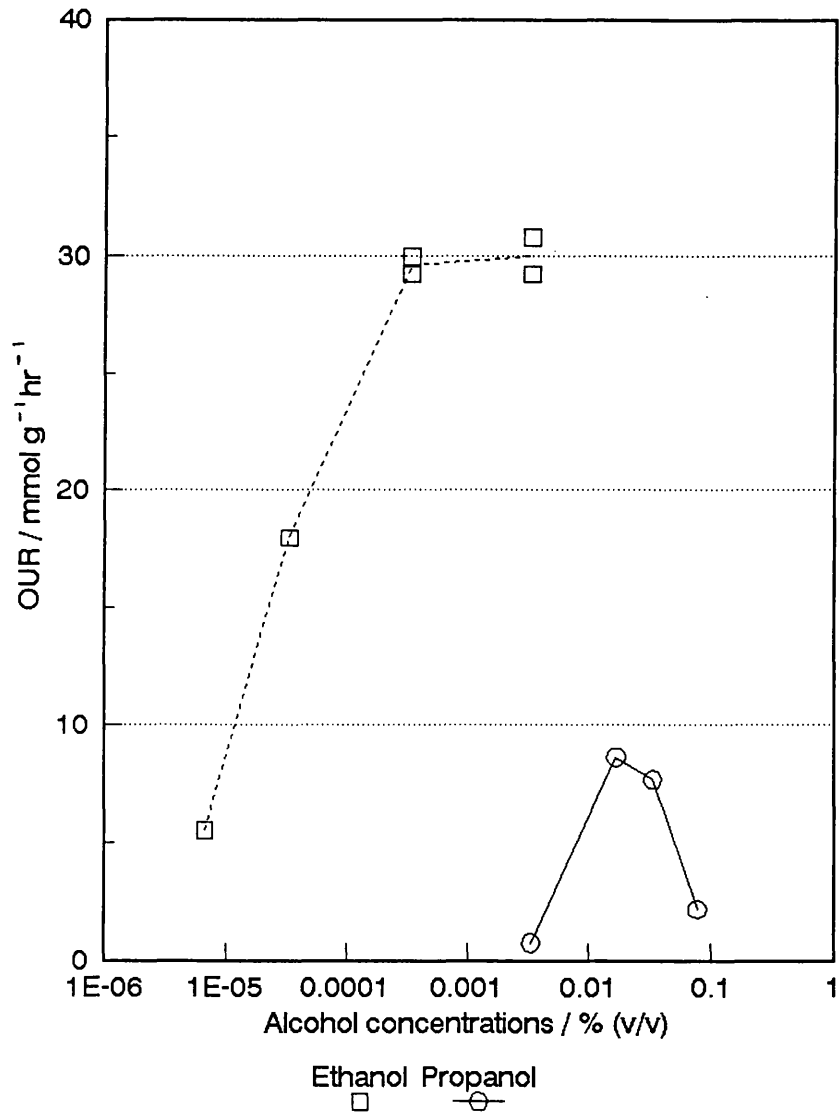


Figure 3.33 - The effect of fluorobenzene concentration upon the specific OUR of the cells.

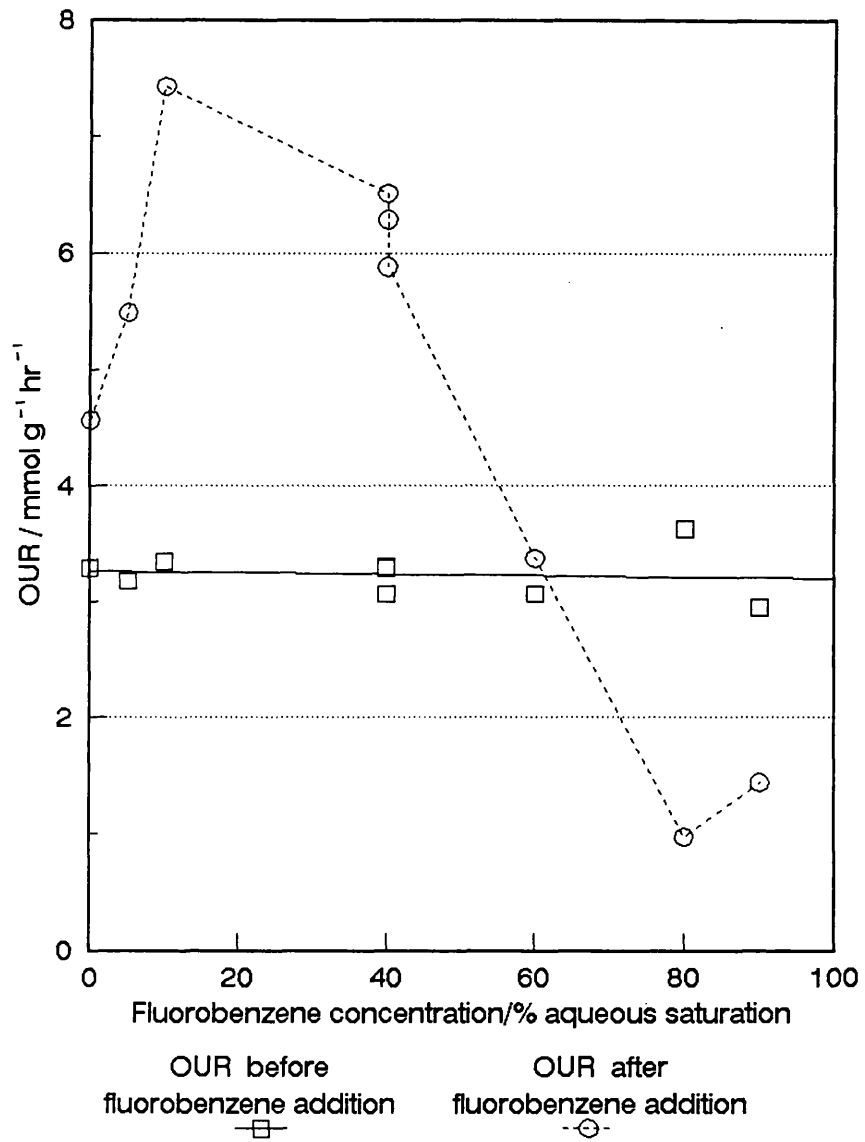
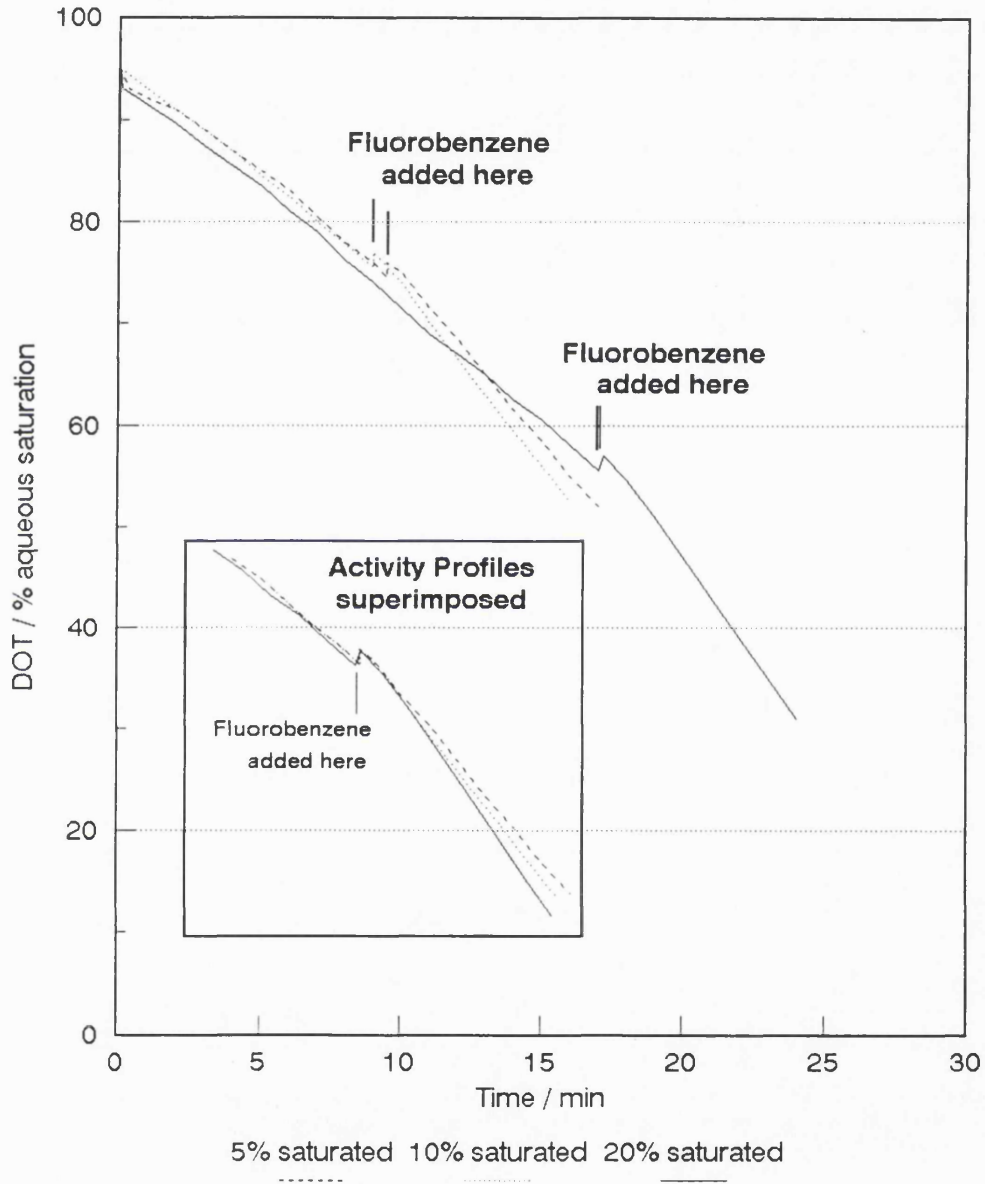


Figure 3.34 - Profiles of the DOT against time of three runs of a single sample with fluorobenzene added at different concentrations.

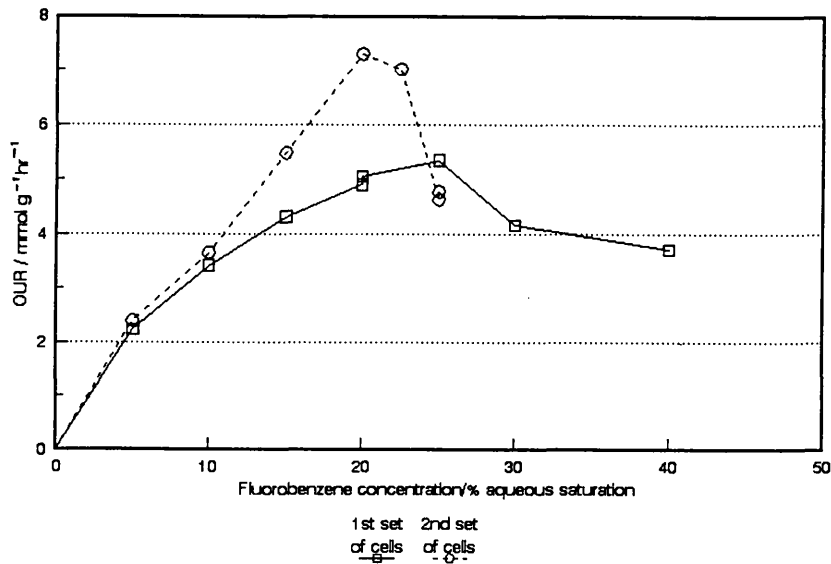


cell would result in a decrease in OUR. In these experiments the fluorocatechol did not accumulate to a sufficiently high level to become toxic. Figure 3.35a shows the profile of the activity against fluorobenzene concentration from 0 to 40% aqueous saturation of two separate shake flask cultures. Optimum activity was between 20 to 25 % aqueous saturation and was reduced below these levels. To analyse the oxidation activity, fluorobenzene was added in propanol (10 $\mu$ l total volume) to a reactor concentration of 20 % aqueous saturation. The benzene oxidation activity of bacteria grown on succinate minimal medium in shaken flask culture was also analysed. The activity upon benzene was much higher and it was also less toxic to the cells than fluorobenzene (Figure 3.35b).

Pentanol and fluorobenzene toxicity was demonstrated by the fact that the OUR which was linear for 10 mins before the solvent addition had reached zero 6 and 9 min after their addition. It is unlikely that the reduction in OUR after fluorobenzene addition was due to fluorocatechol accumulation since a linear profile was obtained for previous runs at lower fluorobenzene concentration (Figure 3.34). Aromatic compounds are known to disrupt the membrane resulting in loss of metabolic activity (Jackson & Demoss, 1965). It was therefore decided to analyse the effect of fluorobenzene upon growth. In minimal medium with succinate as carbon source, high toxicity was only achieved at a fluorobenzene concentration close to saturation (Figure 3.36a). Initial growth rates declined rapidly at near aqueous saturated concentration of fluorobenzene (Figure 3.36b). Further detailed toxicity studies upon fluorobenzene is given in Section 3.3.5.

Figure 3.35 - The effect of fluorobenzene and benzene concentration upon the aromatic oxidation activity

a) The specific oxygen uptake rates due to the addition of fluorobenzene at various concentrations below 40% aqueous saturated concentration.



b) The specific oxygen uptake rate of the cells due to benzene at various concentrations.

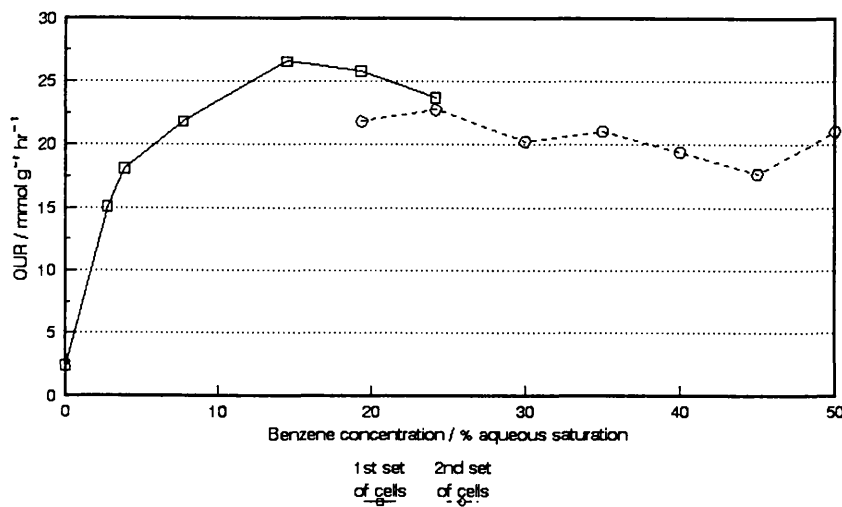
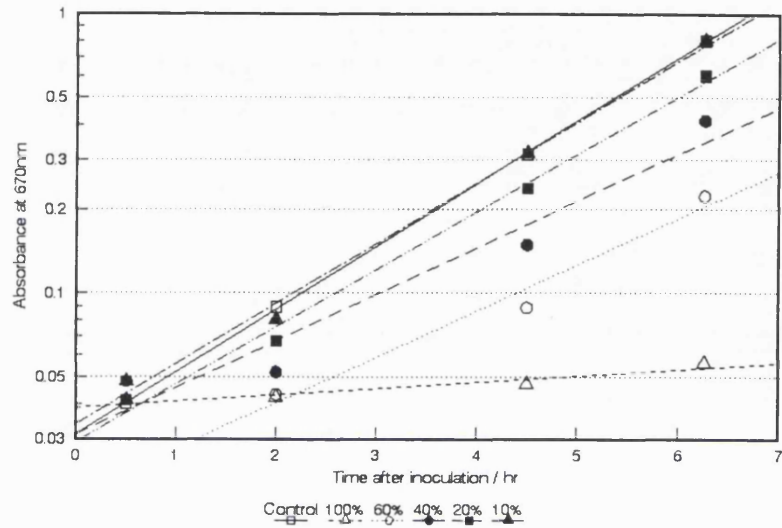
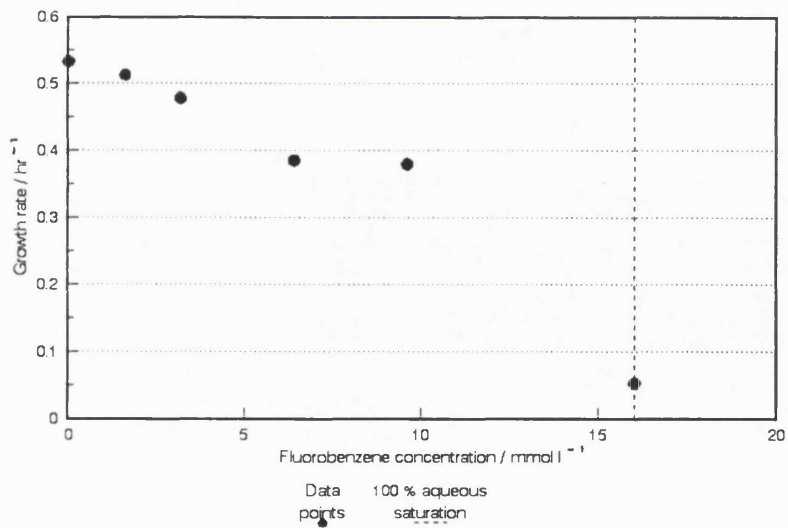


Figure 3.36 - The effect of fluorobenzene concentration upon the growth of *P.putida* ML2 in succinate minimal medium.

a) Growth profiles



b) Growth rate against fluorobenzene concentration with the aqueous saturated concentration of fluorobenzene indicated by a dashed line.



#### 3.2.1.2 - Cell concentration

The effect of cell concentration upon the aromatic oxidation activity was determined so that a suitable range of cell concentrations could be selected for the assay.

Two experiments were carried out with different cell cultures grown on succinate minimal medium. The experiments analysed the cell concentration from 0.08 to 0.48  $\text{gl}^{-1}$  and from 0.14 to 2.18  $\text{gl}^{-1}$ . At cell concentrations below 0.5  $\text{gl}^{-1}$  the activity did not vary significantly (Figure 3.37). At higher cell concentrations the aromatic oxidation activity decreased.

#### 3.2.1.3 - Stability of the Aromatic Oxidation Activity

Cells which had been grown in shake flask culture on succinate minimal medium were harvested, washed and maintained on ice over a number of days, and then analysed for retention of activity. The results are shown in Figure 3.38, black precipitates were observed in the cultures after 7 days.

During the development of the assay 5 other shake flask cultures had been analysed for aromatic oxidation activity. Direct comparison of the aromatic oxidation activity over a number of days could not be obtained due to variations in the assay procedure. In all 5 cases high activity had been retained overnight.

#### 3.2.1.4 - Effect of Propanol upon Activity

Bacteria obtained from a fermentation had lower activities than those obtained by shake flask culture. The calculated value for the aromatic oxidation activity (=OUR after addition of fluorobenzene - endogenous OUR - propanol oxidation activity) in some cases was equal to the calculated value for propanol oxidation activity. The effect of



Figure 3.37 - The effect of cell concentration upon the fluorobenzene oxidation activity.

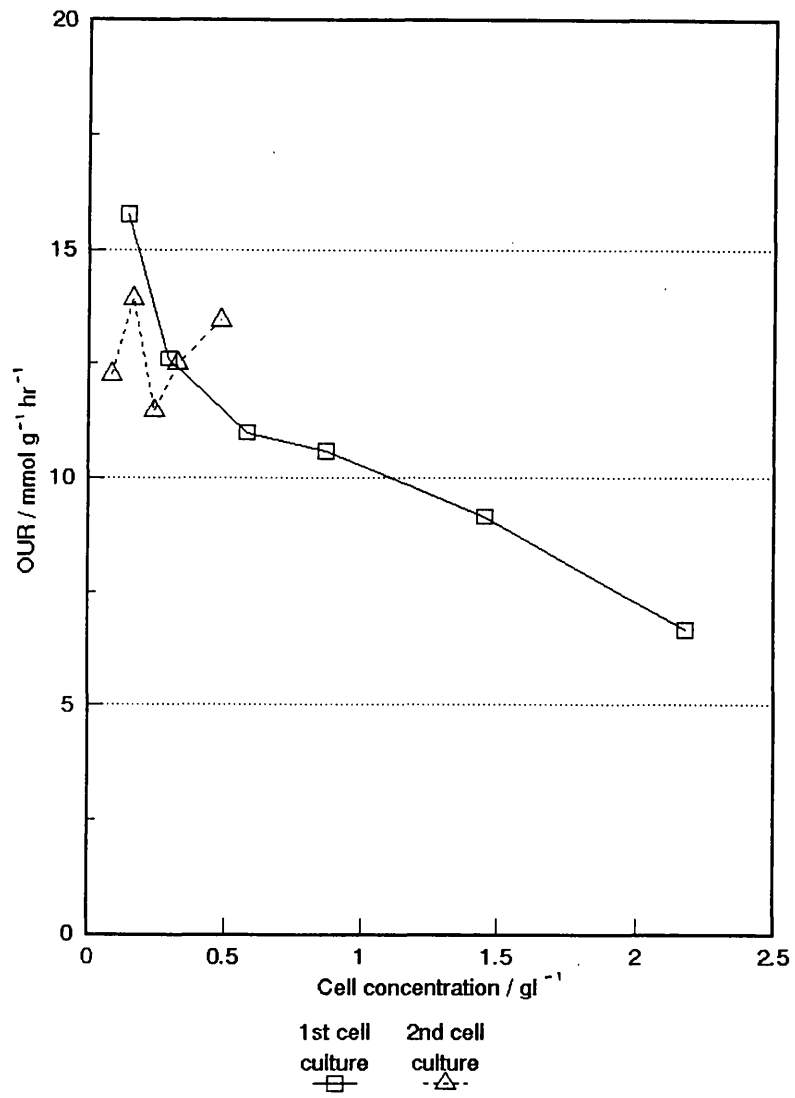
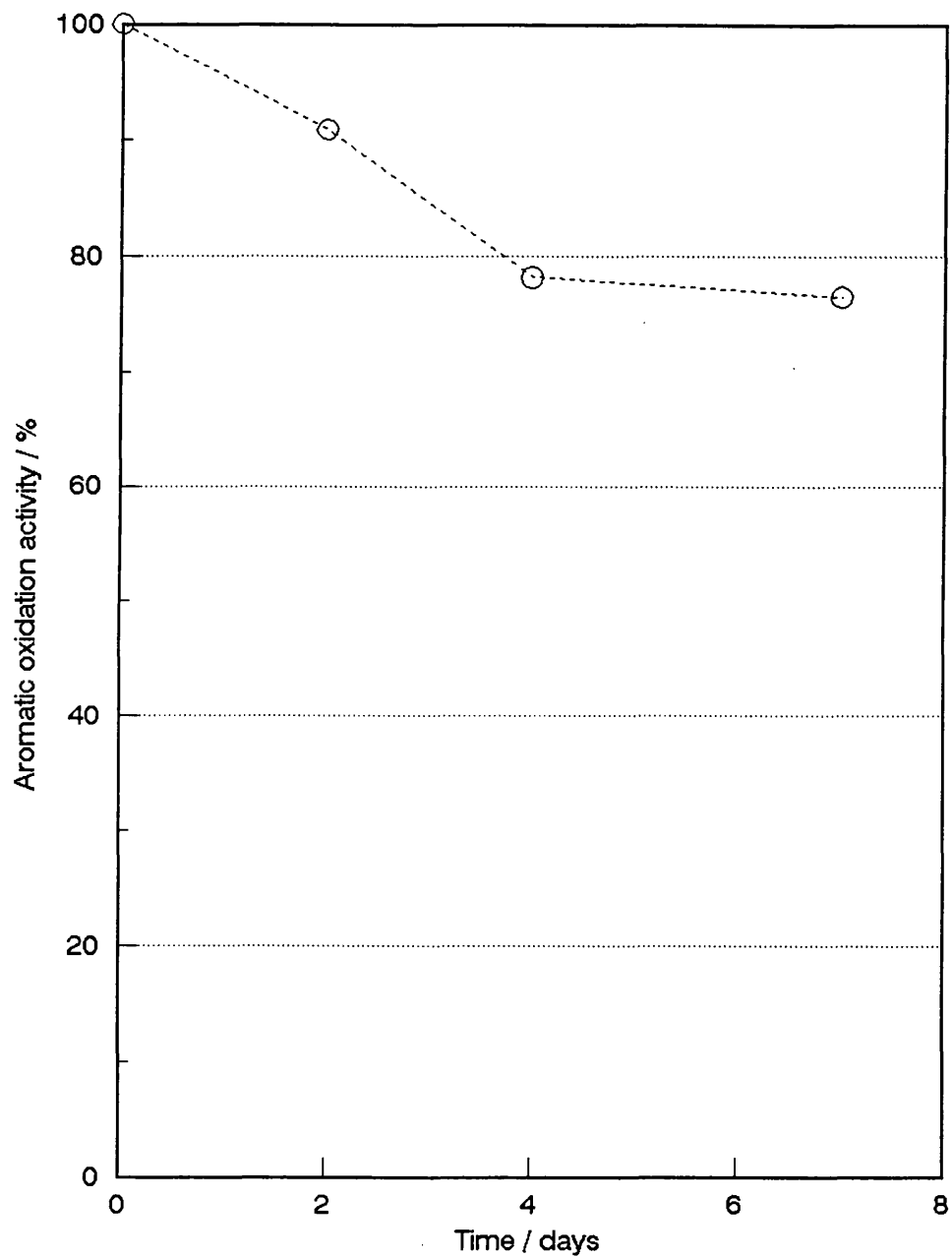


Figure 3.38 - The stability of the fluorobenzene oxidation activity of cells from shake flask culture which were stored on ice.



propanol upon the oxidation of fluorobenzene was therefore determined.

When fluorobenzene was added with propanol to the oxygen electrode chamber there was an immediate increase in DOT of 1% of saturation where as if fluorobenzene was added alone no change was observed. The OUR reached linearity 2 min after addition of fluorobenzene in propanol but took 6 min when the fluorobenzene was added neat to the electrode. This would indicate that the propanol solubilises the fluorobenzene where as when added neat droplets were formed which take time to solubilise.

Fluorobenzene was added to the electrode as a 9.1 % (v/v) solution in propanol (volume = 10  $\mu$ l). Four runs with different additions were done to determine whether propanol had an effect upon the fluorobenzene oxidation activity. In the first run 0.91  $\mu$ l fluorobenzene was added, in the second 9.1  $\mu$ l of propanol was added. The third run had fluorobenzene added as a 9.1 % (v/v) solution to the electrode in propanol, the OUR obtained was 37 % higher than the sum of the first two runs. The fourth run had propanol (9.1  $\mu$ l) added first followed shortly afterwards by fluorobenzene (0.91  $\mu$ l). The sum of the OUR of the first two runs was similar to the fourth run. Propanol did increase fluorobenzene oxidation which could be due to an increase in its rate of transfer into the cell.

As a result of these experiments, addition of fluorobenzene alone was chosen as a standard assay. It would be added close to the stirrer allowing its rapid dispersion. In all subsequent assays 1  $\mu$ l of fluorobenzene was added which corresponds to an initial concentration of 22 % aqueous saturation.

### 3.2.1.5 - Product toxicity

It was important to determine the toxicity of fluorocatechol and the need to control its level during the biotransformation. During the biotransformation, bacteria would be exposed to a rising concentration of fluorocatechol. The effect of fluorocatechol concentration upon the aromatic oxidation activity of the cells was analysed. Complete inhibition of aromatic oxidation activity was observed at a fluorocatechol concentration of  $1 \text{ gl}^{-1}$  (Figure 3.39).

The nature of the aromatic oxidation activity inhibition was determined by analysing the effect of fluorocatechol upon endogenous oxygen uptake rate. Fluorocatechol affected the oxygen uptake rate of the cells when measured in the Clark electrode (Figure 3.39). The effect of increasing fluorocatechol concentration was a rapid rise in toxicity up to  $2 \text{ gl}^{-1}$ . A further increase in concentration only resulted in small increases in toxicity.

The results in Figure 3.39 show the immediate effect of exposure of fluorocatechol on the metabolic activity. Long term effect of fluorocatechol upon the growth of the bacteria was analysed. There was a strong effect of fluorocatechol upon growth on succinate especially at  $0.25 \text{ gl}^{-1}$  or higher concentrations (Figure 3.40a). Growth occurred at a fluorocatechol concentration of  $0.1 \text{ gl}^{-1}$  but at a rate which was 56%<sup>that</sup> of the control. Cells cultured in benzene minimal medium were unable to grow unless the fluorocatechol concentration was below  $0.05 \text{ gl}^{-1}$  (Figure 3.40b). An increase in fluorocatechol concentration resulted in an increase in the lag time of the growth of the bacterial<sup>culture</sup>. At  $0.01 \text{ gl}^{-1}$  of fluorocatechol the lag time was over 20 hr which increased to above 40 hr at a fluorocatechol concentration of  $0.025 \text{ gl}^{-1}$ . It is therefore clear that fluorocatechol inhibited growth more when benzene was the carbon source rather than succinate.

Figure 3.39 - The effect of fluorocatechol upon the endogenous OUR and the specific aromatic oxidation activity of the bacteria expressed as a percentage of the control.

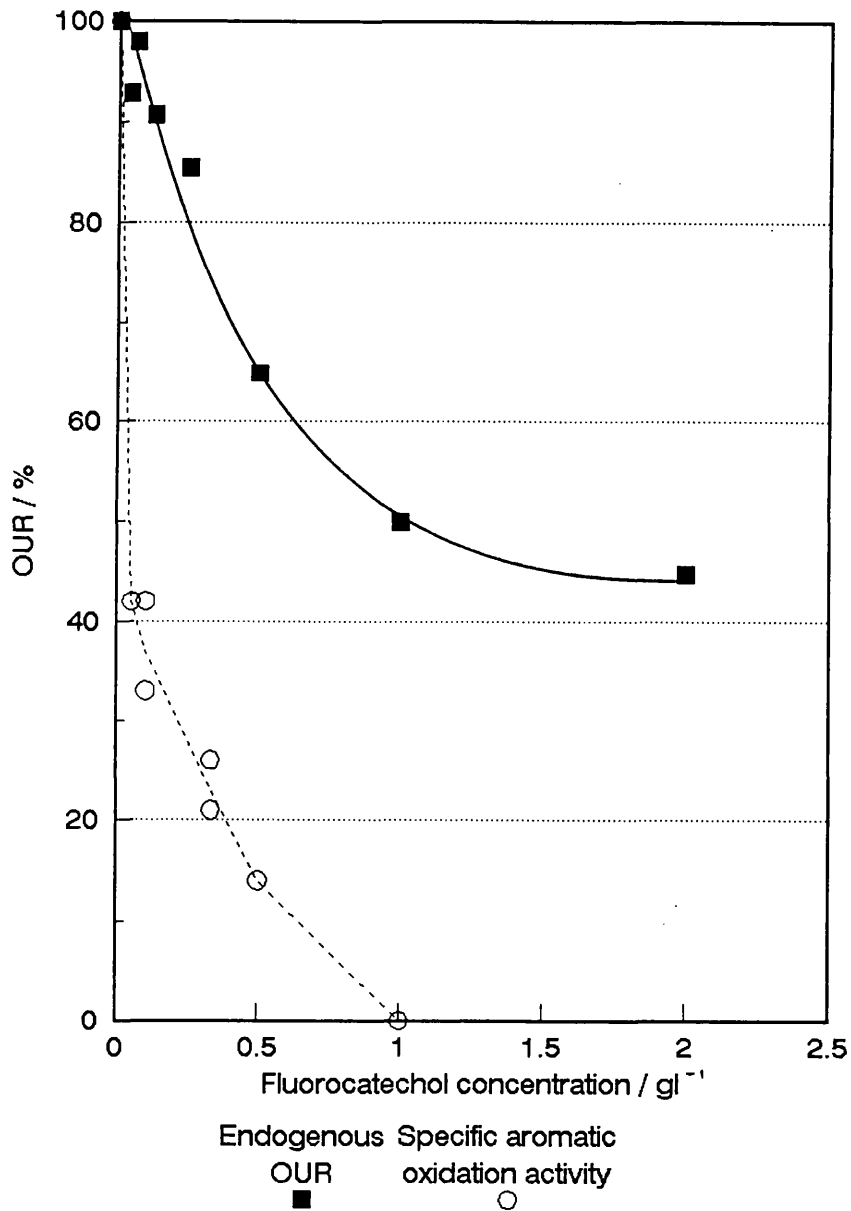
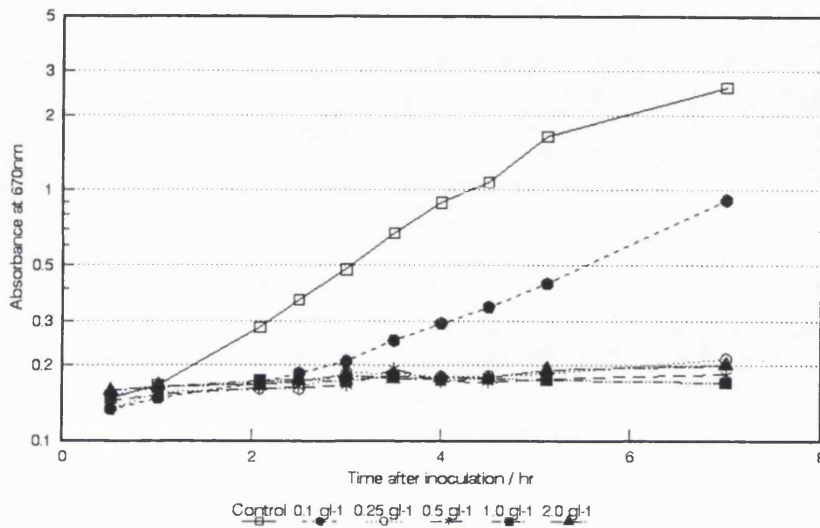
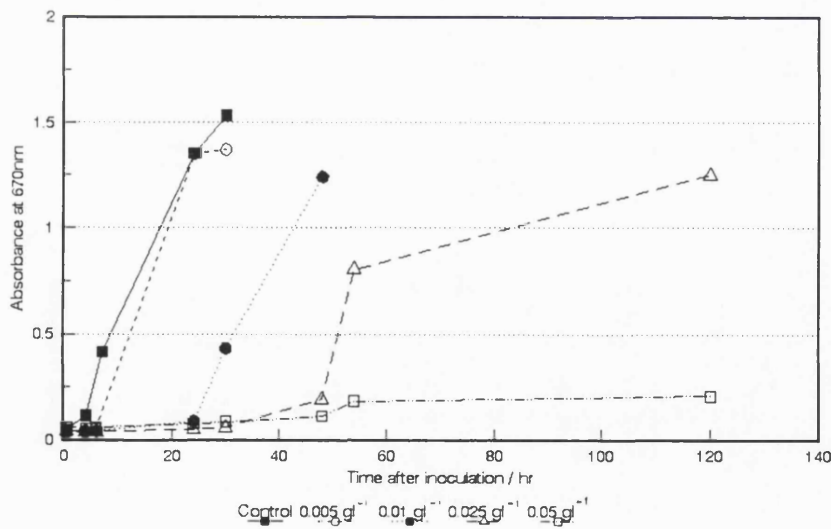


Figure 3.40 - The effect of fluorocatechol concentration upon the growth of *P.putida* ML2 in minimal medium.

a) Succinate as carbon source.



b) Benzene as carbon source.



The long term effect of fluorocatechol on non-growing cells was tested by shaking in 50mM phosphate buffer with various concentrations of fluorocatechol for up to 1.5 hr. The viability of the cells was tested by inoculating 1ml aliquots of the cell suspension into succinate minimal medium containing no fluorocatechol. Figure 3.41 demonstrates that there were no significant changes in the lag phase of the bacterium when incubated in 0.01 and 0.25  $\text{gl}^{-1}$  fluorocatechol. At  $1\text{gl}^{-1}$  the fluorocatechol was toxic to the cells but not until after 0.5 hours.

This would suggest that at concentrations of 0.1 and 0.25  $\text{gl}^{-1}$  the fluorocatechol is inhibitory towards the bacterium since it had difficulties growing in its presence, but not irreversibly toxic.

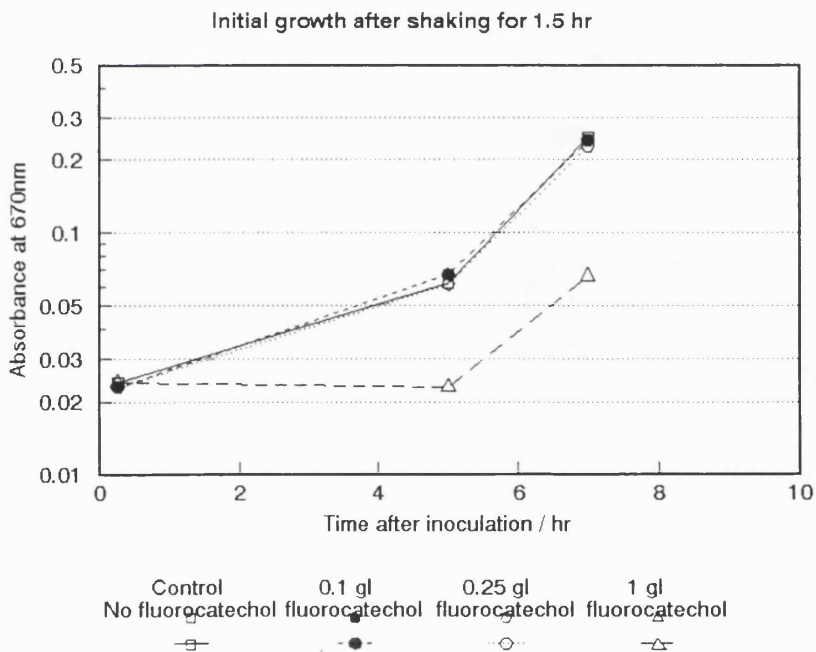
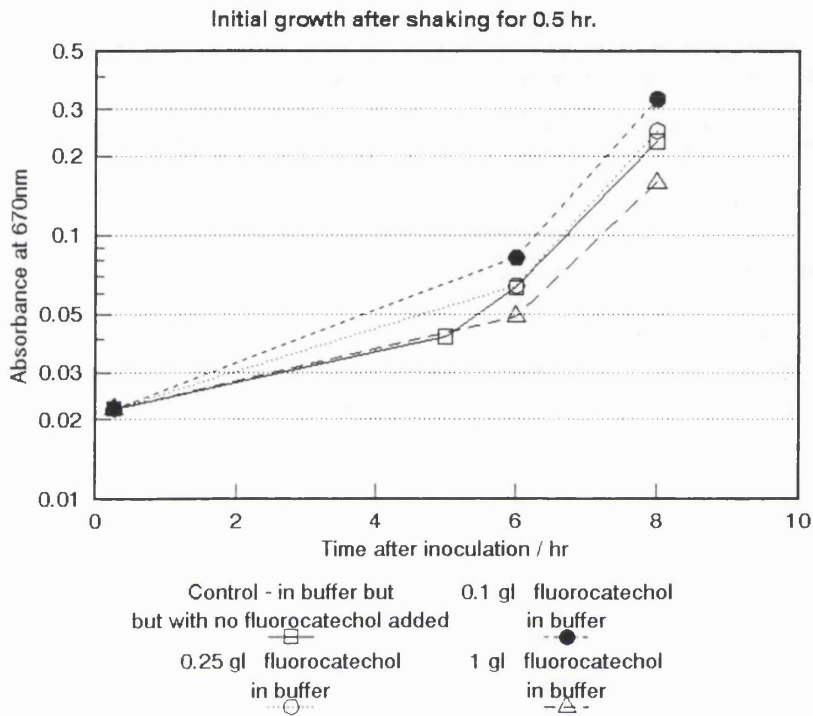
#### 3.2.1.6 - Fluorocatechol Resistance

Fluorocatechol at a concentration above 0.25  $\text{gl}^{-1}$  had inhibited bacterial growth in the first 7 hr (Figure 3.40a); the effect of fluorocatechol upon the bacterium after 24 hr was analysed. The bacterial culture had grown from a  $A_{670}$  value of 0.133 to 0.212 in the first 7 hr, after 24 hr the cells indicated a recovery since a  $A_{670}$  value of 1.95 had been reached.

The increase in absorbance could have been due to fluorocatechol denaturing to a compound which absorb light at 670nm or that the compound had denatured to a non-toxic compound which allowed the cells to grow.

An experiment was set up to determine whether P.putida ML2 which had previously been growing on succinate minimal medium containing fluorocatechol could retain tolerance to fluorocatechol once growing in a medium not containing fluorocatechol. Four different cultures were set up which had originated from the same fermentation sample.

Figure 3.41 - The initial growth of the bacteria in succinate minimal medium after shaking in buffer containing different concentrations of fluorocatechol.





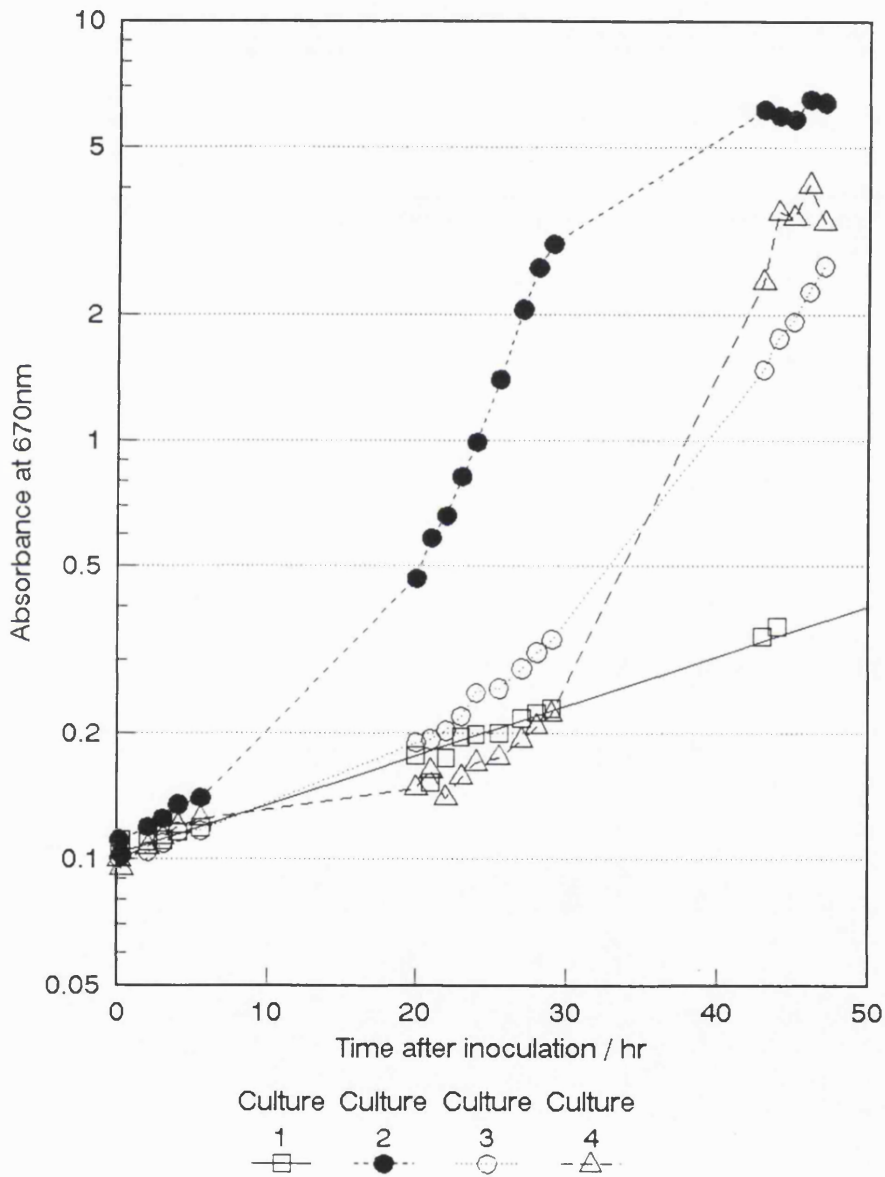
There were four stages to this experiment of which only the first stage was different. In the first stage the cultures were subjected to different concentrations of fluorocatechol. The second stage involved taking 1 ml of the culture and inoculating into benzene minimal medium for three days to allow the cells to recover from the toxicity of fluorocatechol. The third stage involved inoculating the benzene grown culture (1 ml) into succinate minimal medium and growing overnight. Each culture (1 ml) was then inoculated into succinate minimal medium containing  $0.5 \text{ gl}^{-1}$  fluorocatechol to determine whether the bacteria had retained the ability to grow in the presence of fluorocatechol.

The profiles obtained for the four cultures are shown in Figure 3.42. Culture 1 had been grown in succinate minimal medium which did not contain fluorocatechol. The growth profiles indicate that the three cultures which had been grown in succinate minimal medium containing fluorocatechol had overcome the toxicity of fluorocatechol and started to grow at a faster rate. It also demonstrates that the increase in absorbance is not due to the denaturation of the fluorocatechol while being shaken at  $30^{\circ}\text{C}$  to either a less toxic compound or to a compound which absorbs light at  $670\text{nm}$ .

Culture 2 had been grown in succinate minimal medium containing  $0.5 \text{ gl}^{-1}$  of fluorocatechol for 47 hr in stage 1. The growth profile of the bacteria in stage 4 (Figure 3.42) indicates that the cells had retained the ability to grow in the presence of fluorocatechol even when the selective pressure had been removed. A lag-time of approximately 15 hr was observed.

Culture 3 had been grown in succinate minimal medium containing  $0.25 \text{ gl}^{-1}$  fluorocatechol for 52 hr in stage 1. These strains had overcome the toxicity of fluorocatechol at a faster rate than culture 1 but not at the same rate as culture 2.

Figure 3.42 - Growth of four different *P.putida* ML2 cultures in succinate minimal medium containing  $0.5 \text{ gl}^{-1}$  fluorocatechol but had different inoculum development.



Culture 4 was formed from culture 3 in that 1 ml of culture 3 which had been cultured for 5 hr in succinate minimal medium containing  $0.25 \text{ gl}^{-1}$  fluorocatechol was subcultured into succinate minimal medium containing  $0.5 \text{ gl}^{-1}$  fluorocatechol for 47 hr. This culture had therefore initially been subjected to a low fluorocatechol concentration before moving to a higher concentration. This would allow an initial faster growth of the cells before moving to a high selective pressure. The growth profile was similar to culture 3 indicating that the same tolerant strain could of been selected.

The concentration of fluorocatechol in the first stage did not seem to affect which strains were selected if it was present at a concentration of either  $0.25$  or  $0.5 \text{ gl}^{-1}$  since culture 2 and 4 had different profiles. This would indicate that the strain selected in culture 2 was not the same as the strain with lower fluorocatechol tolerance in culture 3 or 4.

It was shown that the strain of bacterium had retained the ability to grow in the presence of fluorocatechol even though the selective pressure had been removed while the cells had been growing in benzene minimal medium followed by growth in succinate minimal medium. This would strongly suggest that this is a genetic selection of the bacterium to tolerate fluorocatechol.

#### 3.2.1.7 - Plasmid Stability

The genes coding for the proteins which contribute to the oxidation of fluorobenzene to fluorocatechol are carried on a plasmid (Tan & Mason, 1990). Before undertaking fermentation studies it was essential to check plasmid stability.

The cells were subcultured from one shake flask to another and then a diluted sample was plated on indole agar

to test for the ability to convert indole to indigo. After 13 generations all the colonies were green indicating there was no loss of benzene dioxygenase activity. After 43 and 66 generations 5 and 26% of the cells were white indicating that they had lost the benzene dioxygenase activity. Loss of benzene dioxygenase activity is usually initiated by the loss of the plasmid.

Fewer than 10 generations will occur in a small scale<sup>batch</sup> fermentation therefore it is unlikely that there will be problems due to plasmid instability.

### 3.2.2 - Fermentation controlled at 20 % DOT and at 30°C

Before examining the effects of temperature and DOT on the growth and biotransformation activity of the bacteria, it was necessary to establish the behaviour and reproducibility of fermentations carried out under standard conditions. These were selected as 30°C and 20 % DOT.

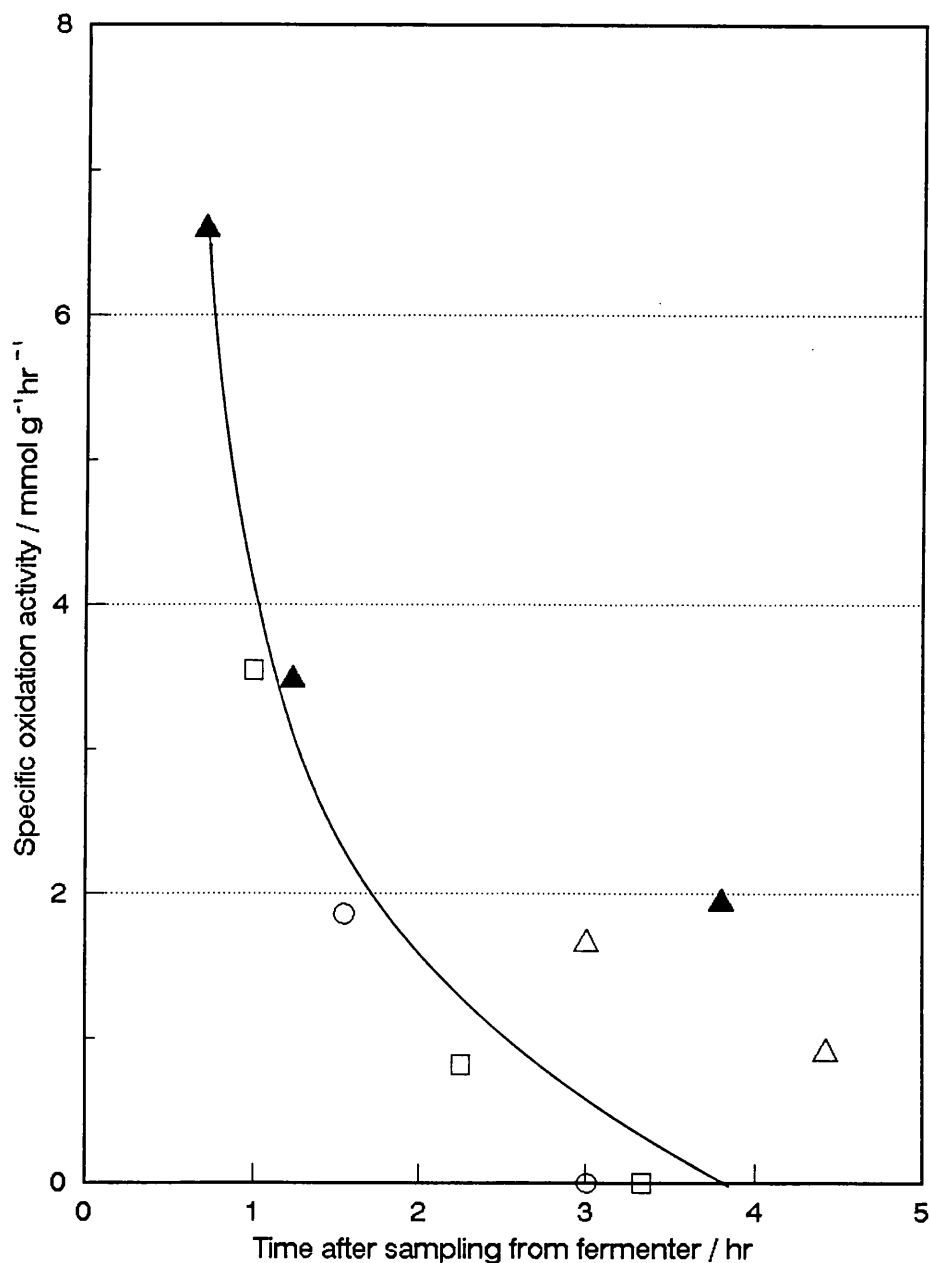
#### 3.2.2.1 - Stability of Aromatic Oxidation Activity

Initially the samples taken from this type of fermentation had shown no fluorobenzene oxidation activity. Upon further analysis it was observed that samples analysed soon after sampling from the fermenter had activity which was lost rapidly. It was clear from these fermentations that the <sup>degree of</sup> stability of the aromatic oxidation activity was crucial.

Four samples from four different fermentations were analysed for retention of activity by sampling on two or three different occasions after storage on ice. The data from these samples are given in Figure 3.43. Two of the samples had lost all their activity after 200 min which is completely different from shake flask cultures where at least 60 % of the activity can be maintained even after a number of days storage on ice (Figure 3.38). It was envisaged that combining all the data available from different samples from all the fermentations available could provide an accurate mathematical expression for the rate of loss of activity since a large amount of data was available. This was not observed due to the variations in activity of the cells which was related to the growth stage of the cells analysed.

It was therefore decided it would be essential to assay the samples as quickly as possible before activity was lost. All data presented were obtained from assays in which fluorobenzene was added within 60 minutes of sampling from the fermenter.

Figure 3.43 - Effect of storing the bacterium at 0°C on the specific fluorobenzene oxidation activity of cells from four different fermentations controlled at 30°C and 20% DOT, which were assayed after different storage times.



Time was between sampling from fermenter and adding fluorobenzene to the cells  
Data from four different samples are shown.

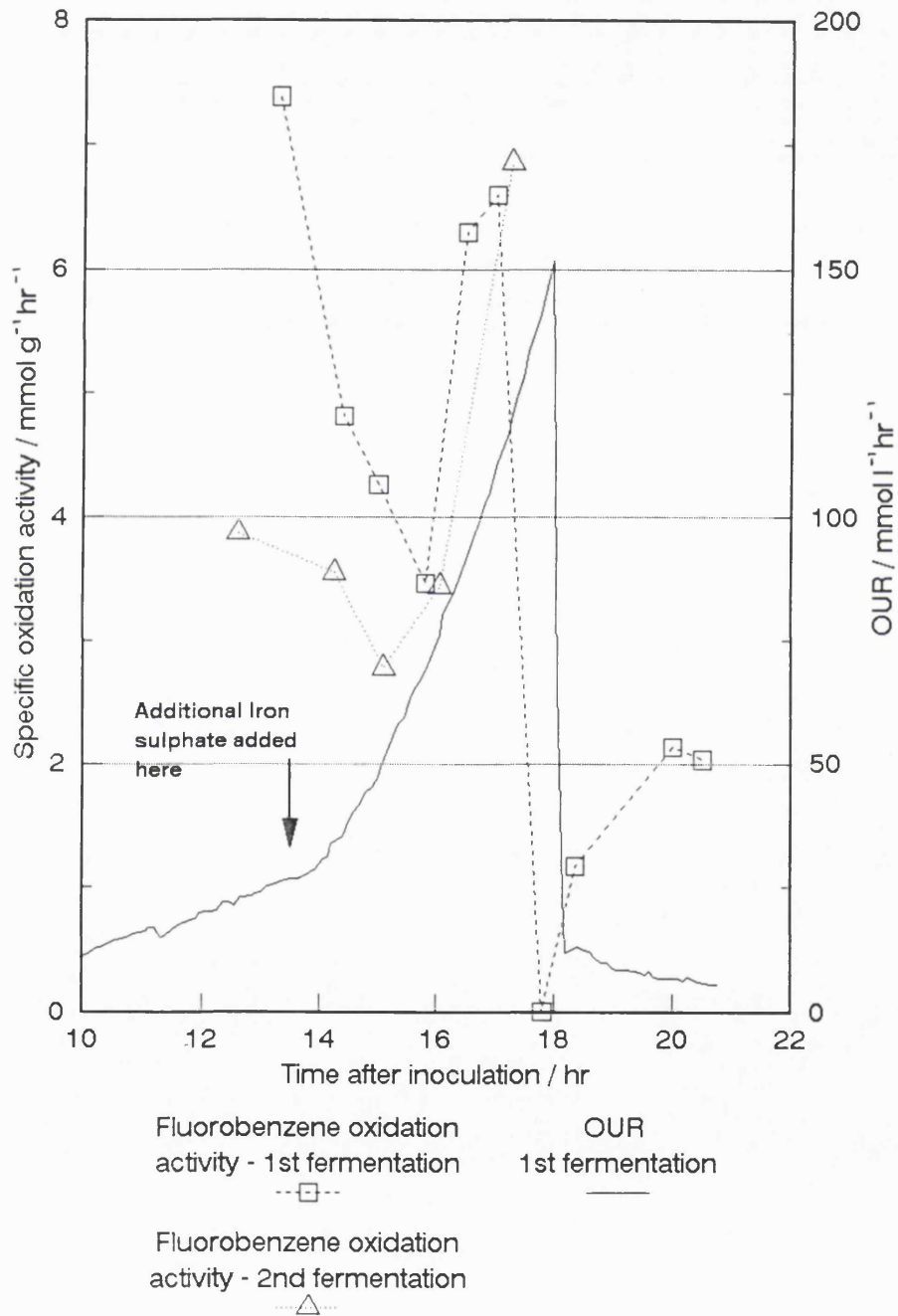
#### 3.2.2.2 - Fermentation Profile

Figure 3.44 shows the profile of the specific fluorobenzene oxidation activity of a fermentation at 30°C and 20 % DOT, the fermentation culture oxygen uptake profile from 10 hours onwards is also shown. This fermentation had become iron limited since once iron sulphate had been added the OUR increased rapidly. This resulted in the specific aromatic oxidation activity decreasing which is likely to be due to the increase in metabolic activity of the cells. Near the end of the growth phase an increase in enzyme activity is observed which could be due to nutrient limitation but once the cells have been starved of carbon all the activity is lost. Activity is regained but at a lower level when the cells are in the death phase. Figure 3.44 also shows the aromatic oxidation activity of the culture in the growth phase of a second fermentation. This fermentation is described in section 3.1.4.2. It is seen in this case that the specific fluorobenzene oxidation activity is fairly constant initially and at around 16 hr the value is similar to the first. There is again a sudden increase in activity near the end of the growth phase.

Samples taken from other fermentations include one in mid growth phase which had activity of  $5.38 \text{ mmol g}^{-1} \text{ hr}^{-1}$  and another where no detectable activity was observed soon after the end of the growth phase.

It is therefore clear from the data that the level of activity can change rapidly during the fermentations and once the cells are harvested.

Figure 3.44 - Oxygen uptake rate profile of cells grown at 30°C and 20% DOT and the fluorobenzene oxidation activity of samples taken from the fermentation.





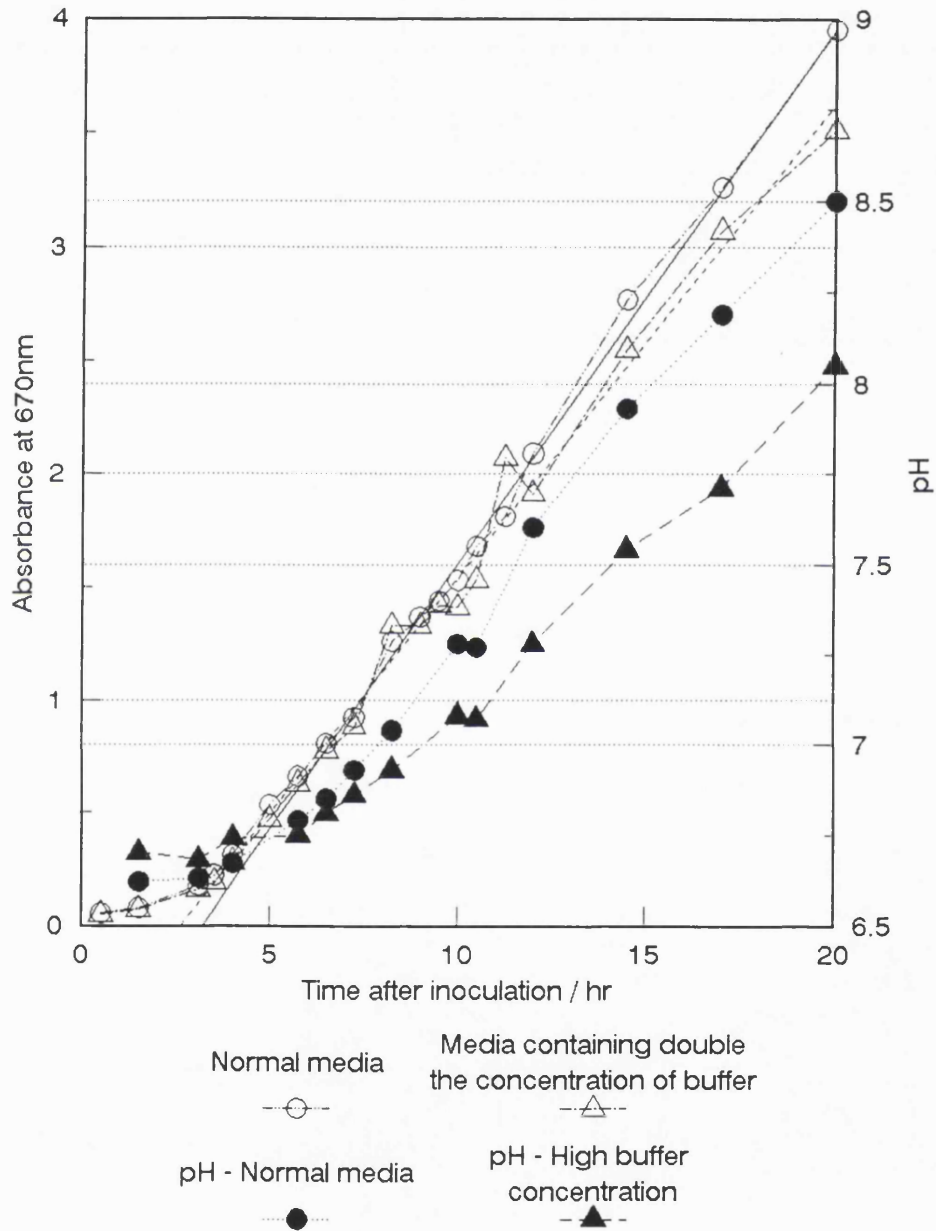
### 3.2.3 - Aromatic Oxidation Activity of P.putida in Shake Flask Culture

The rapid changes in biotransformation activity during the fermentations in the previous section and the instability of the activity posed a real problem. Shake flask cultures had retained high activity even over a number of days (Section 3.2.1.3). The growth of the bacterium in the shaken flask was therefore analysed further to determine whether the growth conditions influenced the enzyme stability. Such growth factors could include oxygen limitation, pH, changes in growth rate or a slow steady increase in biomass rather than a rapid growth phase.

Two cultures were set up with succinate minimal medium but with one culture containing double the quantity of buffer to improve pH control. Flasks were inoculated with 1 ml of culture which had grown on benzene overnight. The growth of the cells was exponential from 1.5 to 5 hr after inoculation. The specific growth rate was similar for both cultures at  $0.55 \text{ hr}^{-1}$ . Figure 3.45 shows the growth of the bacteria in shake flask with the two different media. It is seen that there is very little difference in the yield and the growth profile between the two cultures. The linear growth observed after five hours indicated that the cells had become oxygen limited. Changes in pH were observed but did not seem to have a major effect upon the rate of increase of biomass. The rates obtained were  $0.236$  and  $0.209 A_{670} \text{ hr}^{-1}$  which is equivalent to  $0.109$  and  $0.096 \text{ gl}^{-1} \text{ dry cell mass hr}^{-1}$ . The lower rate of increase in biomass was for cultures grown in medium containing<sup>a</sup> higher buffer concentration. This could be due to slower transfer of oxygen due to the higher salt concentration or the specific yield of cells per mole of oxygen was lower.

The stability of the oxidation activity of the cells which were stored on ice was also analysed for both cultures.

Figure 3.45 - Growth of *P.putida* ML2 in succinate minimal medium with normal and double amounts of buffer to analyse the effect of pH upon growth.



Activity was detected in the cultures even after approximately 150 min storage on ice. It was observed that there were large variations in the fluorobenzene oxidation rates. These variations were thought to be due to the concentration and washing stage since the cells had to be centrifuged in a Microcentrifuge rather than the refrigerated MSE Highspeed centrifuge. The samples also had to be concentrated 24 fold to obtain a high enough cell concentration for the assay. This had resulted in large variations in OUR observed for endogenous respiration and activity upon propanol. The sample taken 10.5 hr after inoculation had activities of 5.2, 6.7 and 8.2 mmol g<sup>-1</sup> hr<sup>-1</sup>. The OUR due to endogenous respiration and propanol were high at 77, 77 and 84% of the total oxygen uptake after addition of fluorobenzene in propanol.

The stability of the aromatic oxidation activity in shake flask culture was likely to be due to the cells becoming oxygen limited. The experiment was not repeated but a number of fermentations would be carried out to determine the effect of varying the oxygen concentration upon the growth and aromatic oxidation activity. In a fermentation a larger number of variables such as DOT, OUR and CER could also be analysed to allow better characterisation of the metabolism.

#### 3.2.4 - Changes in Dissolved Oxygen Tension at 30°C

##### 3.2.4.1 - Fermentation controlled at 5% DOT

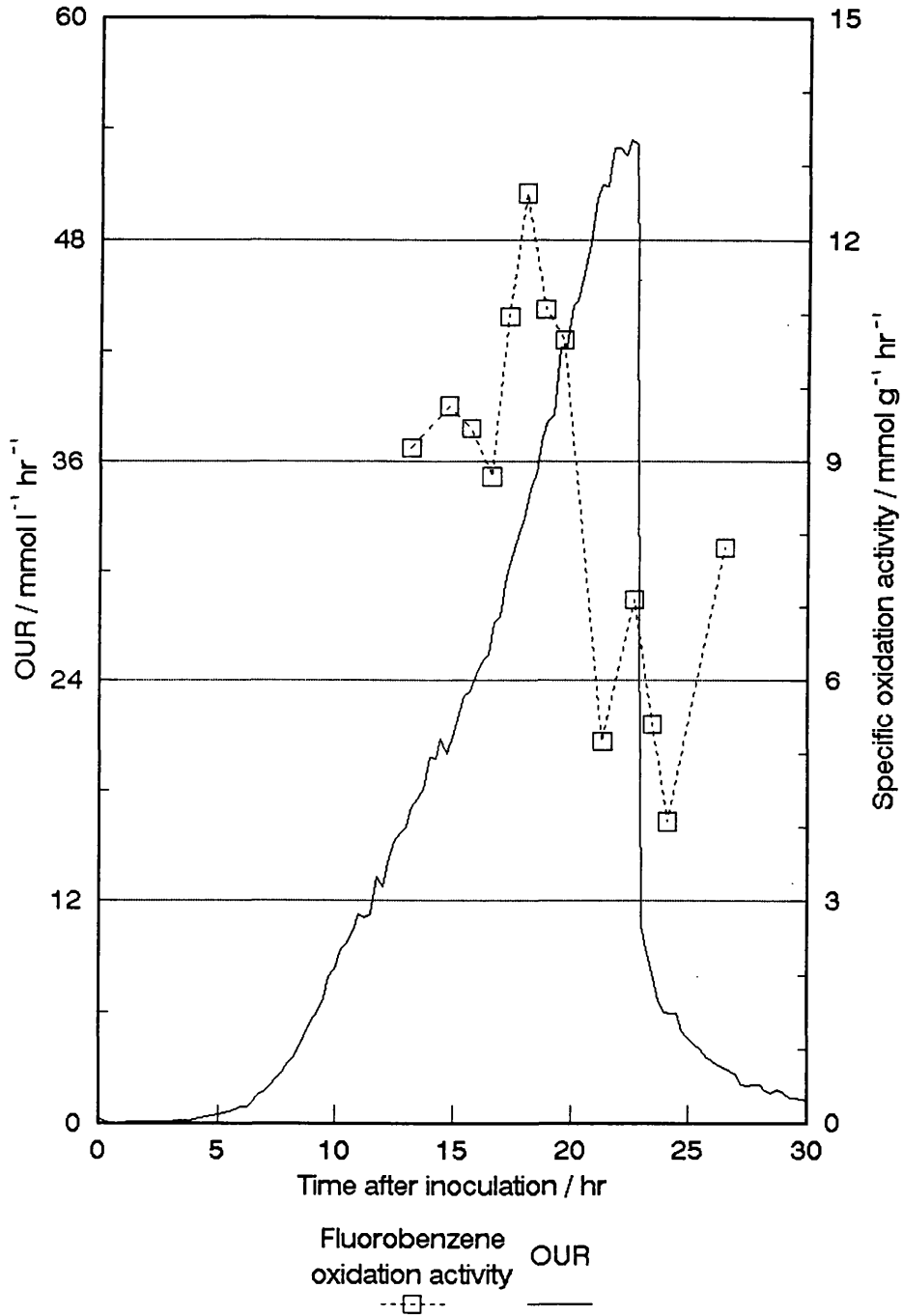
It was shown in section 3.1.4.1 that there were two phases to the growth of the bacterium when the DOT was controlled at 5% aqueous saturation. The second phase of growth started around 15 hr after inoculation. The profile was otherwise similar to the fermentations controlled at 20% in that the growth phase comes to an abrupt halt once the carbon source has been exhausted.

Figure 3.46 demonstrates that the growth conditions and growth phase have a major effect upon the enzyme activity. Close to the end of the growth phase the enzyme drops dramatically from 12.6 to 5.2 mmol g<sup>-1</sup> hr<sup>-1</sup>. During the growth phase in a period of 3.4 hours the activities of the cells were between 8.79 and 9.75 mmol g<sup>-1</sup> hr<sup>-1</sup> but afterwards the activity increased to 12.6 mmol g<sup>-1</sup> hr<sup>-1</sup>. During the end of the growth phase and continuing into the death phase the activity of the cells did change but the reasons why such changes were seen would be difficult to explain. A sample taken 39 hours after inoculation had a fluorobenzene oxidation activity of 5.87 mmol hr<sup>-1</sup> g<sup>-1</sup>.

Two samples had been analysed to determine whether growth at 5 % DOT (30°C) had increased the stability of the aromatic oxidation activity. The sample taken 13.2 hr after inoculation had an aromatic oxidation activity of 9.18 mmol g<sup>-1</sup> hr<sup>-1</sup> with fluorobenzene added 53 mins after sampling. The sample had been kept on ice and analysed 248 mins after sampling and had an activity of 2.93 mmol g<sup>-1</sup> hr<sup>-1</sup> which is 32 % of the activity 53 mins after sampling. The sample taken 26.5 hr after inoculation was analysed 40 mins after sampling to give an activity of 7.81 mmol g<sup>-1</sup> hr<sup>-1</sup> which then dropped to 50 % of this value (3.9 mmol g<sup>-1</sup> hr<sup>-1</sup>) 93 mins after sampling. Significant increase in stability of the aromatic oxidation activity had not been observed.

Variations in the aromatic oxidation activity were observed due to the difference of the controlled oxygen concentration.

Figure 3.46 - Fluorobenzene oxidation activity of *P.putida* ML2 samples taken from fermentation controlled at 30°C and 5 % DOT, the fermentation OUR profile is also shown.



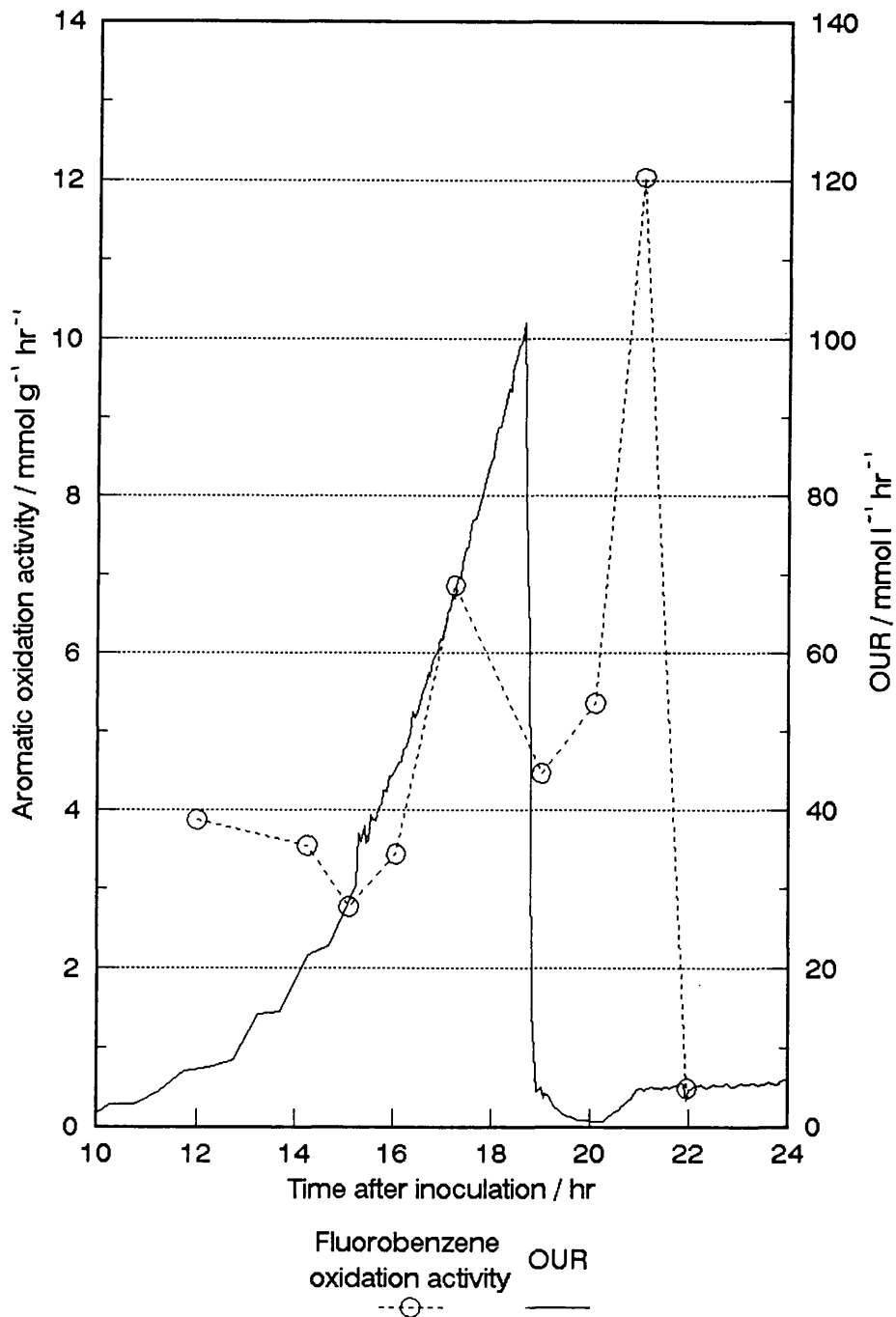
3.2.4.2 - Aromatic oxidation activity of the cells grown at 30°C and 20 % DOT with transition to zero DOT at the end of the growth phase.

Cells grown in a shake flask became oxygen limited as shown by a linear growth curve (Figure 3.45) <sup>from 5 hr after inoculation onwards.</sup> A fermentation was set up to look at the effect of zero DOT upon the aromatic oxidation activity of the cells. The cells were initially grown at 20 % DOT to obtain high biomass prior to moving down to zero. The fermentation is described in detail in Section 3.1.4.2.

The samples taken from the fermentation 12 to 22 hr after inoculation were analysed for aromatic oxidation activity (Figure 3.47). The activities in the first 18 hr have already been compared to the profile obtained from the control in Section 3.2.2.2. In the first 2.5 hr after the DOT was decreased to zero the aromatic oxidation activity increased from 4.5 to 12.0 mmol g<sup>-1</sup> hr<sup>-1</sup>. Once the DOT was increased from 0 to 0.1 % aqueous saturation the aromatic oxidation activity decreased from 12.0 to 0.5 mmol g<sup>-1</sup> hr<sup>-1</sup>. The DOT was controlled at 0.1 % DOT and the aromatic oxidation activity 43 hr after inoculation ( 22.9 hr after DOT moved to 0.1 % ) was 2.242 mmol g<sup>-1</sup> hr<sup>-1</sup>.

A second fermentation was also analysed for aromatic oxidation activity after taking the DOT from 20 % to 0. The average activity of the three samples prior to decreasing the DOT to 0 was 2.803 mmol g<sup>-1</sup> hr<sup>-1</sup>. The activity of the cells 16.35 hr after moving the DOT to zero was 2.725 mmol g<sup>-1</sup> hr<sup>-1</sup>. No major changes in activities were observed.

Figure 3.47 - OUR profile of fermentation controlled at 20 % DOT which is then dropped to zero and the resulting effect upon the fluorobenzene oxidation activity of the samples taken.



#### 3.2.4.3 - The effect upon the aromatic oxidation activity of moving from 20 % DOT to a low $K_a$ during the growth phase.

The fermentation was set up to look at the effect of low oxygen transfer rates upon the aromatic oxidation activity. The fermentation characterisation is discussed in detail in Section 3.1.4.3. The OUR and the aromatic oxidation profile is shown in Figure 3.48. The activity is initially high since it was iron limited but once this limitation had been removed the activity goes to zero. An increase in the aromatic oxidation activity was observed once the cells had become oxygen limited. The activities from 18.1 to 21.5 hr after inoculation then decreased to zero, at this stage DOT spikes were observed (Figure 3.21). The aromatic oxidation activity had once again increased, 22 hr after inoculation, the DOT spikes at this stage had decreased to a small and similar size.

The aromatic oxidation activity has been shown to be sensitive to changes in the metabolism of the cells.

#### 3.2.4.4 - Unstable DOT

One of the fermentations at 34°C had poor DOT control. Large oscillations in the DOT were observed which were regularly spaced and spanned a range of 20 % DOT. The DOT profile obtained between 15 and 25 hr after inoculation is shown in Figure 3.49. This fermentation was followed further to determine the effect of the DOT oscillations upon the growth and aromatic oxidation activity of the cells without them becoming oxygen limited.

The oscillating CER and OUR values of the fermentation were totaled to obtain a correlation between the total oxygen consumption, total carbon evolved and the biomass achieved. The cells consumed 90.3 mmol oxygen (g biomass)<sup>-1</sup>



Figure 3.48 - The aromatic oxidation activity of the samples taken and the OUR of the fermentation initially at 20 % DOT but is taken to low oxygen transfer rates near the end of the growth phase.

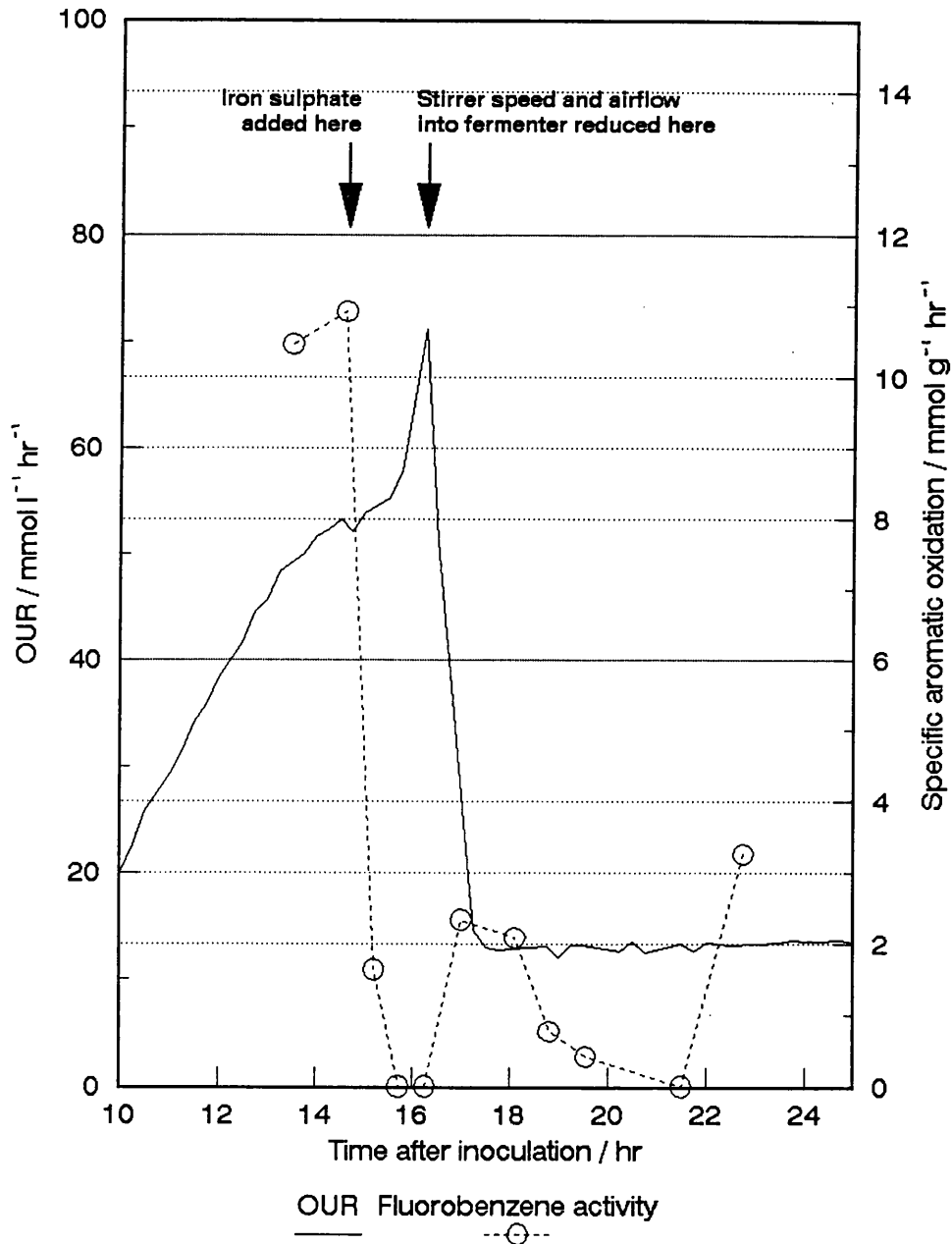
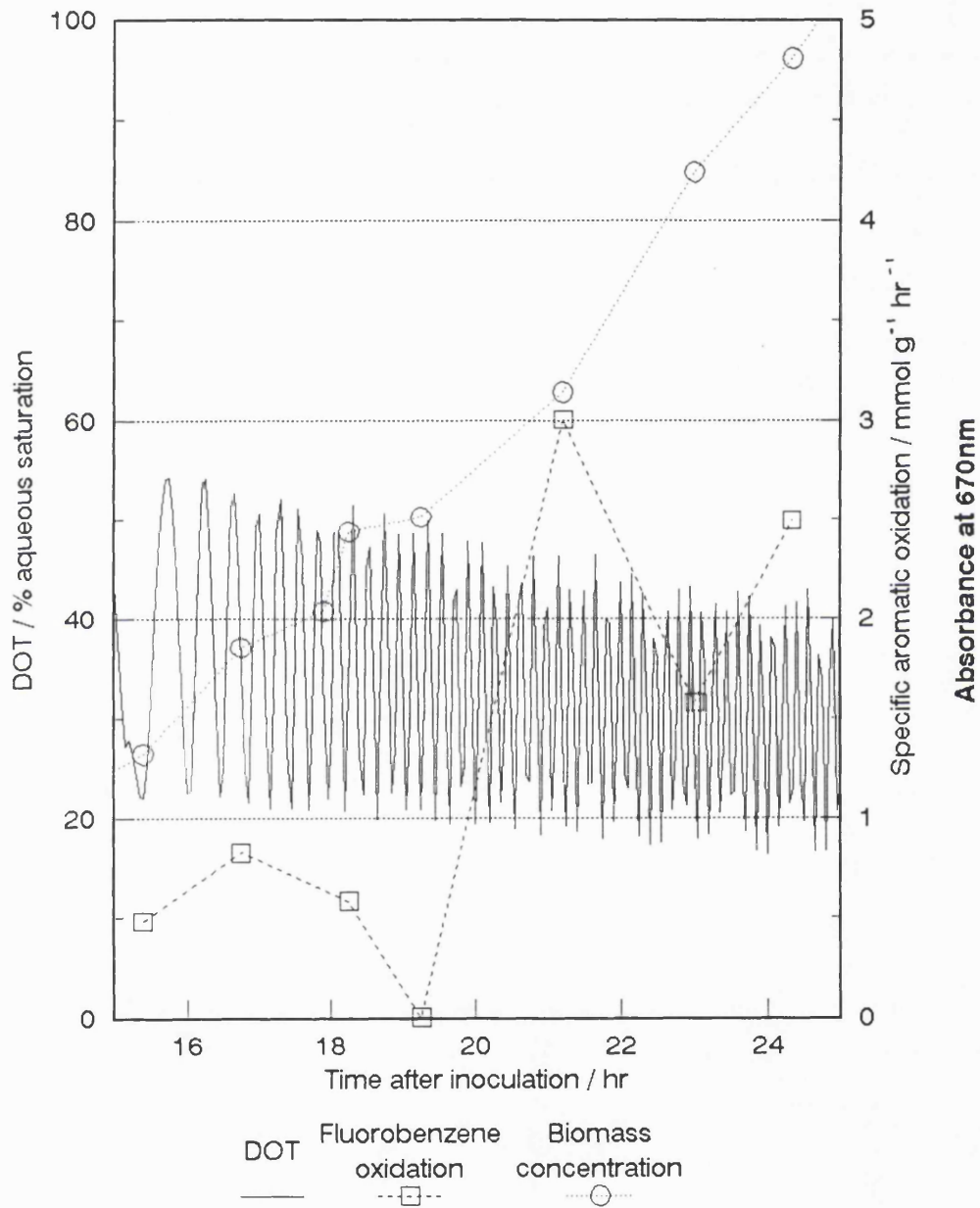


Figure 3.49 - The DOT, specific aromatic oxidation activity and biomass profile from 15 to 25 hr after inoculation of the fermentation at 34°C and with oscillating DOT.



and evolved 120.3 mmol carbon dioxide (g biomass)<sup>-1</sup>. An RQ value of 1.33 was therefore obtained.

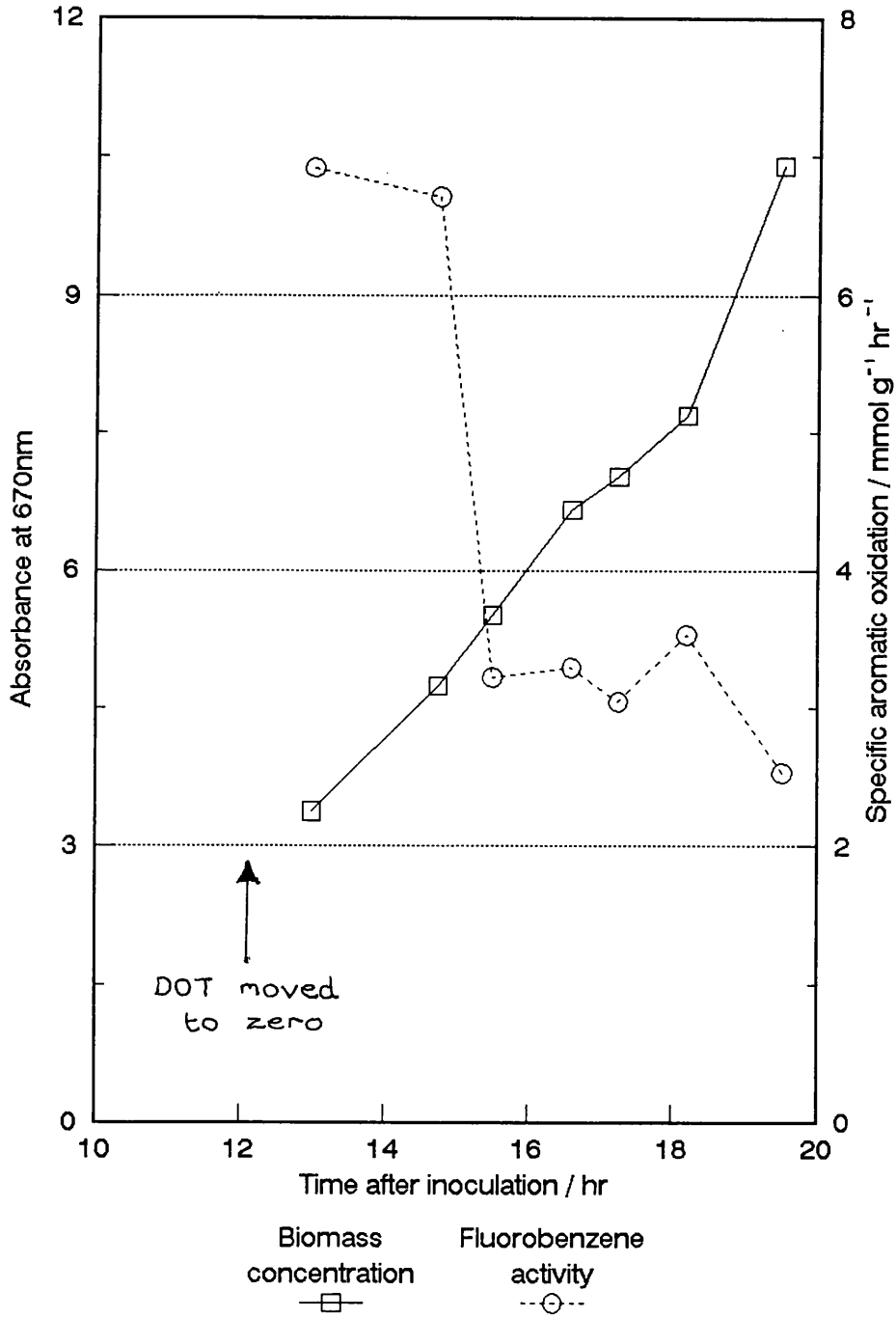
The aromatic oxidation activities of the cells sampled from 15 to 25 hr after inoculation are shown in Figure 3.49. The activity of the cells are unstable which could be a result of the changes in cellular metabolism due to the oscillating DOT. The aromatic oxidation activity of the cells sampled 47.25 hr after inoculation was 4.2 mmol g<sup>-1</sup> hr<sup>-1</sup>. The sample was reanalysed 24 hr later after storage upon ice and had aromatic oxidation activity of 5.6 mmol g<sup>-1</sup> hr<sup>-1</sup>. The activity had therefore increased which was a result of the cold storage. Instability in the growth of the cells could be a factor in achieving stable oxidation activity.

#### 3.2.4.5 - The aromatic oxidation activity of the cells from the fermentation at 20 % DOT with transition to zero DOT for a short period of time during the growth phase.

In the first fermentation with glucose and fructose as carbon source the DOT had reached zero before increasing again to 40 %. The effect of taking the DOT to zero from 20 % and then returning to 20 % DOT was analysed to determine the effect of the stress upon the aromatic oxidation activity and growth of the cells.

During the fermentation the culture was taken to zero DOT for a short period of time (less than 6 mins) in the middle of the growth phase (12.15 hr after inoculation). It was unlikely that this short period of anaerobic condition would lead to cell death or the accumulation of byproducts. The biomass and the specific aromatic oxidation rate profiles are shown in Figure 3.50. Growth was much slower than in the fermentations controlled at 20 % DOT throughout the growth phase since these had stopped around 17 hr after inoculation. The growth rate from 13 to 16.6 hr after inoculation was 0.19

Figure 3.50 - The biomass and specific aromatic oxidation activity of the samples from the growth phase of the fermentation controlled at 20 % DOT which was reduced to 0% DOT for a short period of time.



hr<sup>-1</sup> which is 54 % lower than the fermentation controlled at 20 % DOT. The specific activity of the cells was high soon after the DOT had been moved down to 0, but after 3 hr the activities had dropped to 3 mmol g<sup>-1</sup> hr<sup>-1</sup> which is similar to cells grown at 20 % DOT throughout.

The fermentation showed that the activity of the cells increased dramatically when the cell growth had been inhibited for a short period of time. The activity decreases rapidly afterwards and returns to a value similar to the fermentations controlled at 20 % DOT.

### 3.2.5 - Effect of changing temperature

#### 3.2.5.1 - 26°C growth temperature

The growth of the cells at 26°C and 20% DOT was analysed in detail in Section 3.1.5.1. The growth curve was smooth and similar in shape to the fermentations controlled at 30°C and 20% DOT. The profile of the specific fluorobenzene oxidation activity is shown in Figure 3.51 and is compared to the OUR of the fermentation culture. The shape of the fluorobenzene activity is similar to that obtained at 30°C. In the fermentation at 30°C the bacteria had aromatic oxidation activity ( $6.59 \text{ mmol g}^{-1} \text{ hr}^{-1}$ ) upto 0.95 hr before the end of the growth phase where as in this fermentation the activity had reached zero at an earlier time of 2.15 hr before the end of the growth phase.

The reason for the drop in activity before the end of growth phase could be due to a short period of time in which DOT control was lost. Figure 3.51 shows that during this time (i.e. between 14 and 16 hr after inoculation) improvements in the DOT control was obtained. This was due to the tuning of the proportional, integral and derivative values of the controller. The increase in activity corresponded to the decrease in DOT which could be due to the cells entering a less favourable growth condition. The activity then decreased dramatically once the cells had come out of the less favourable growth due to the improvement in DOT control. It is likely that these variations in DOT levels are not the sole reason for the changes in enzyme activity observed but could of induced the earlier drop in activity before the end of the growth phase.

#### 3.2.5.2 - 34°C growth temperature

The fermentations are described in detail in section 3.1.5.2. A profile for this type of fermentation was not obtained but the samples analysed of the two fermentations are shown in Figure 3.52 and compared to the CER profile.

Figure 3.51 - OUR and DOT profile of the fermentation at 26°C and 20 % DOT and the fluorobenzene oxidation activity of the samples taken from the fermentation.

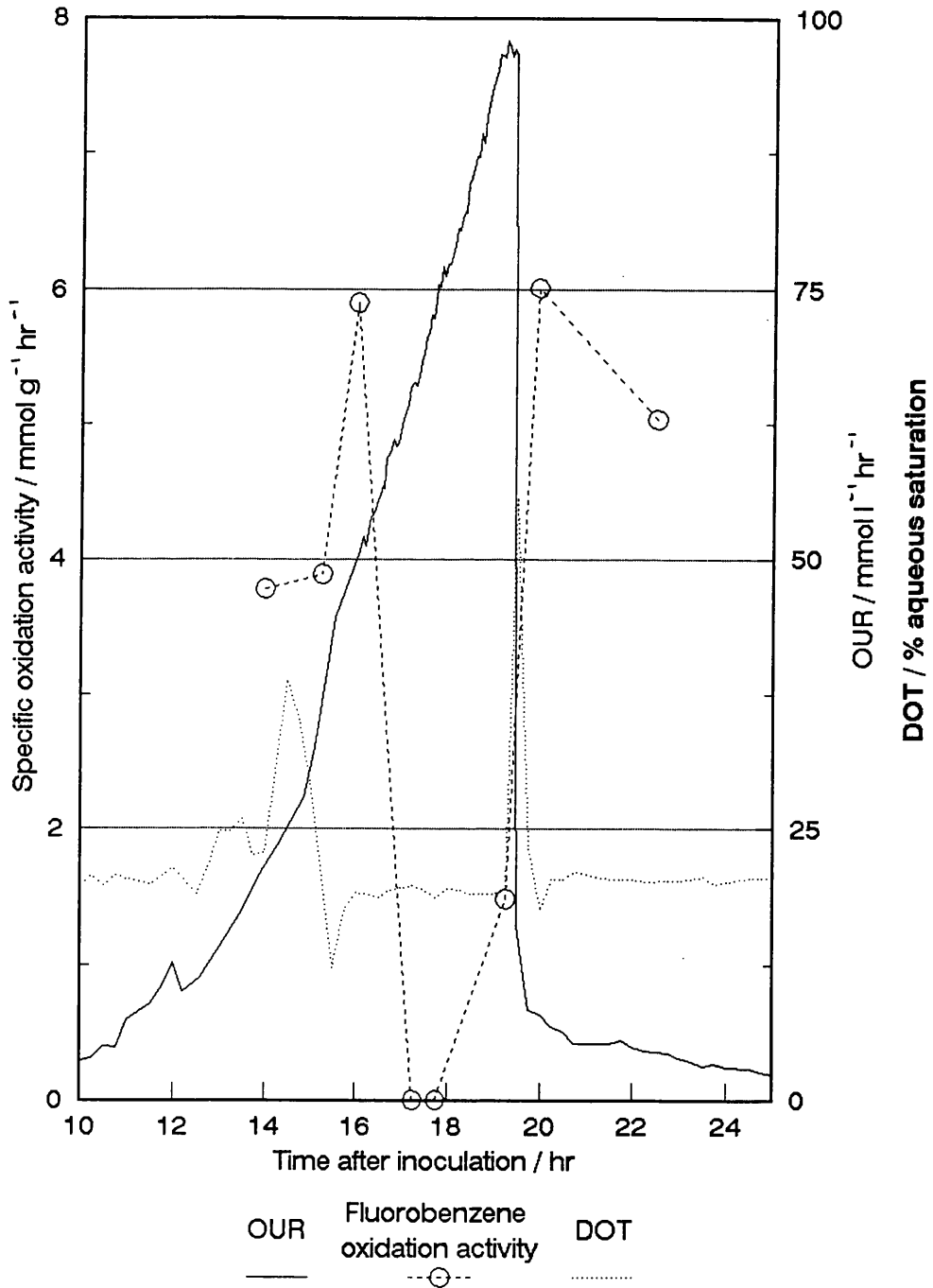
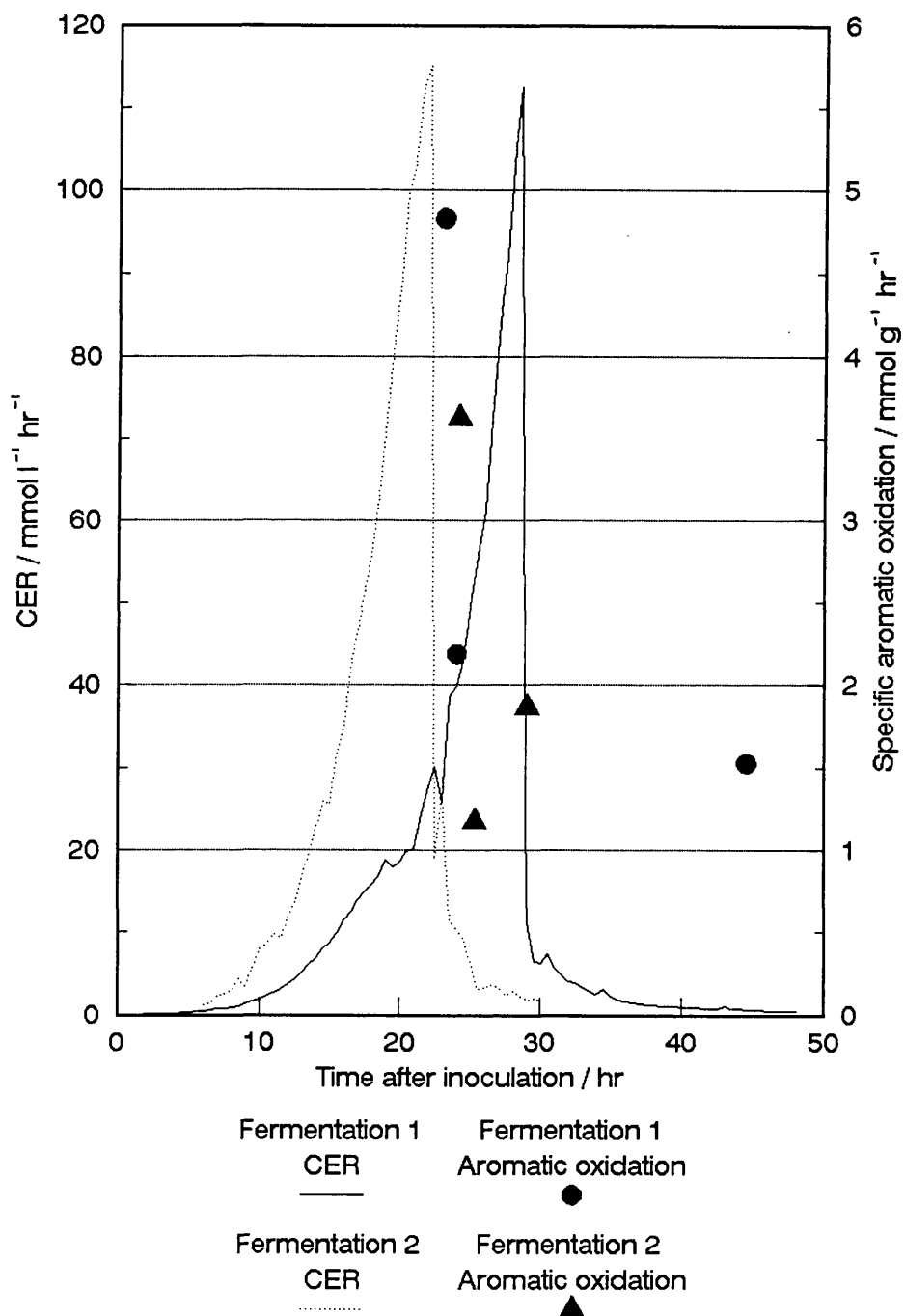


Figure 3.52 - Profile of the aromatic oxidation activity of samples taken from the two fermentations controlled at 20% DOT and 34°C.





These values are similar to the fermentations at 30°C and 20 % DOT.

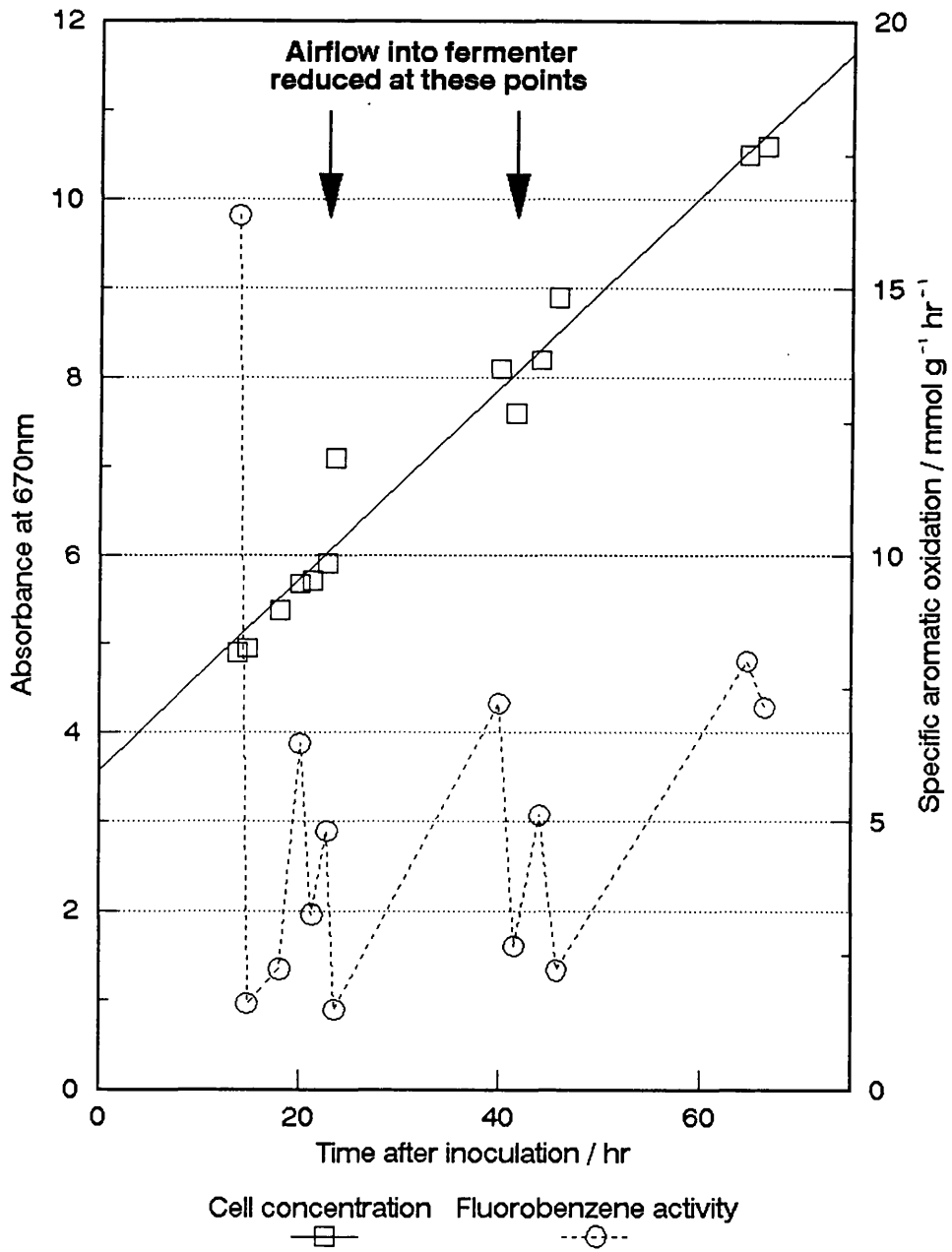
#### 3.2.6 - Carbon limited growth

The carbon limited fermentation was described in detail in Section 3.1.6. The system was not stable due to the neutralised succinate being pulsed into the fermenter and not at a steady flow. Significant variations in the metabolism of the cells were then observed.

The aromatic oxidation activity of the cells are shown in Figure 3.53 with the biomass profile of the fermentation culture. These values were similar to cells grown with succinate added in bulk and controlled at 20% DOT and 30°C. The activities are seen to be very unstable indicating that the cell metabolism influence the activity enormously. The changes in metabolism are a result of the pulsing of succinate into the fermenter but it is also seen that the metabolism is also likely to be affected by the DOT level. The first sample analysed had high activity which could of been due to the higher initial instability observed since the controller was attempting unsuccessfully to control the DOT at 20%. The stirrer speed and airflow rate were then fixed to reduce this instability.

The effect of the oxygen transfer rate upon the activity of the cells was also analysed. No major differences were observed when the airflow rate was reduced 22.75 hr after inoculation. The activity profiles were similar although the DOT profile had changed significantly therefore the instability of the enzyme was a result of the pulsing of the succinate into the fermenter rather than the resulting variation in DOT levels. After 60 hours the enzyme activity of two samples were high which could be due to the low airflow rate since the OUR of the fermentation culture had also decreased. It was seen that these activities were not

Figure 3.53 - Profile of the cell concentration of the carbon limited fermentation controlled at 30°C and the aromatic oxidation activity of the samples taken.



as high as those observed for the fermentation controlled at 5 % DOT.

The carbon limited cells had retained aromatic oxidation activity and therefore carbon starvation may not be the only reason why the cells lose their activity at the end of the growth phase of a normal batch fermentation. The fermentation proves again that the activity of the cells is highly dependent on their metabolic activity.

### 3.2.7 - Iron limitation

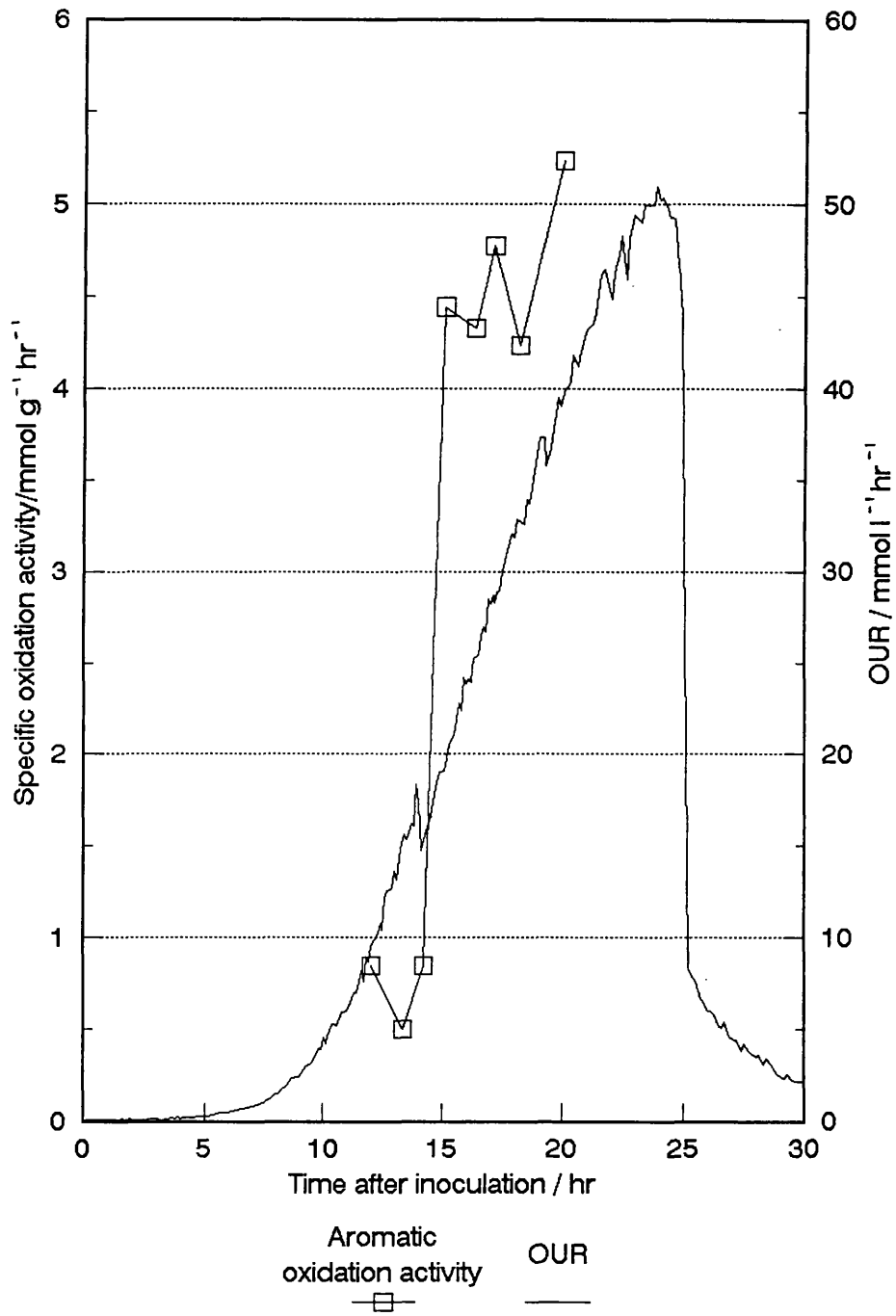
In some fermentations growth had been limited by another nutrient since the OUR profiles were different from the fermentations controlled at 30°C and 20 % DOT. This nutrient limitation was seen to be iron sulphate since upon its addition to the culture a rapid increase in OUR readings were obtained. The OUR profiles of two such fermentations are shown in Figure 3.54. Once iron sulphate had been added to the fermentations the aromatic oxidation activity of the cells decreased rapidly. The initial aromatic oxidation activities of these cells were higher than the cells not limited by iron.

The OUR and biomass profile of a third fermentation had a similar shape to the OUR profile of the fermentations which were not limited by iron but had finished after 25 hr and not 17 hr after inoculation. The aromatic oxidation activity of the cells clearly indicates that the cells have low activity at the start of the growth phase which increases to high level at the end (Figure 3.55). The activities of cells before 15 hr after inoculation were lower than  $1 \text{ mmol g}^{-1} \text{ hr}^{-1}$  but afterwards the activities increased to above  $4 \text{ mmol g}^{-1} \text{ hr}^{-1}$ .

The fermentation OUR profile was broken by a rapid decrease of 3 to  $4 \text{ mmol l}^{-1} \text{ hr}^{-1}$  at around 14 hr after



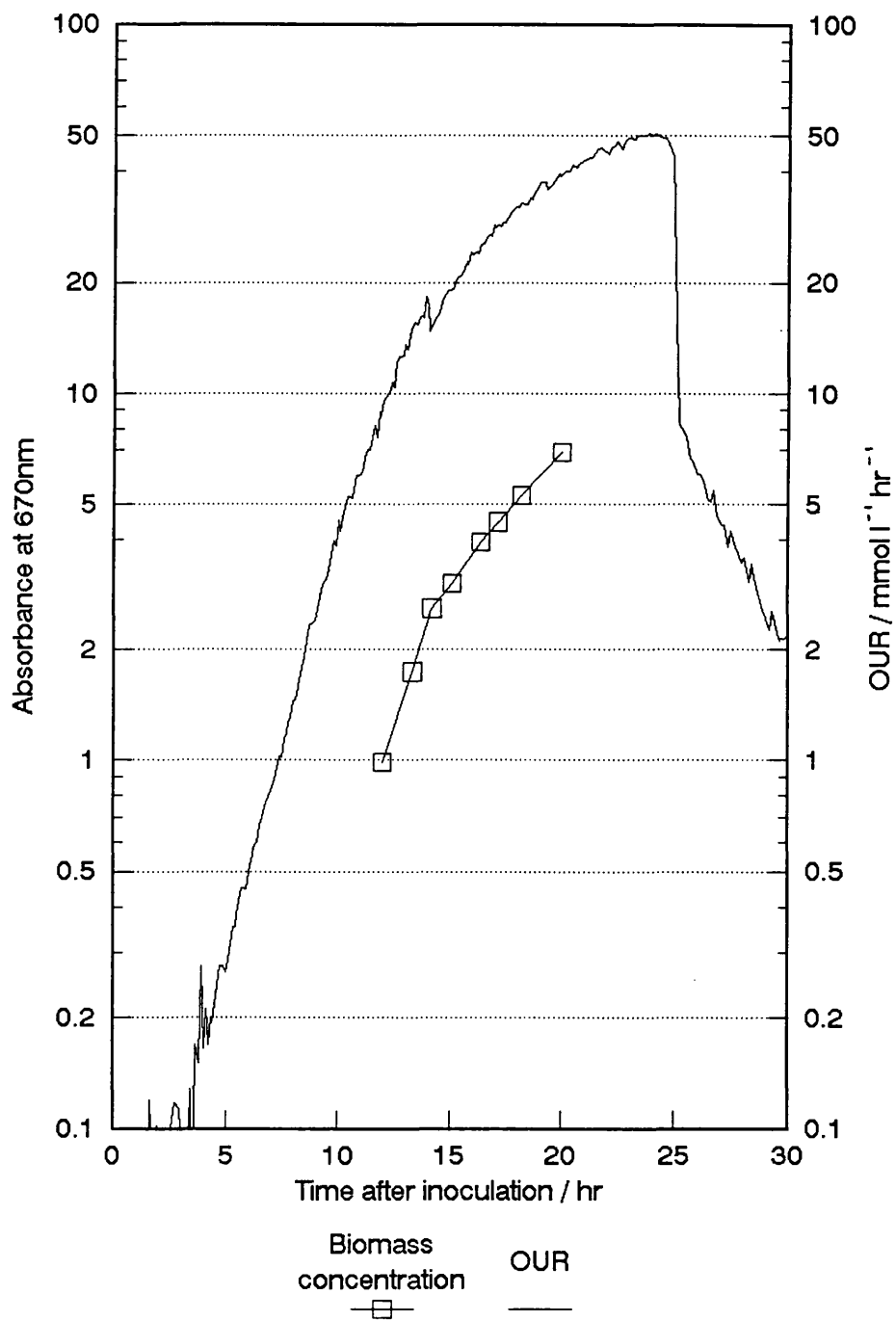
Figure 3.55 - The specific aromatic oxidation activity and the OUR profile of the iron limited fermentation at 30°C and 20 % DOT.



inoculation. The OUR then continued to increase from this point onwards. This strongly suggests that there is a change in the metabolism around 14 hr after inoculation. The OUR and biomass profile were plotted on a logarithmic scale (Figure 3.56) and clearly demonstrate the change in carbon metabolism of the bacteria from 15 hr after inoculation onwards.

Iron limitation had slowed down the growth of the cells resulting in an increase in the aromatic oxidation activity of the cells.

Figure 3.56 - The OUR and biomass profile of the iron limited fermentation at 30°C and 20 % DOT expressed on a logarithmic scale to detect changes in metabolism.



### 3.3 - Solvent tolerance

The biotransformation capability of the organism under different growth conditions has been studied in Section 3.2. The growth profiles from the fermentations have also been analysed in detail in Section 3.1. This section will consider how the growth conditions has affected the solvent tolerance of the bacterium.

#### 3.3.1 - Assay development

The solvent tolerance of an organism has previously been studied by analysing the loss of biocatalytic activity due to the solvent toxicity. Both the naphthalene hydroxylation (Harrop,1990) and steroid dehydrogenation (Hocknull,1989) involved substrate and products which were non-toxic. A number of solvents with a log P lower than 4 inhibited these reactions. The Gram - bacterium was shown to have greater solvent tolerance than the Gram + bacterium for solvents with a log P from 4 to 10 (Harrop et al,1989). The fermentation conditions and the point of harvesting for each organism were similar which resulted in a similar biotransformation activity. In this study the fermentation conditions were changed and the biocatalyst harvested at different stages in the fermentation. The assay of fluorobenzene oxidation activity was seen to be unsuitable for this part of the work due to the following reasons:-

- a) Variations in the stability and level of the aromatic oxidation activity have already been seen which are due to the changes in the fermentation. These variations would have to be taken into consideration when calculating the loss of activity and would lead to numerous problems.
- b) The assay is complex and time consuming and therefore the number of samples that could be taken would be restricted. It would not be possible to store the organism since cells from different stages of growth could change



differently under the same storage conditions.

This assay was deemed as unsuitable for this type of work.

Since it was not possible to analyse the solvent tolerance of the bacteria by the effect of solvents upon their biocatalytic activity, it was necessary to develop an alternative assay. Ideally such an assay would be simple and not manually exhaustive so that a large amount of data could be obtained by running a large number of assays at the same time. Suitable parameters (e.g. viability) had to be selected instead of biocatalytic activity as indicators of the loss of cellular activity. A number of parameters were selected before the assay could be fully developed around them.

Viability<sup>testing</sup> is a common microbiological technique and was therefore considered first. Three types of assays were looked at but they had the following problems:-

- a) In all three assays the maintenance of a sterile environment is a hindrance and it would be difficult to use the small reactors as described by Hocknull (1989). Suitable alternative reactor vessels would include shake flasks or Universal bottles.
- b) The standard viable cell count is based on the ability of a single viable cell to form a colony upon solid agar plate. The method requires accurate dilution of the cells prior to spreading onto the solid agar. It is therefore labour intensive and would result in a small amount of data upon the solvent tolerance of the bacteria from each fermentation. A total cell count would also be required so that a % viability could be obtained.
- c) The slide culture method initially developed by Postgate et al (1961) but further developed by Fry

and Zia (1982) was considered. This had an advantage over the standard viable cell count in that the majority of the procedure occurred some time after the fermentation had finished and would give a % viability. The culture was mixed with melted agar and then plated on a glass slide and covered with a cover slip. The next day the number of single cells and microcolonies were counted by analysing the slide by phase contrast microscopy and would correspond to the number of non-viable and viable cells. Problems were encountered with the small size of the bacteria in that it was difficult to distinguish between the non-viable cells and particulate matter.

- d) Viability measured by dye uptake was considered but it was envisaged that the solvents would have a major effect in disrupting the assay. Dye uptake due to changes in membrane permeability alone and not the complete loss of metabolic activity is considered further on.

Viability was not considered as a suitable parameter to analyse.

During the fermentation succinate uptake could be indirectly measured by the acid addition to the fermenter to control pH. In these assays pH could not be maintained but activity of the cells could be analysed by the changes in pH due to succinate uptake. The increase in pH could be determined by either taking readings with a pH probe or following the change in colour of a universal pH indicator. The following problems made the assay unsuitable:-

- a) Problems were encountered with the development of a suitable medium. It was not buffered so as to allow a large shift in the pH. It was kept simple but resulted in cells unable to grow in this medium. Succinate uptake was therefore low and rapid results could not be obtained.

- b) The assay had to be carried out in sealed containers (e.g. shake flasks or universal bottles) so as to prevent solvent loss. Due to the necessary high uptake of succinate to give a sufficient pH change the cells became starved of oxygen.
- c) Sampling of the bottles to obtain pH readings could only occur once since this would result in a change in oxygen concentration once the sealed environment had been broken. The colour variation from one pH to another was small therefore errors would occur in visibly determining the pH.

Proteins have been shown to be released from cells once they have been disrupted with solvents (DeSmet et al,1978). The effect of a solvent upon a bacterium could be determined by analysing the amount of protein released into the supernatant. Examination of the absorption spectra of the supernatant from 220 to 280nm was considered. The protein concentration can be calculated from the absorption values at 228.5 and 234.5nm since nucleotides absorb the same amount of light (Ehresmann et al,1973). This was unsuitable since buffer saturated with hexanol had an  $A_{220}$  and  $A_{240}$  value of 0.62 which would interfere with this assay. Assay based on dye binding such as the commercially available 'BioRad' protein assay are also affected by solvents.

The uptake of crystal violet by P.putida has been shown to increase once the permeability of the membranes had been increased with solvents (Harrop, 1990). The dye uptake was therefore considered as a suitable assay for general use. In this assay 1 ml of a cell suspension was added to 9 ml of the dye solution (10 mg l<sup>-1</sup>) in a Universal bottle and shaken for 30 min at 30°C in an orbital shaker. The cells were removed by centrifugation for 3 min at high speed in a Microcentrifuge. The dye concentration of the supernatant was calculated from its absorbance (Dye concentration (mg l<sup>-1</sup>))

= 8.686  $A_{584}$  ) and the calculated dye uptake by the cells was obtained by subtracting this value from the initial dye concentration. Problems were encountered with the crystal violet becoming attached to the surface of both the Universal bottle and Eppendorf<sup>tube</sup>. Controls were set up to analyse the rate and the quantity removed. Four controls were set up with duplicate samples centrifuged in the Eppendorf. The values were not constant with  $14 \pm 2 \mu\text{g}$  of dye becoming attached to the glass and Eppendorf. The dye uptake of the cells was calculated by subtracting the dye concentration remaining in the sample from the amount remaining in the control after adhesion of the dye to the glass and Eppendorf. Six samples were set up with the same cell concentration and with duplicate samples spun down in Eppendorfs. 0.339 mg of cells took up an average of 6  $\mu\text{g}$  of crystal violet. Upon completion of the assay 23 % of the dye had been removed from the supernatant. The large amount of dye removed from solution by the control as compared to the amount taken up by the cells alone deemed the assay unsuitable for general use.

It had been shown previously that high concentrations of fluorobenzene and pentanol had decreased the OUR of the cells (Section 3.2.1.1). The OUR of the cells was therefore considered as a suitable parameter to analyse. This could also be correlated to loss of biotransformation activity since molecular oxygen is a substrate in the reaction. This would be the basis to the assay. Cells would be allowed to reach a linear OUR before solvent would be added to the electrode in propanol. The decrease in OUR due to the toxicity of the solvent would then be followed. First of all it was necessary to select the toxic solvent.

The selection of the solvent was based on the following criteria:-

- a) Toxic at below aqueous saturated concentration so that a supply of the solvent from a second liquid phase was not necessary to observe an inhibitory

effect. Cells become attached to an interphase and the number of cells could vary depending on the growth conditions.

- b) Solvents selected should have a low volatility since the concentration should not change dramatically during the assay. With non-volatile compounds it is also possible to control accurately their concentration.
- c) The toxicity of hydrophobic compounds in other research areas could be used as comparison to this work. In medical studies the transmembrane diffusion of some hydrophobic substances has been studied (Nikaido, 1976). The accumulation of organic pigments in fish has also been studied (Anliker & Moser, 1987). In both cases  $\log P$  was selected as the parameter to describe the hydrophobicity of the compounds and could therefore be compared to other compounds.

Fluorobenzene and other similarly substituted benzenes were not considered since they could be converted to other chemicals by the aromatic oxidation system. This could result in a product which was either less or more toxic than the substrate and a change in the DOT profile. If the compound acted as a substrate then the increase in OUR could be seen before its toxic effect became apparent. This would lead to a confusing profile. Alcohols and alkanes were not selected due to their low toxicity at below aqueous saturated concentration.

Previous work on two liquid phase biotransformations has concentrated on solvents which had low if negligible aqueous saturated concentration. This was unsuitable for this assay. The selection of the toxic hydrophobic compound was therefore based on disinfectants which have high toxicity at concentration below aqueous saturation. A large number of disinfectants are based on ionic interactions but the phenolic compounds are hydrophobic compounds and could

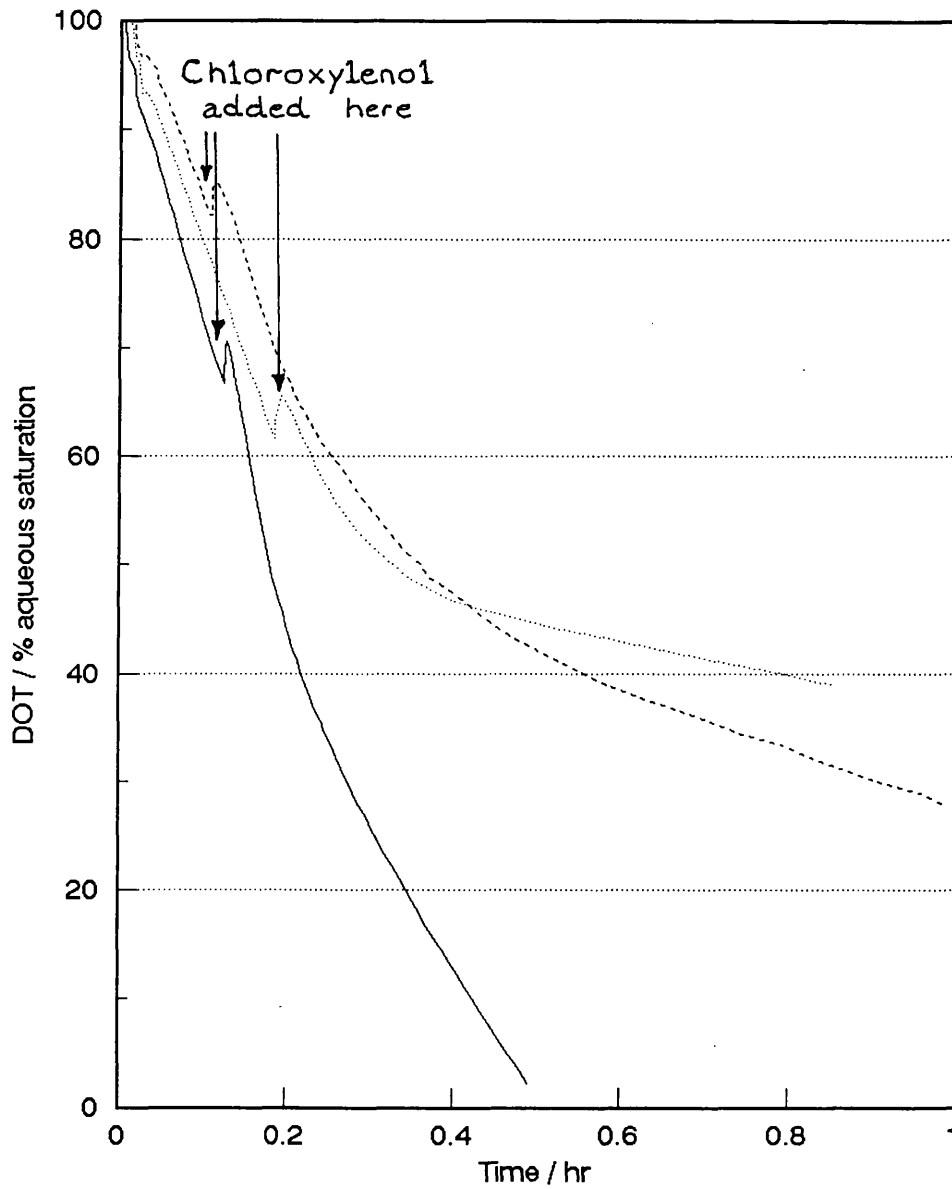
therefore be considered for this assay. The phenol which was selected was 4 chloro 3,5 dimethyl phenol (chloroxylenol) which is the active compound in 'Dettol' the disinfectant produced by Reckitt and Coleman. It was selected on the criteria set above and on the basis that it has low toxicity towards human beings by inhalation. At 25°C it has a solubility of 3.44  $\text{gl}^{-1}$  in 6 mM  $\text{KH}_2\text{PO}_4$ , pH=5.1 (Blackman et al,1955).

Chloroxylenol was added to the oxygen electrode in a similar way to how fluorobenzene was initially added to the oxygen electrode in the development of the aromatic oxidation activity assay in that they were added in propanol which help to dissolve the compounds in water. The cell concentration was higher so that the optimum would give a large amount of data covering a range of DOT values and a long period of time (approximately 30 mins). The initial concentration of chloroxylenol in the reactor was approximately 10 % of aqueous saturation and resulted in the OUR going immediately to zero. The chloroxylenol concentration was decreased and added in 25  $\mu\text{l}$  of propanol and not 50  $\mu\text{l}$ . The profiles obtained at three concentrations are shown in Figure 3.57. This shows clearly that at different concentrations of chloroxylenol the rate of drop in OUR changes. Upon addition of propanol the DOT increased due to the addition of oxygen in the propanol and an increase in the OUR was observed due to the oxygen requirements for propanol consumption. This increase in OUR was reduced by adding a smaller volume of propanol (10  $\mu\text{l}$ ). Data was taken every 1 % DOT unit from the original curve obtained on the chart recorder. The curve indicates that the time taken to drop 10 % DOT units increased with the time the bacterial<sup>culture</sup> had been subjected to chloroxylenol.

A ratio could be obtained between the time to drop the second 10 % DOT units and the first 10 % DOT units. The idea of relating how the DOT decreased by taking the ratio of the times was used to describe mathematically the curvature of the profile. A central point was selected from

Figure 3.57 - The effect of chloroxylenol upon the oxygen uptake of *P. putida* ML2.

(Chloroxylenol was added in 25 $\mu$ l of propanol)



Final chloroxylenol concentration: 0.037 g l<sup>-1</sup> 0.074 g l<sup>-1</sup> 0.107 g l<sup>-1</sup>

which these ratios could be calculated at different DOT values away from it and would give a linear curve when the time ratios were plotted against DOT units. The value of the gradient would describe the toxicity of the compound, the gradient should tend towards zero as the compound becomes non-toxic or to a concentration which is non-toxic since the OUR should be linear and therefore the time ratio will always be one.

The assay was therefore selected as the main assay to determine the tolerance of the bacterium towards a hydrophobic compound. The values obtained in the form of time ratio was deemed as unsuitable since the gradient of the curves would be obtained with units of DOT<sup>-1</sup> and not in time. By obtaining the gradient of the curves in time it is possible to compare to work by other researchers with data obtained from the loss of biotransformation activity and loss of capacitance readings measured with the bugmeter. Initially values were obtained from the curves in which time was kept constant and a ratio of DOT values were obtained. The DOT ratios were plotted against time and it was observed that the curve increased exponentially. The graph was replotted on a logarithmic scale and a straight line was obtained but a large scatter of points around the line was observed.

It was therefore necessary to obtain an equation which would describe the way that the OUR decreased with time. The loss of viability due to phenol toxicity has been described as a first-order chemical reaction (Chick,1908) as shown below:-

$$-dN/dt = cN$$

where N = number of viable organisms present

t = time in the presence of phenol

c = specific death rate.



The above equation can be integrated to the following expression:-

$$N_t/N_0 = \exp^{-ct}$$

where  $N_t$  = number of organisms at time  $t$

$N_0$  = number of organisms at the start

The loss of OUR was also thought to decrease exponentially since the DOT profiles observed had suggested this and plotting the ratio of the DOT values against time had given an exponentially increasing curve. The equation above was modified with OUR being analysed rather than the number of bacteria to give the equation below:-

$$OUR_t / OUR_0 = \exp^{-ct}$$

The equation was then converted to DOT against time with  $OUR_0$  and  $c$  staying constant.  $OUR_t$  was then converted to  $-d(DOT)/dt$ , it is negative since positive OUR results in a decrease of DOT with time. The equation was then expressed as:-

$$-d(DOT) / dt = OUR_0 \cdot (\exp^{-ct})$$

$$- \int d(DOT) = OUR_0 \int \exp^{-ct} dt$$

which when integrated between the limits:-

$$DOT = DOT_0 \text{ at } t = 0$$

and  $DOT = DOT_t$  at  $t = t$

gives the solution:-

$$-(DOT_t - DOT_0) = OUR_0 \cdot ((-\exp^{-ct}/c) - (-\exp^0/c))$$

$$(DOT_0 - DOT_t) = (OUR_0/c) \cdot (1 - \exp^{-ct})$$

where at  $t = 0$ , chloroxylenol was added.

The data obtained from the assay could now be fitted to the equation. Curve fitting routine was performed on the 'Origin' programme with best fit based on least square rule.

Figure 3.58 shows the profile obtained when 2.91 gl<sup>-1</sup> of cells were subjected to a chloroxylenol concentration of 0.2 gl<sup>-1</sup> in the reactor. 10μl of a 60 gl<sup>-1</sup> solution of chloroxylenol in propanol was added to the reactor once the OUR had reached a steady value. The above equation was fitted to the data giving the parameter values of:-

$$\begin{aligned} \text{OUR}_0 &= 801 \text{ \% DOT hr}^{-1} \\ c &= 19.06 \text{ hr}^{-1} \end{aligned}$$

The data obtained from the assay could therefore be described as an exponential decay curve. Problems were encountered in that if the number of data points analysed was changed the fitted curves would also change. These fitted curves are shown in Figure 3.59 and values of the specific death rate constant increased as the number of datapoints decreased. The assay does indicate that the cellular OUR decreases exponentially but since it does not fit exactly the same when different datapoints are considered it must be assumed that another factor needs to be considered.

The assay was modified by removing the plunger to allow oxygen to be transferred into the culture. This would allow the use of the equation below without integration:-

$$\text{OUR}_t / \text{OUR}_0 = \exp^{-ct}$$

It could be considered that OUR would be equal to the OTR if equilibrium was reached due to high oxygen transfer rate. The following equation could then be used :-

$$\text{OUR} = \text{OTR} = K_L a \cdot (C^* - C_L)$$

We can then transfer this equation into the exponential decay equation as below:-

$$\text{OUR}_t / \text{OUR}_0 = \exp^{-ct} = K_L a (C^* - C_{L_t}) / K_L a (C^* - C_{L_0})$$

Figure 3.58 - The  $(DOT_0 - DOT_t)$  profile obtained after cells are subjected to  $0.2 \text{ gl}^{-1}$  reactor chloroxylenol concentration.  $(DOT_0 - DOT_t) = (801/19.06) \cdot (1 - \exp^{-19.06 \cdot t})$  was fitted to the datapoints .

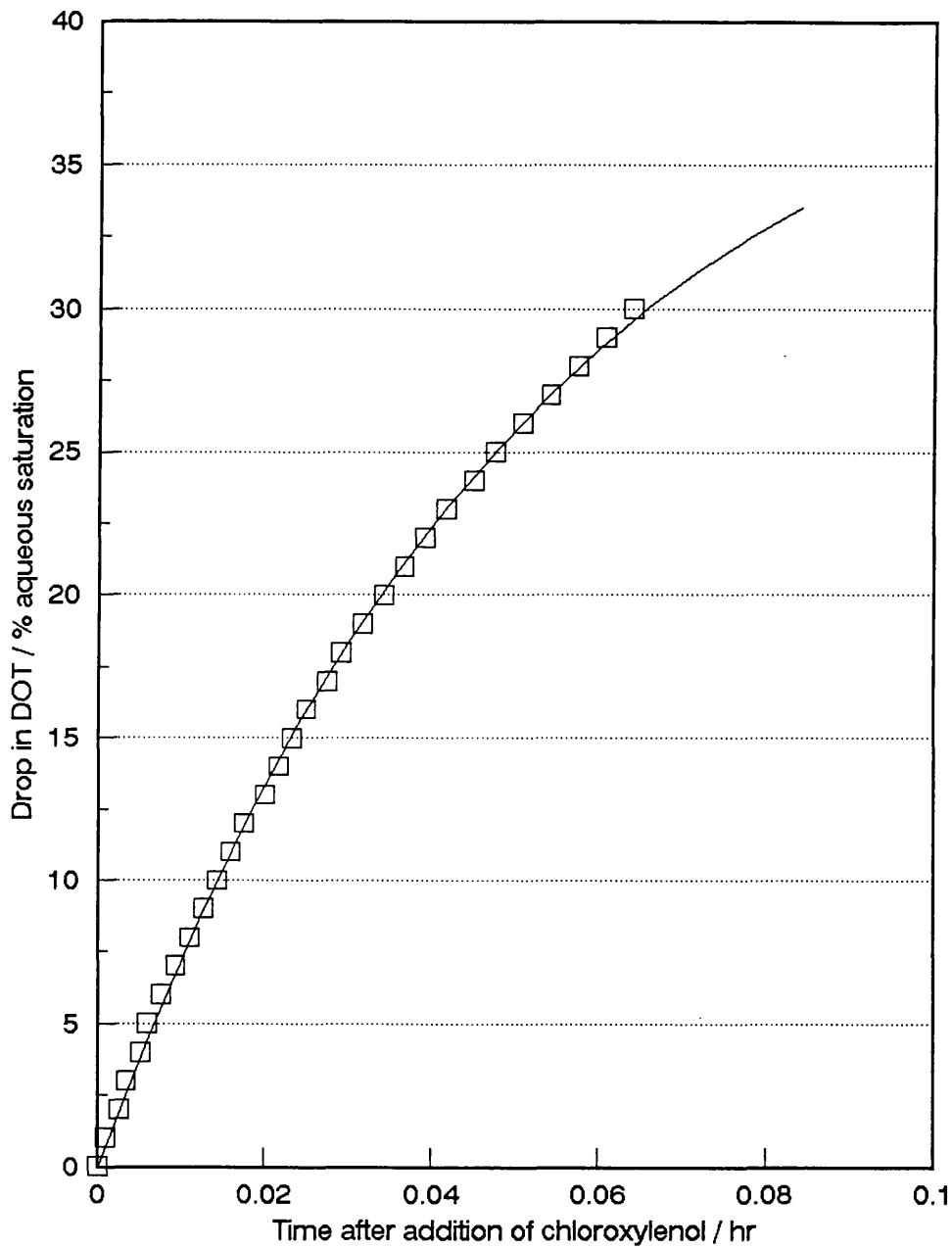
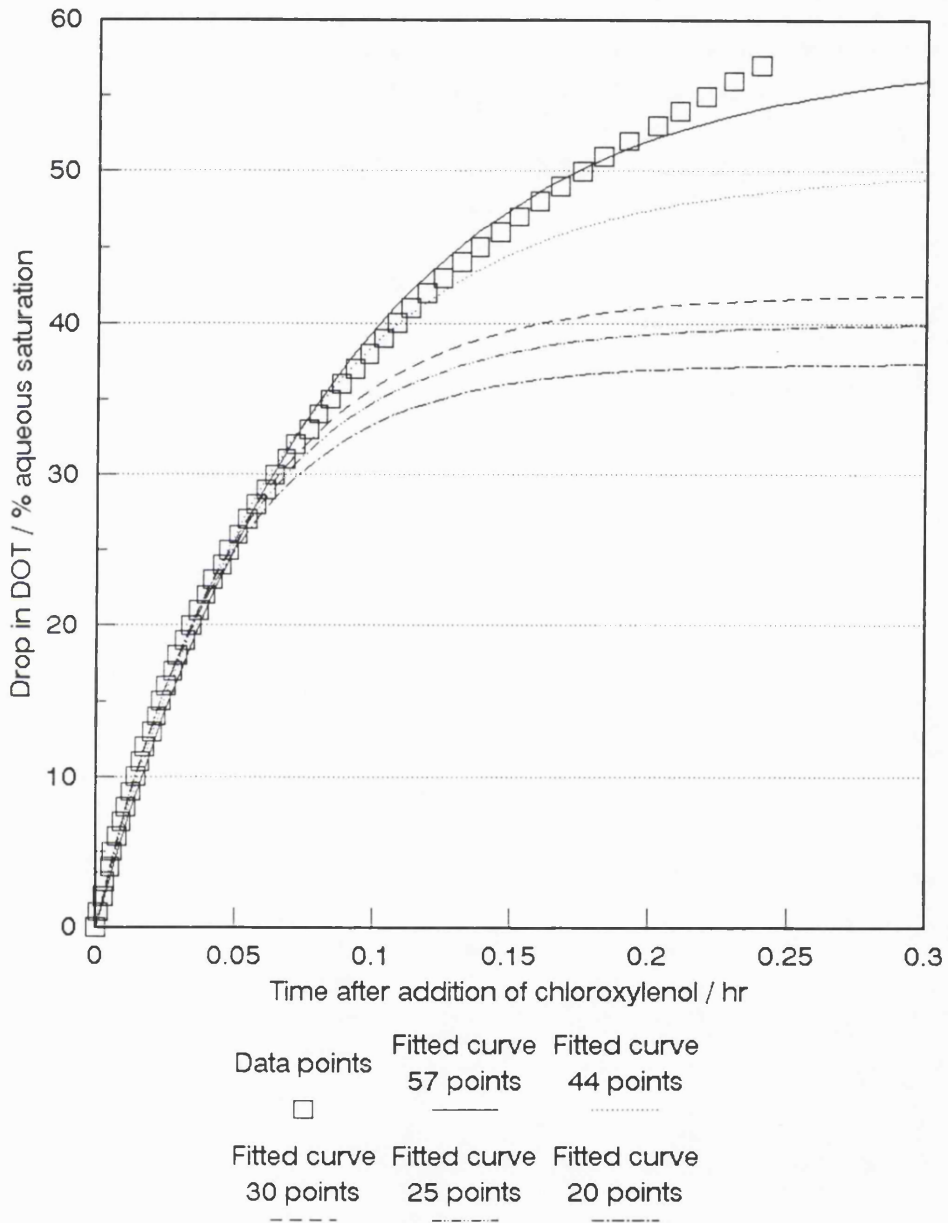


Figure 3.59 - The effect of changing the number of datapoints analysed upon the curve profile obtained after fitting to the equation  $(DOT_0 - DOT_t) = (OUR_0/c) \cdot (1 - \exp^{-ct})$ .



From this expression the following equation can be obtained:-

$$(\text{DOT}^* - \text{DOT}_t) / (\text{DOT}^* - \text{DOT}_0) = \exp^{-ct}$$

At this stage I decided to change the loading solvent from propanol to ethanol. During the development of the aromatic oxidation assay the profiles of the OUR due to propanol and ethanol consumption were obtained (Figure 3.32). Ethanol had an advantage over propanol in that a plateau was obtained in which the specific oxygen uptake rate was constant once a minimum level of ethanol had been reached. This level was below 10  $\mu\text{l}$  added to 3 ml of buffer. This meant the addition of a second 10  $\mu\text{l}$  volume of ethanol did not result in an increase in the OUR. Ethanol could be added at the start to obtain a steady OUR value before the addition of chloroxylenol in ethanol.

The assay was therefore set up with 2.4  $\text{gl}^{-1}$  of cells and 5  $\mu\text{l}$  of ethanol added initially without the plunger placed in the top so that oxygen transfer could occur. An exponential type decay of activity was observed when 10  $\mu\text{l}$  of 45  $\text{gl}^{-1}$  of chloroxylenol in ethanol was added to the culture, but the curve had not gone towards 100 % DOT but towards 60 % DOT. This indicates that the OUR had not reached zero but approximately 40 % of the original OUR value. At the higher chloroxylenol concentration the rate at which DOT increased was close to the oxygen transfer to buffer (containing no cells) after it had been sparged free of oxygen with nitrogen gas. It is clear that this assay would be unsuitable since the oxygen transfer was slow and it is doubtful that OUR could be considered to be equal to the STR and therefore the equation to describe the rate of cell death would be more complex. The profile obtained for the culture at 0.15  $\text{gl}^{-1}$  chloroxylenol concentration had reached a constant OUR and not gone to zero. This would indicate that the cells had come to a steady OUR value which is unaffected by chloroxylenol.

This resulting OUR at the end of the assay was termed as  $OUR_f$ . The OUR which is available to be lost due to chloroxylenol toxicity was therefore  $(OUR_0 - OUR_f)$ . The rate of exponential decay of the metabolic activity from  $OUR_0$  to  $OUR_f$  can be described by the equation:-

$$(OUR_t - OUR_f) / (OUR_0 - OUR_f) = \exp^{-ct}$$

To obtain the profile of the decrease in oxygen uptake in terms of DOT and time, the equation was integrated as follows:-

$$(OUR_t - OUR_f) = (OUR_0 - OUR_f) \cdot (\exp^{-ct})$$

$$OUR_t = (OUR_0 - OUR_f) \cdot (\exp^{-ct}) + OUR_f$$

$$OUR_t = -d(DOT)/dt$$

$$- \int d(DOT) = (OUR_0 - OUR_f) \int \exp^{-ct} dt + \int OUR_f dt$$

which when integrated between the limits:-

$$DOT = DOT_0 \text{ at } t = 0$$

and

$$DOT = DOT_t \text{ at } t = t$$

gives the solution:-

$$(DOT_0 - DOT_t) = (-1/c) \cdot (OUR_0 - OUR_f) \cdot (\exp^{-ct} - 1) + (OUR_f \cdot t)$$

$$(DOT_0 - DOT_t) = ((OUR_0 - OUR_f)/c) \cdot (1 - \exp^{-ct}) + (OUR_f \cdot t)$$

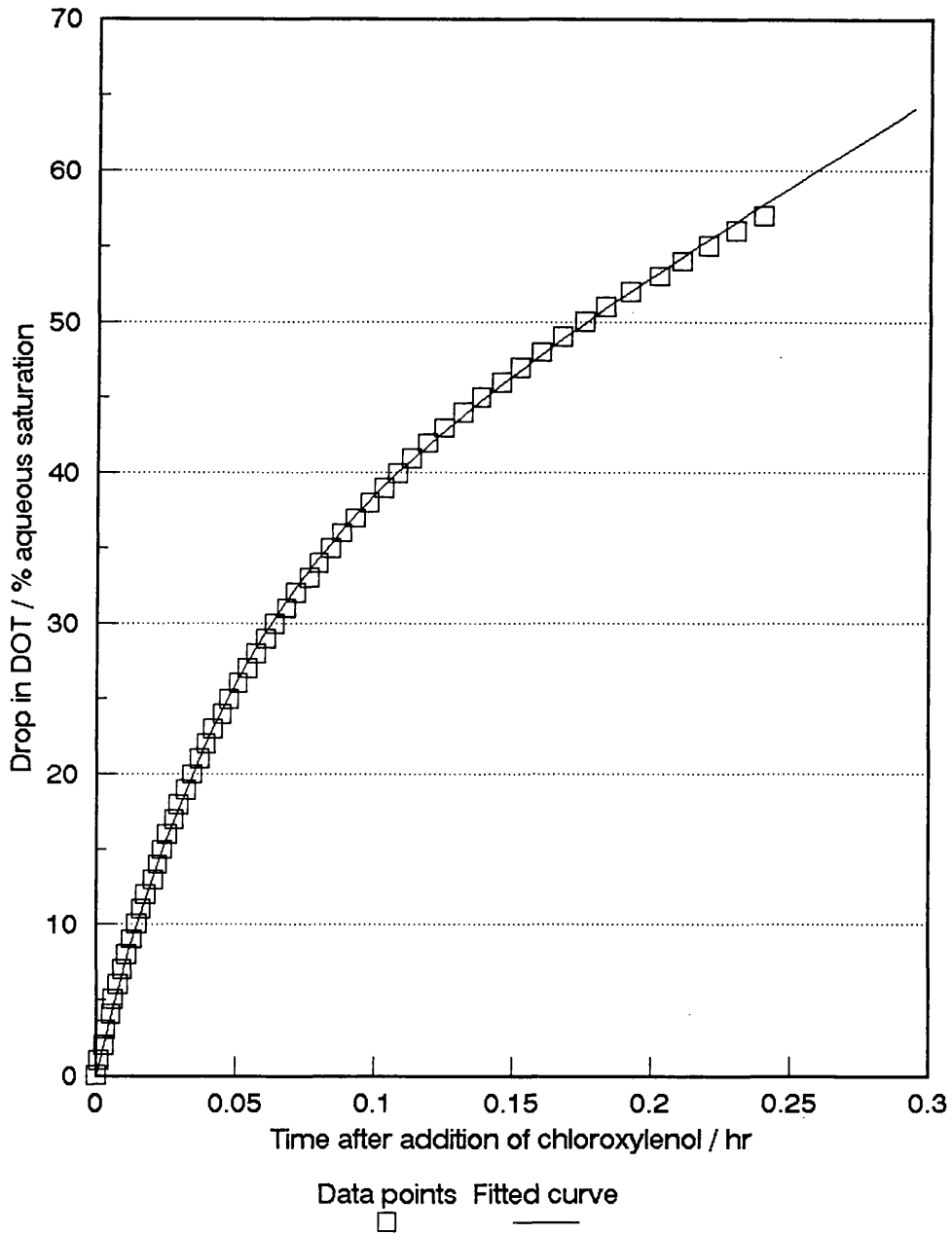
where at  $t = 0$ , chloroxylenol was added.

Data obtained previously were re-examined to see if a better fit could be obtained with this equation. Data shown in Figure 3.59 were fitted to the above equation in Figure 3.60. It is clear that a better fit was obtained. Values for the death rate constants at a reactor chloroxylenol concentrations of 0.15, 0.175 and 0.2  $gl^{-1}$  were 7.1, 15.6 and 26.9  $hr^{-1}$ .

Figure 3.60 - Re-examination of the data shown in Figure 3.59

but with data fitted to the equation

$$(\text{DOT}_0 - \text{DOT}_t) = ((\text{OUR}_0 - \text{OUR}_f) / c) \cdot (1 - \exp^{-ct}) + (\text{OUR}_f \cdot t)$$

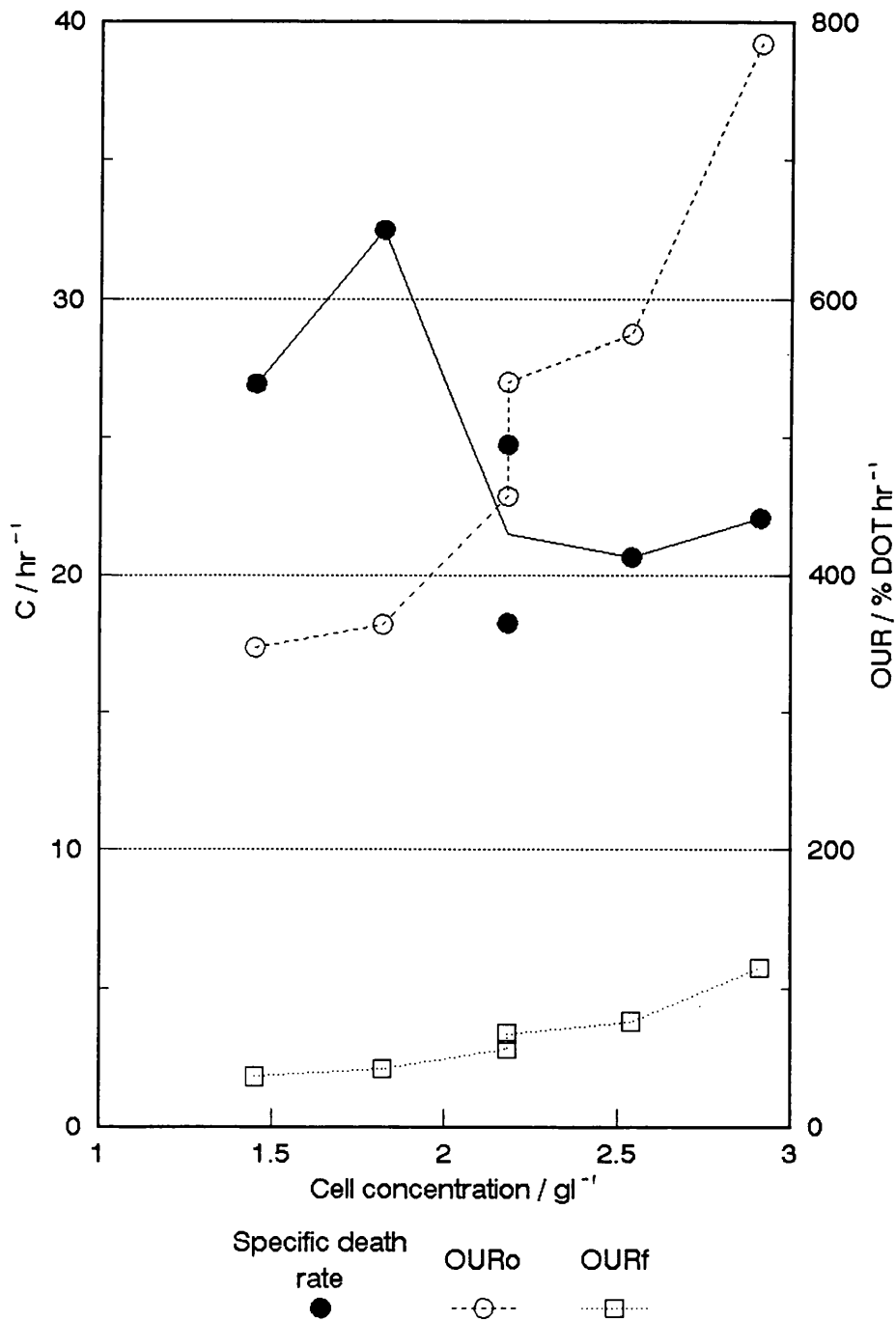


The effect of cell concentration upon chloroxylenol inactivation was analysed to determine if the concentration of chloroxylenol per bacterial cell was an important factor to consider in this study. The C and OUR values were calculated for the assays with cell concentration from 1 to 3  $\text{gl}^{-1}$  (Figure 3.61). The C values obtained did not indicate a trend, this suggested that the chloroxylenol concentration per cell was not a factor to consider at this range of biomass concentration. The variation in C values had indicated that at these low biomass concentrations the assay was prone to error which was a result of a poor data analysis and curve fitting routine rather than an experimental error. The  $\text{OUR}_0$  and C values are highly dependent on the theoretical profile at the start of the bacterial inactivation and therefore accurate estimation of these parameters can only be obtained if a large number of data points had been obtained at this early stage of the curve fitting routine. At low cell concentrations the OUR of the bacterial culture resulted in only a small decrease in DOT before complete inactivation of the culture had occurred which corresponds to a small number of data points, and makes the estimation of  $\text{OUR}_0$  and C by the curve fitting routine prone to error. In this study the calculation of C values was dependent upon the ideal curve being obtained for the curve fitting routine in that a large amount of data points immediately after chloroxylenol addition would lead to accurate evaluation of the C values. The assay was not performed at the same cell concentration but would vary depending on the specific OUR due to ethanol consumption. The C values obtained for the fermentations at 5 % DOT and 30°C (Section 3.3.3.1) and at 20 % DOT and 26°C (Section 3.3.4.1) were compared to the biomass concentration and the specific OUR due to ethanol consumption in later sections to see if a correlation could be obtained.

Development of this assay had indicated a number of factors had to be considered before the accuracy of the



Figure 3.61 - The effect of cell concentration upon the calculated values of  $C$ ,  $OUR_0$  and  $OUR_f$ .



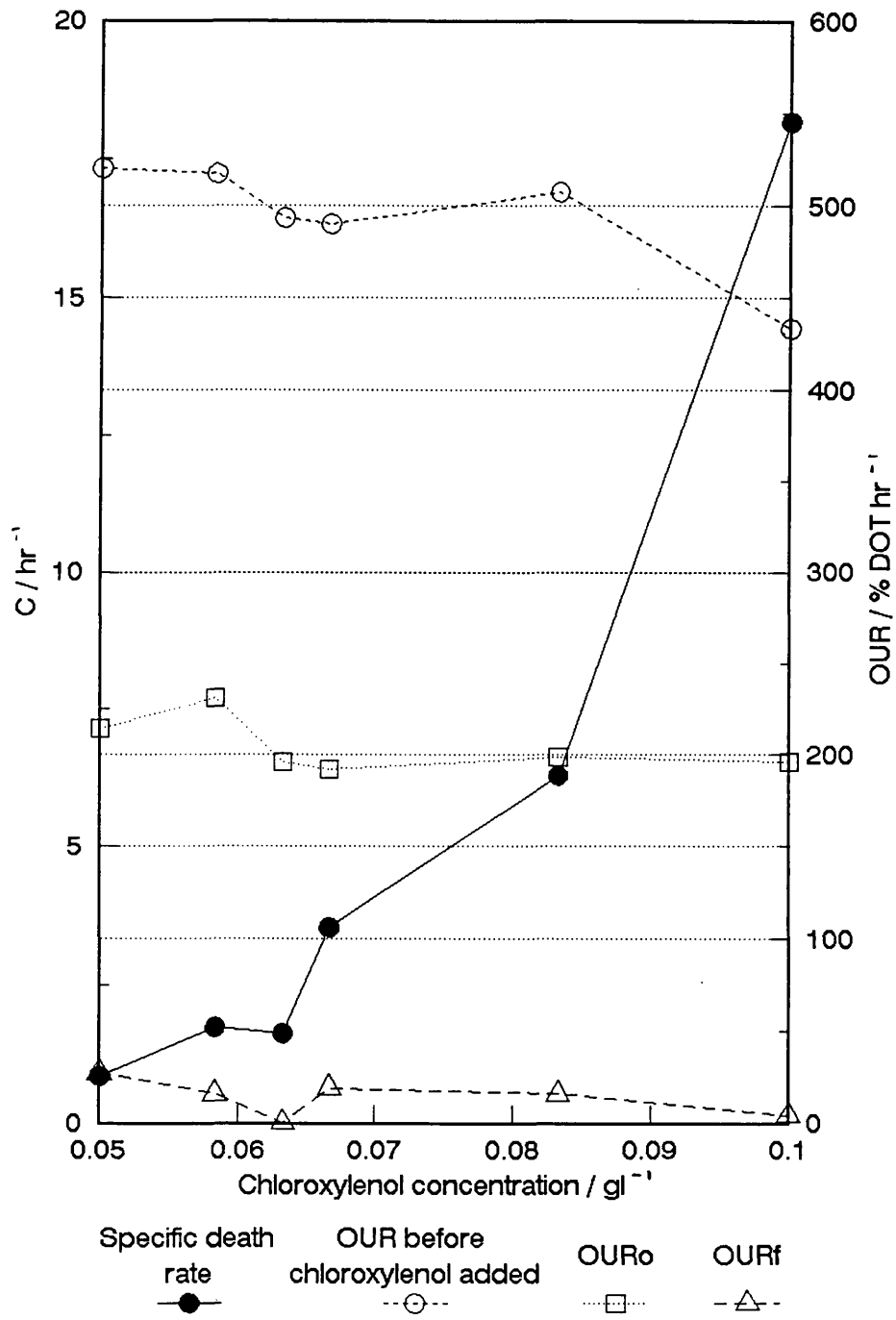
calculated C values could be guaranteed. The addition of chloroxylenol in propanol had resulted in an initial increase in OUR (Figure 3.57) which would cause problems with the curve fitting routine in that the initial profile would contain a stage of increase of OUR due to propanol consumption and a decrease in OUR due to chloroxylenol toxicity. In this assay only the chloroxylenol toxicity was to be considered. During the development of the aromatic oxidation activity assay it was shown that the OUR due to ethanol consumption had reached a maximum level at a low ethanol concentration (Figure 3.32). The assay was therefore set up with ethanol and bacterial<sup>culture</sup>, initially added to the electrode and allowed to reach a steady OUR value before chloroxylenol was added in ethanol. The OUR had reached a maximum level after the first ethanol addition therefore the second ethanol addition had not resulted in an increase in the OUR therefore making the OUR profile an indicator of chloroxylenol toxicity alone.

The effect of chloroxylenol concentration (added to the electrode in ethaol) was examined and the  $OUR_0$ ,  $OUR_f$ , OUR before chloroxylenol addition and C values are shown in Figure 3.62. Toxicity was greater when ethanol was the substrate and changes in chloroxylenol concentration had resulted in significant variations in the C values but not the  $OUR_0$  and  $OUR_f$  values.

The values obtained from the profiles showed that the  $OUR_0$  (calculated by the curve fitting routine) was not equal to the OUR before the second quantity of ethanol containing chloroxylenol was added, this indicated an immediate toxic effect of chloroxylenol upon the OUR of the bacterial culture.

The assay had therefore been developed with chloroxylenol preferably added in ethanol rather than propanol and initial OUR high enough to allow a full range of DOT values to be recorded.

Figure 3.62 - The effect of chloroxylenol concentration upon  $C$ ,  $OUR_0$  and  $OUR_f$ .



### 3.3.2 - Fermentation at 20 % DOT and 30°C

The effect of chloroxylenol concentration upon the solvent tolerance of the cells from the two fermentations described in Section 3.1.3 was determined. A sample taken from the growth phase was subjected to various concentrations of chloroxylenol and the C value and the percentage of  $OUR_0$  to  $OUR$  before chloroxylenol addition was calculated. Figure 3.63 shows the value of C increasing with increase in chloroxylenol concentration which is similar to the bacteria grown in a shake flask (Figure 3.62). The  $OUR_0$  ratio of this sample had decreased when the chloroxylenol concentration had increased but this was not observed for the bacteria grown in a shake flask since the  $OUR_0$  was similar at different chloroxylenol concentration. More than 60 % of the activity of the cells was lost immediately when chloroxylenol was added to a concentration of  $0.1 \text{ gl}^{-1}$ . Decreasing the concentration to  $0.033 \text{ gl}^{-1}$  had resulted in the chloroxylenol reaching a concentration that had little toxicity. Changes in the chloroxylenol concentration had resulted in a rapid increase in toxicity. The bacteria from the shake flask culture were more tolerant to chloroxylenol than the ones grown in a fermenter at 20 % DOT and 30°C as indicated by the difference in C values at the same chloroxylenol concentration.

Samples taken from the fermentation were subjected to a reactor chloroxylenol concentration of  $0.067$  and  $0.1 \text{ gl}^{-1}$  to give the C profile in Figure 3.64. The decrease in C value with time after inoculation indicate an increase in tolerance of the bacteria, this was confirmed in the second fermentation (Figure 3.65).

In all subsequent fermentations the effect of  $0.067 \text{ gl}^{-1}$  of chloroxylenol upon the cells would be determined. The assay was improved by logging the data from the electrode every 5 s with a BBC computer.

Figure 3.63 - The effect of chloroxylenol concentration upon the solvent tolerance of the cells obtained from the end of the growth phase of a fermentation controlled at 20 % DOT and 30°C.

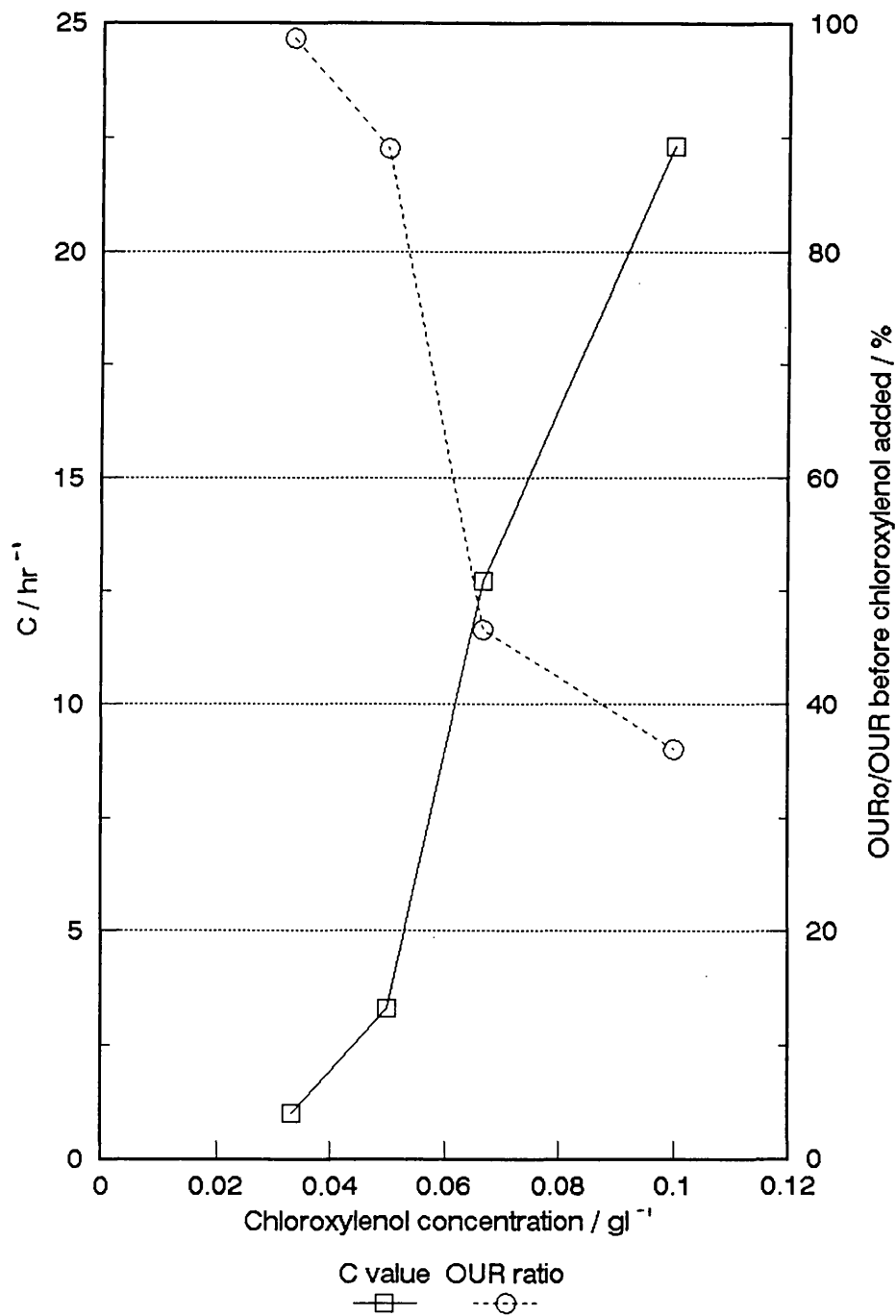


Figure 3.64 - The fermentation profile for cells grown at 30°C and 20 % DOT and the calculated C value obtained for cells at two chloroxylenol concentrations.

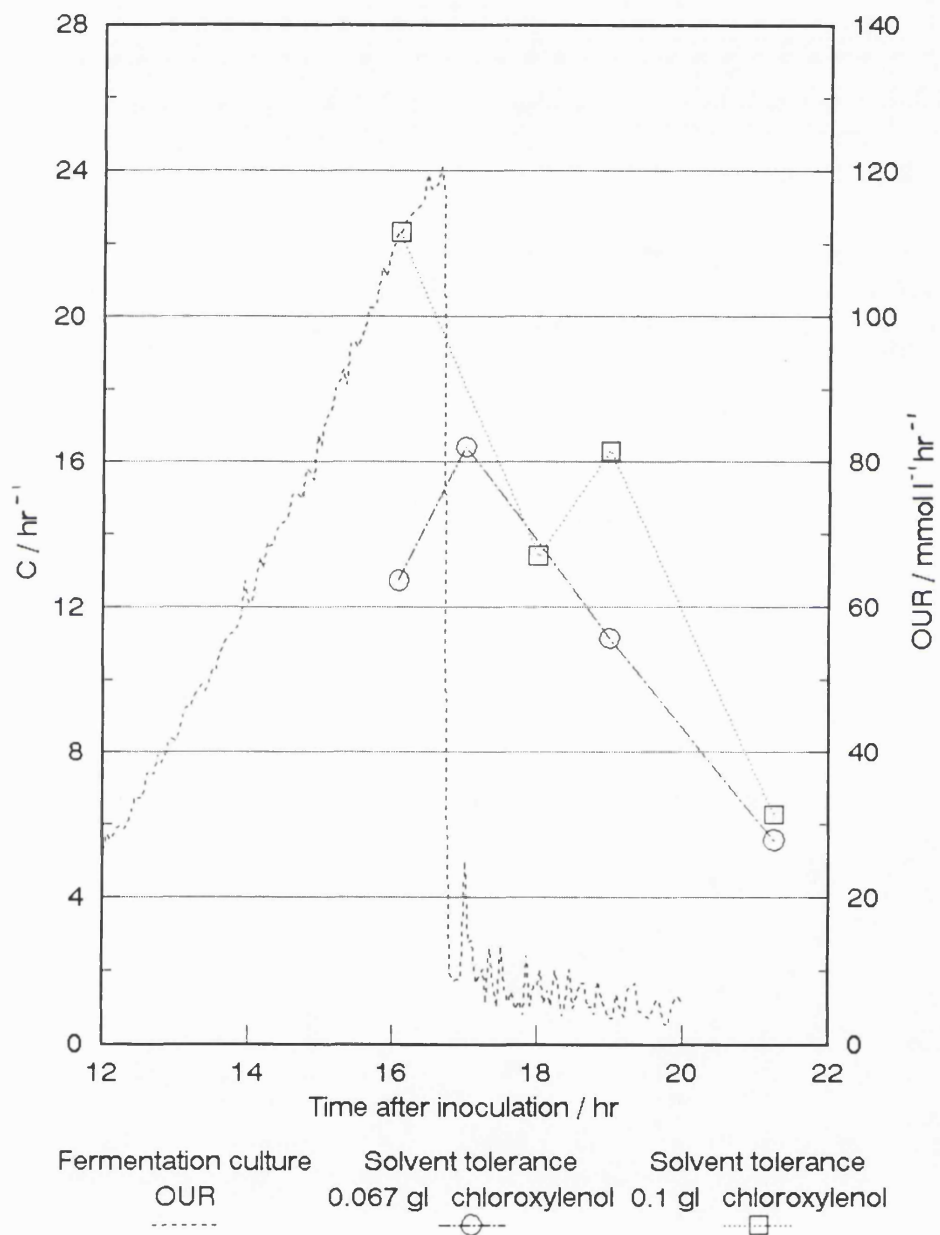
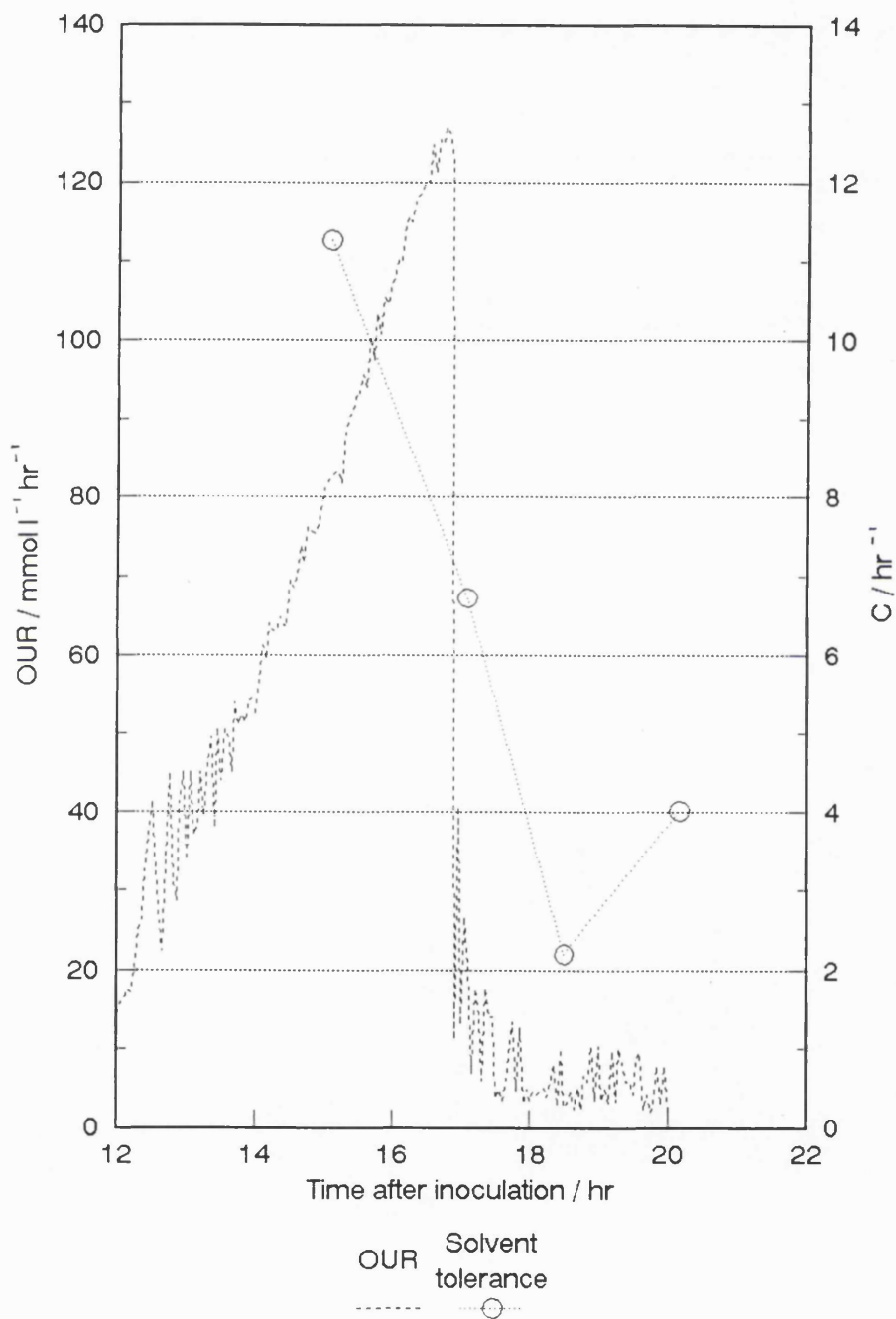


Figure 3.65 - The fermentation profile for cells grown at 30°C and 20 % DOT for the second fermentation analysed with the calculated C value obtained for cells at a reactor chloroxylenol concentration of 0.067 gl<sup>-1</sup>.



### 3.3.3 - Fermentation at 30°C but at different oxygen concentrations.

#### 3.3.3.1 - Fermentation at 5 % DOT and 30°C.

The fermentation profile and the C values obtained are shown in Figure 3.66. A clear profile was not obtained but it had been shown previously in Section 3.1.4.1 that the metabolic activity of the bacterial<sup>culture</sup> had changed during the growth phase, this could explain these variations in the C value. The ratio of the  $OUR_0$  to the OUR before chloroxylenol addition had been calculated with 10 out of the 13 values between 37.0 and 49.4 %, the other three being higher. These values were not significantly different from that obtained at 20 % DOT and 30°C (Figure 3.63) but a correlation between these values and C had not been obtained.

The heterogeneity of the C values was analysed further to determine whether this could be due to variations in the specific OUR due to ethanol consumption or due to differences in the biomass concentration analysed in the electrode. The large amount of scatter obtained when the OUR and biomass concentration were plotted against C (Figure 3.67) had indicated that these factors do not affect the calculation of the C value and therefore the obtained C value is due to variations in the growth of the cells. No correlation had been obtained between the biomass concentration, the OUR due to ethanol consumption and the ratio of  $OUR_0$  to the OUR before chloroxylenol addition.

The C value obtained for cells sampled 39 hr after inoculation was  $2.95 \text{ hr}^{-1}$ . This, as well as the values obtained from 25 hr after inoculation onwards, indicated that the cells had become more tolerant to chloroxylenol soon after the end of the growth phase.



Figure 3.66 - The fermentation profile for cells grown at 30°C and 5 % DOT and the calculated C value obtained for cells at a chloroxylenol concentration of 0.067 gl<sup>-1</sup>.

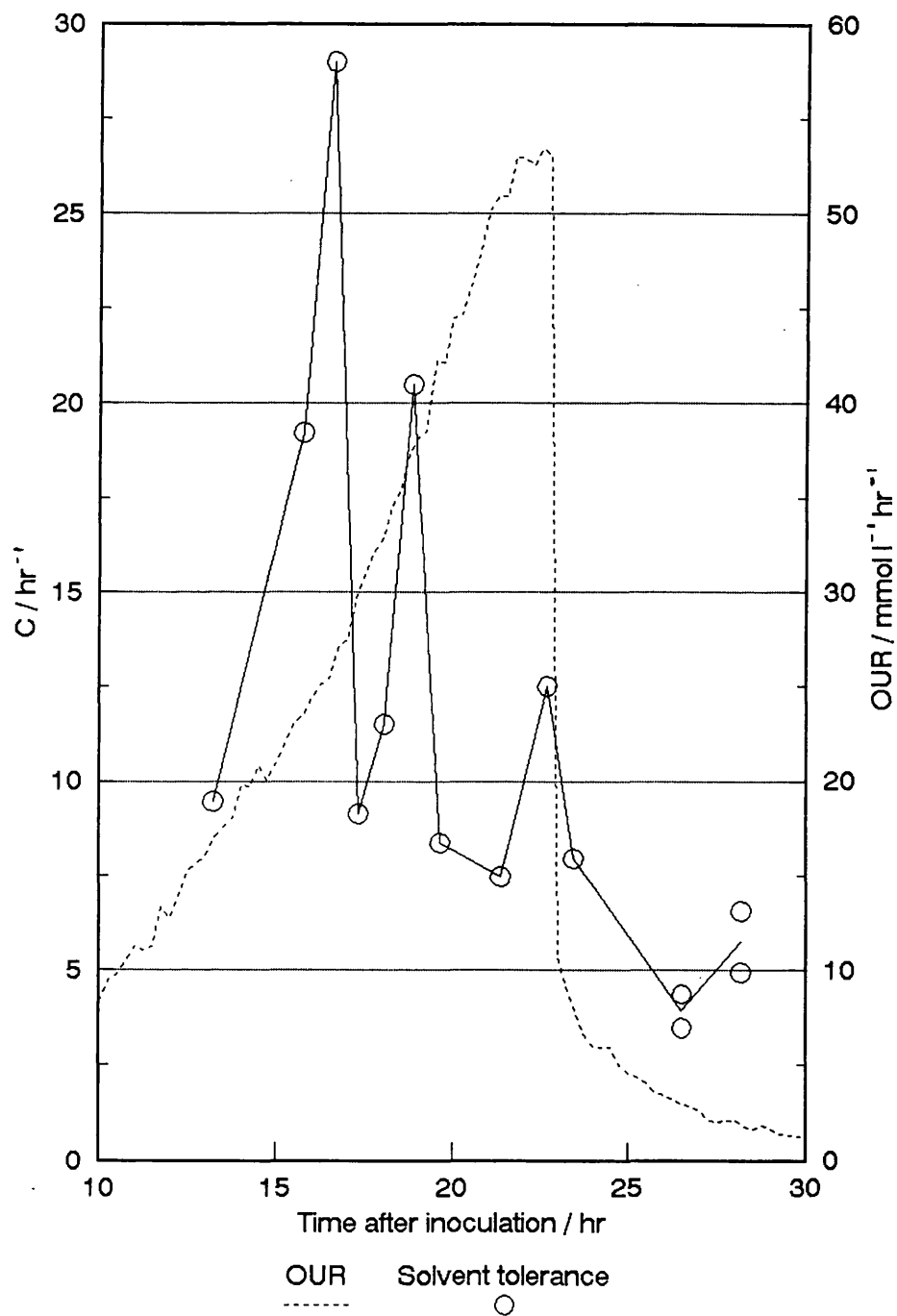
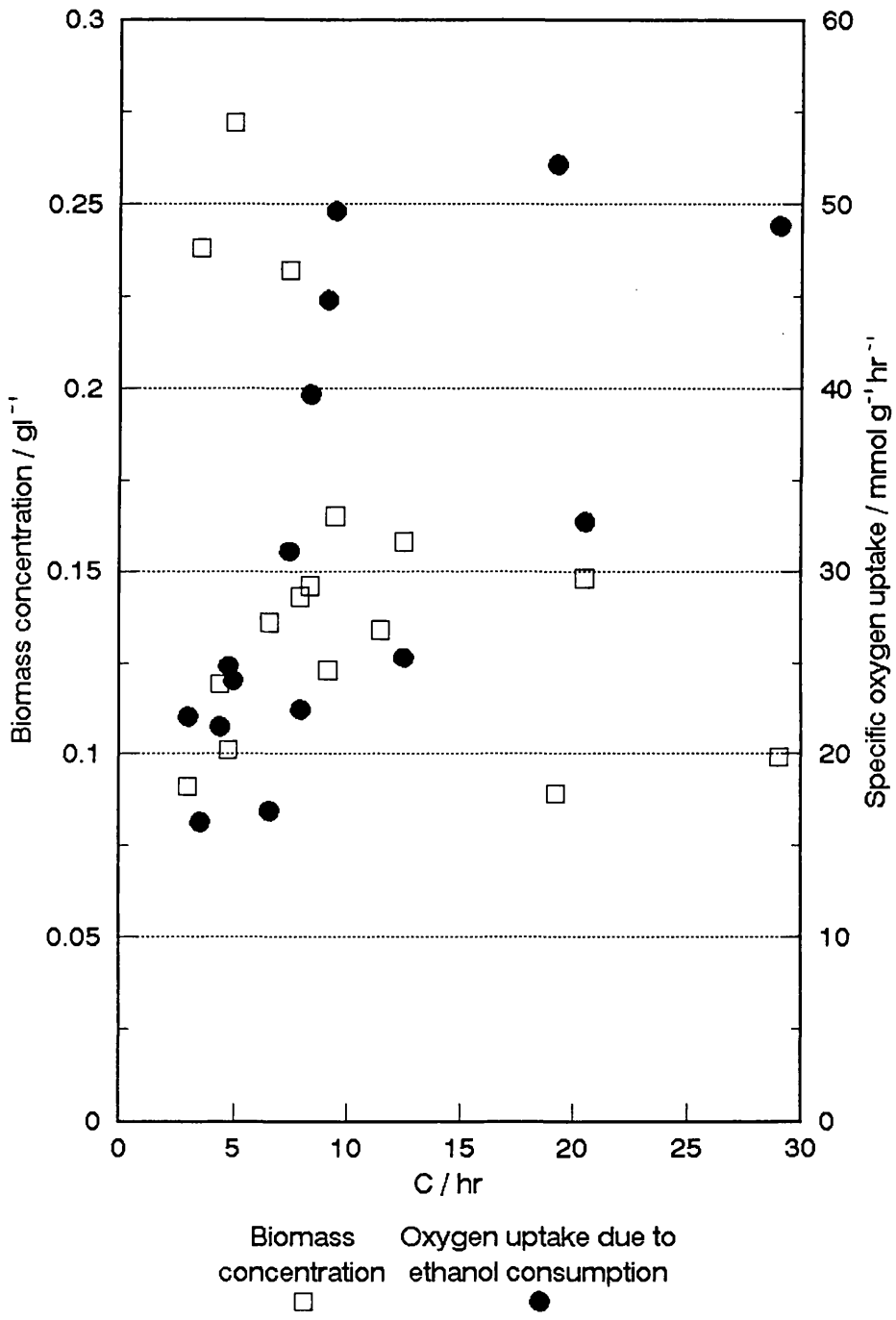


Figure 3.67 - Comparison of the specific OUR due to ethanol consumption, biomass concentration in the reactor and the calculated C value.



### 3.3.3.2 - Fermentation at 30°C and 20 % DOT with transition to 0%DOT at the end of the growth phase.

The fermentation was described in detail in Section 3.1.4.2. The fermentation profile and the calculated C values obtained (Figure 3.68) indicate that the values are either below 2 or above 3.5. A reduction in the DOT had resulted in a decrease in metabolic activity as indicated by a decrease in the fermentation culture OUR. The tolerance of the bacteria towards chloroxyleneol had increased as indicated by a decrease in the C values.

The DOT setpoint had been moved from 0 to 0.1 % aqueous saturation since the oxygen transfer rates had reduced to a low value. This had resulted in an increase in oxygen transfer rate resulting in a higher OUR level and an increase in chloroxyleneol susceptibility as indicated by the increase in C values.

This would strongly suggest that the solvent tolerance increases once the metabolic activity of the cells had been reduced.

### 3.3.3.3 - Fermentation at 30°C with transition of DOT from 20 % aqueous saturation to zero for a short period of time during the growth phase.

The fermentation was described in detail in Section 3.2.4.5. The fermentation profile and the C values are shown in Figure 3.69. It is clear from this profile that as the fermentation proceeds the solvent tolerance of the bacterium in general increases. The values obtained for  $OUR_0 / OUR$  before chloroxyleneol addition for the 6 samples analysed were between 51.2 and 81.9 %. The value of  $OUR_f$  to the  $OUR$  before chloroxyleneol addition was from 0 to 10 %. These two types of ratios did not correlate to the C values.

Figure 3.68 - The fermentation profile and calculated C value for cells initially grown at 20 % DOT with transition to zero at the end of the growth phase.

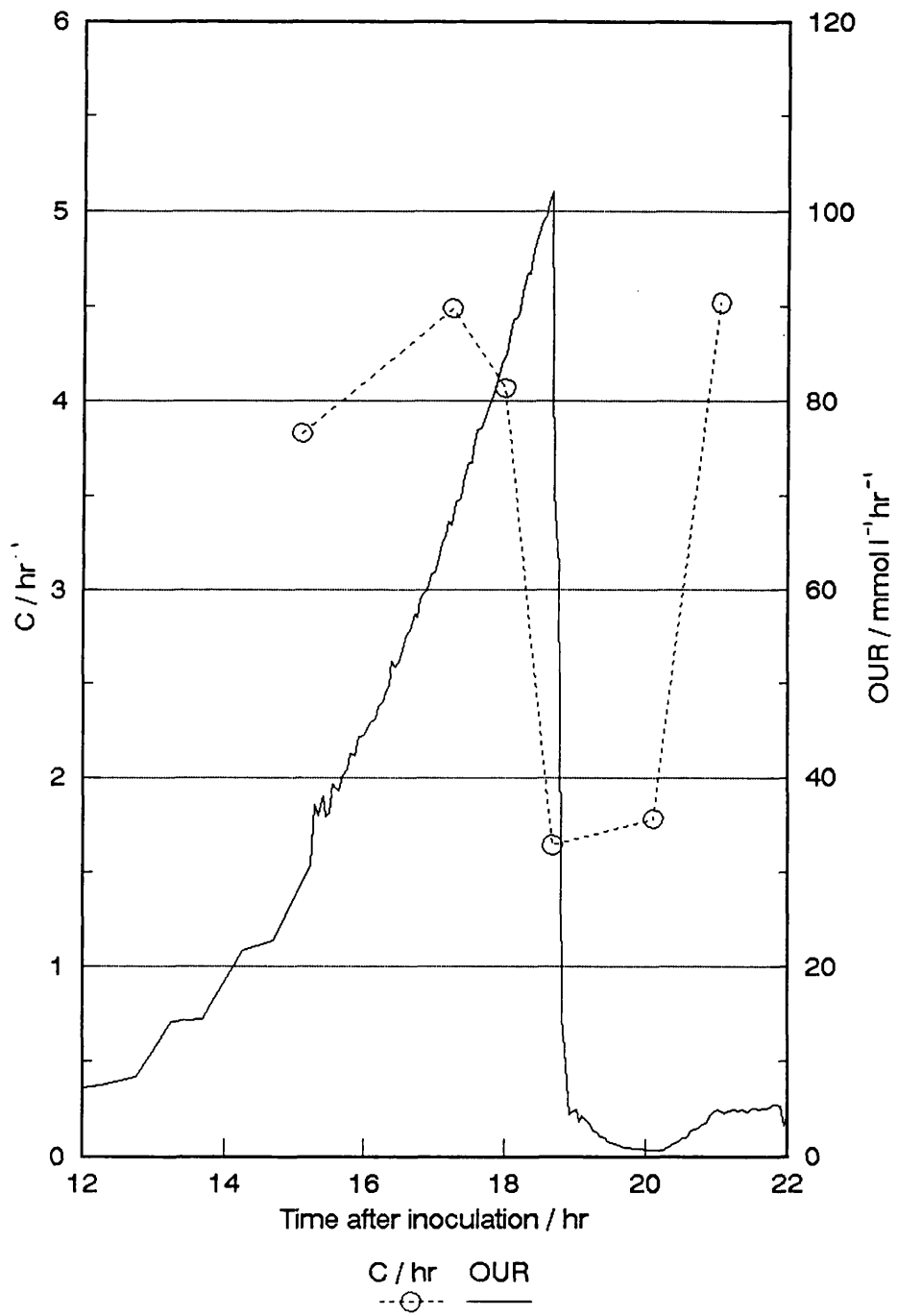
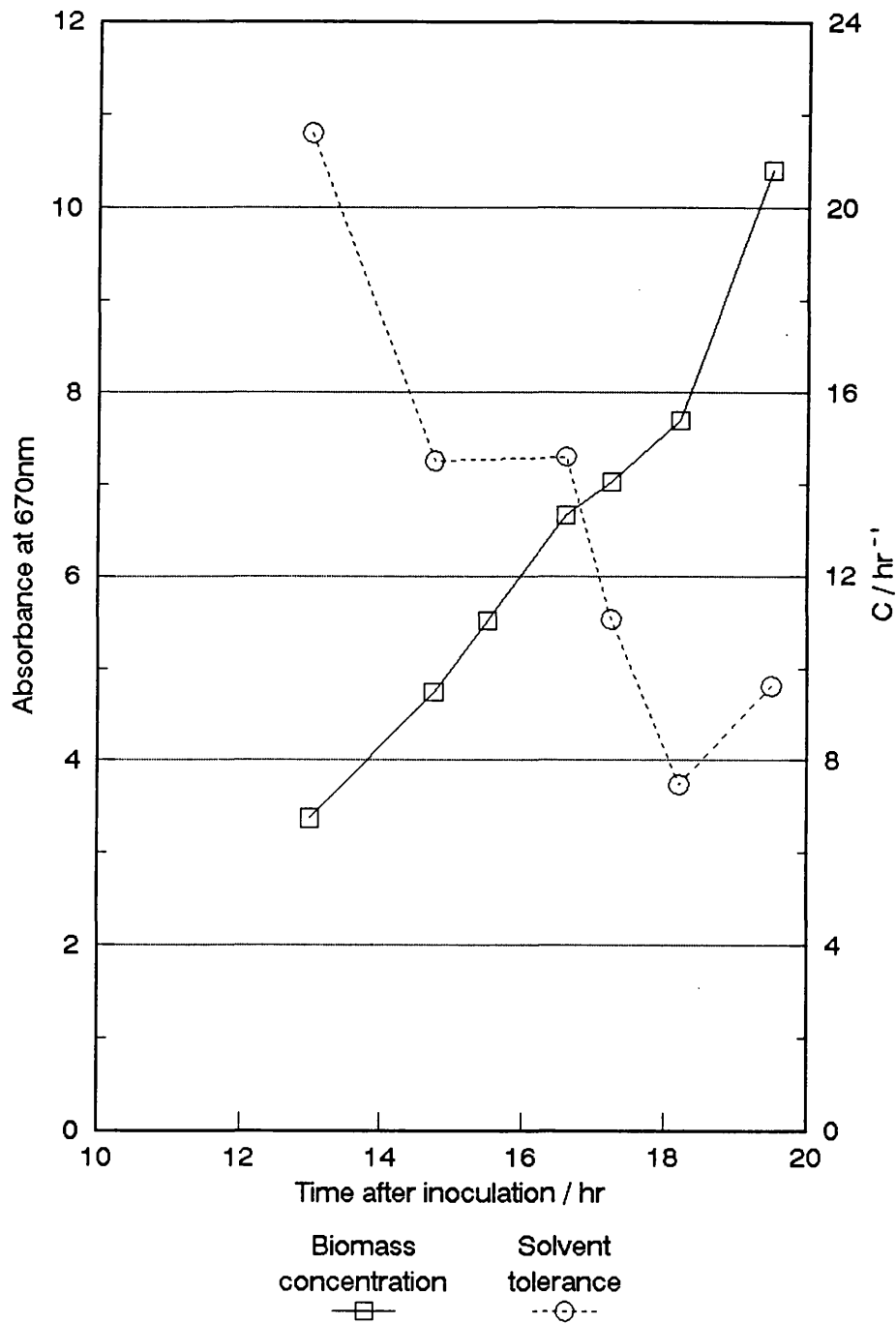


Figure 3.69 - The biomass profile and calculated C value for the bacteria from the fermentation controlled at 20 % DOT with transition to zero for a short period of time during the growth phase.



### 3.3.4 - Fermentations at 20 % DOT but at different temperatures.

#### 3.3.4.1 - 26°C

The fermentation was described in detail in Section 3.1.5.1. The fermentation OUR profile and the calculated values for C are shown in Figure 3.70. The profile indicates that the solvent tolerance of the bacterial <sup>culture</sup> generally increases with time. The biomass in the oxygen electrode during the assay and the specific oxygen uptake due to ethanol consumption was compared to the calculated C values in Figure 3.71. No correlation was obtained indicating that all the variations in the C values were due to variations in solvent tolerance brought about by changes in the growth of the bacteria. The profile of  $OUR_0 / OUR$  before chloroxylenol addition is shown in Figure 3.72.

The main difference between this fermentation and the ones at 30°C was that the  $OUR_f$  was always zero indicating total loss of cellular oxygen uptake.

#### 3.3.4.2 - 34°C

The profile of the drop in DOT against time after addition of chloroxylenol of 3 samples (one in duplicate) are shown in Figure 3.73. Strange values were obtained for the parameters when fitting these curves to the mathematical function since they were close to linearity. The loss of cellular activity was very low, 3 runs not shown in Figure 3.73 had a calculated C value close to zero. For the curve fitting routine to operate the C value cannot be zero and therefore both negative and positive values were obtained.

Due to the linearity of the curves this strongly indicates that growing the cells at a higher temperature had increased their tolerance to chloroxylenol.

Figure 3.70 - The calculated C value for solvent tolerance of the bacteria from the fermentation controlled at 26°C and 20 % DOT which is compared to the fermentation OUR profile.

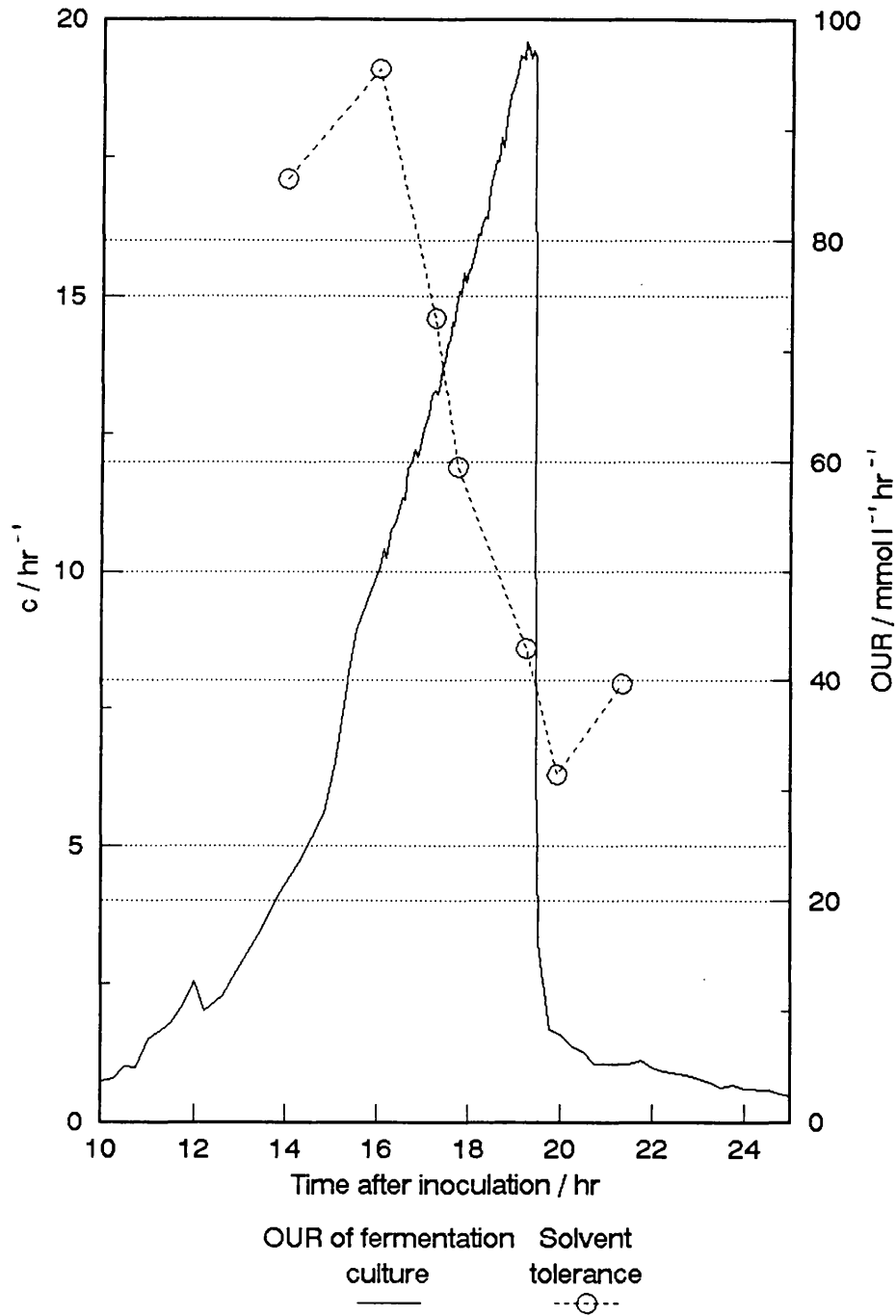


Figure 3.71 - Comparison of the specific oxygen uptake rate due to ethanol consumption, biomass concentration in the reactor and the calculated C value of the bacteria from the fermentation at 26°C and 20 % DOT.

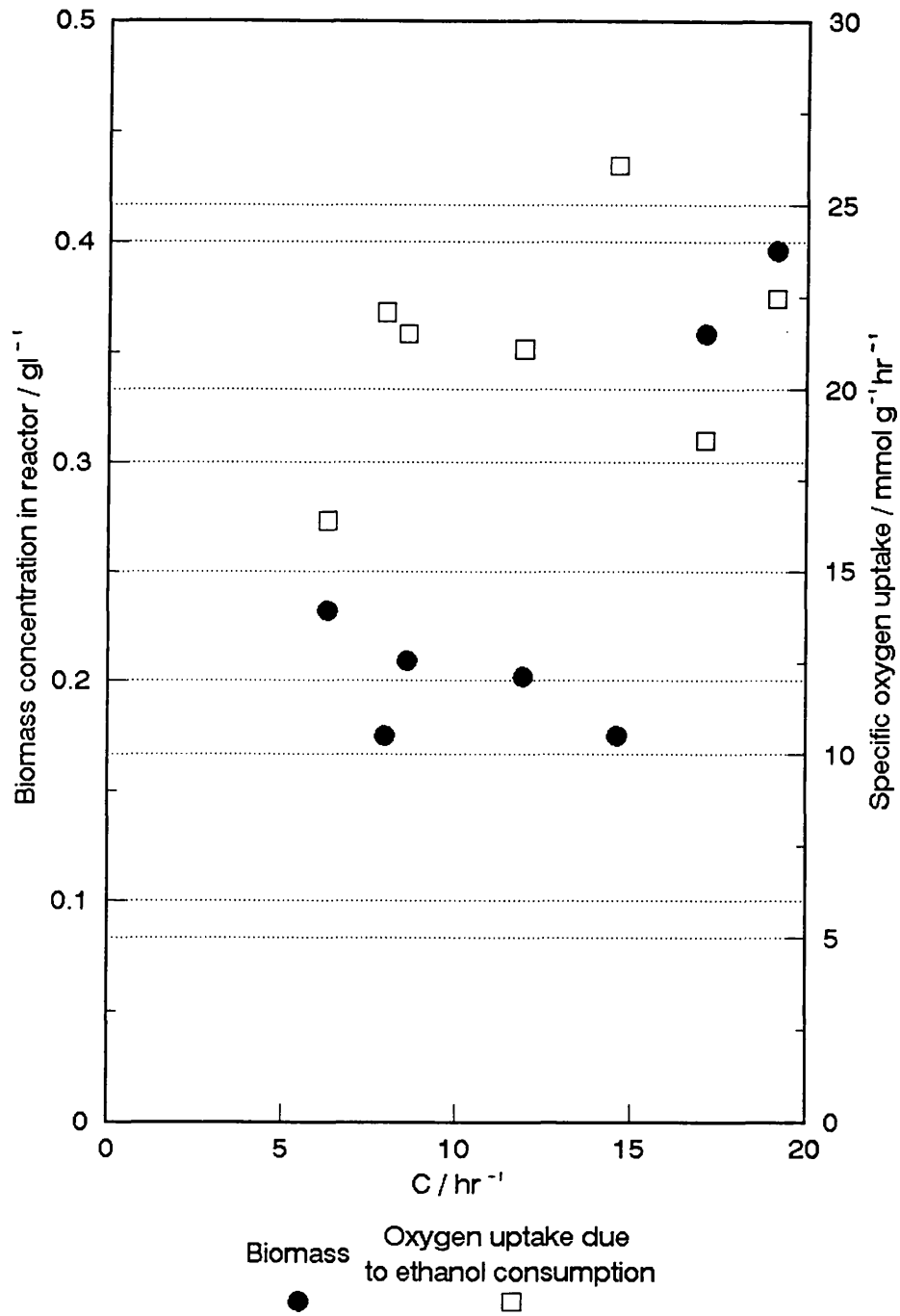




Figure 3.72 - Comparison of the OUR profile of the fermentation at 26°C and 20 % DOT to the calculated value of  $OUR_0 / OUR$  before chloroxyleneol addition.

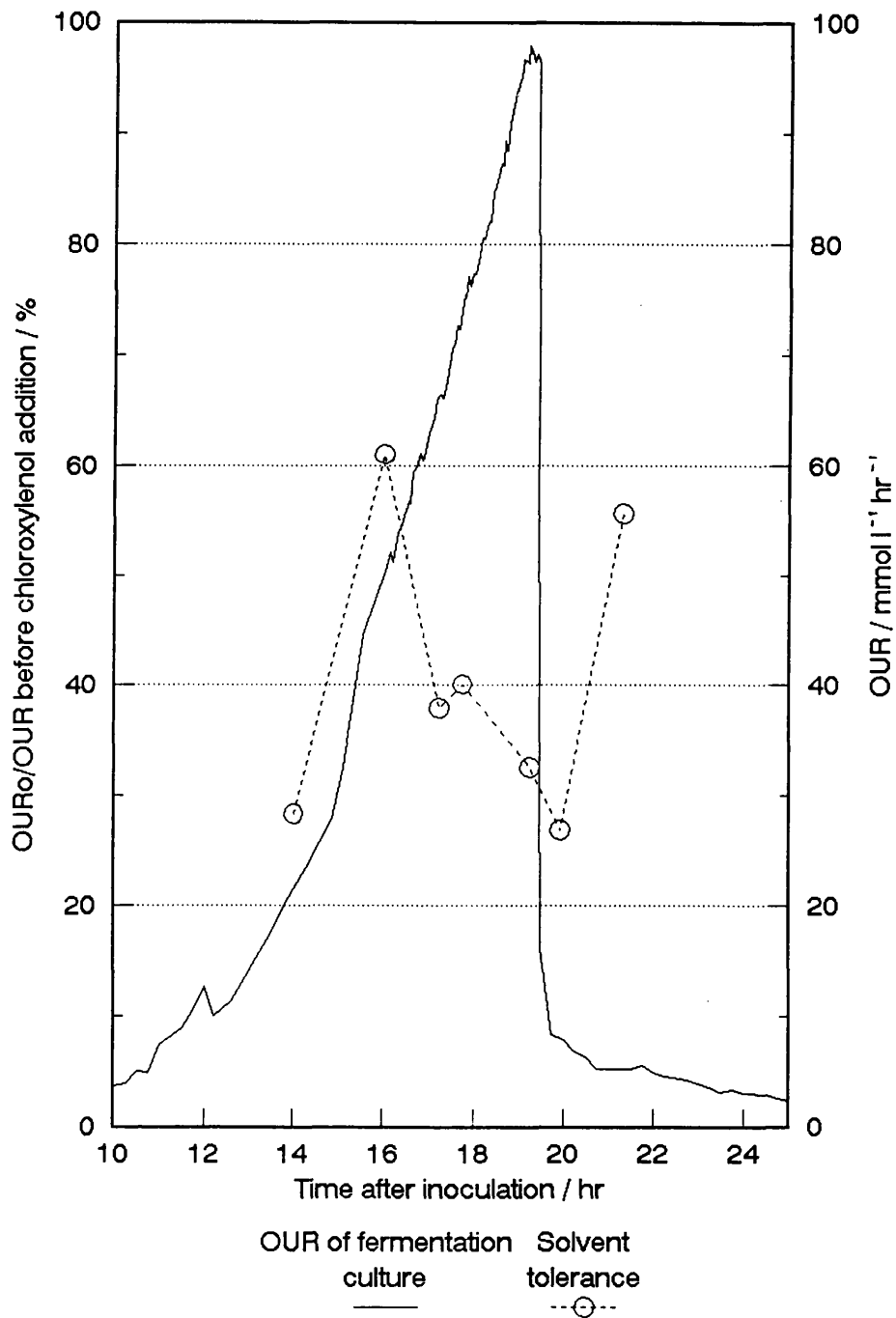
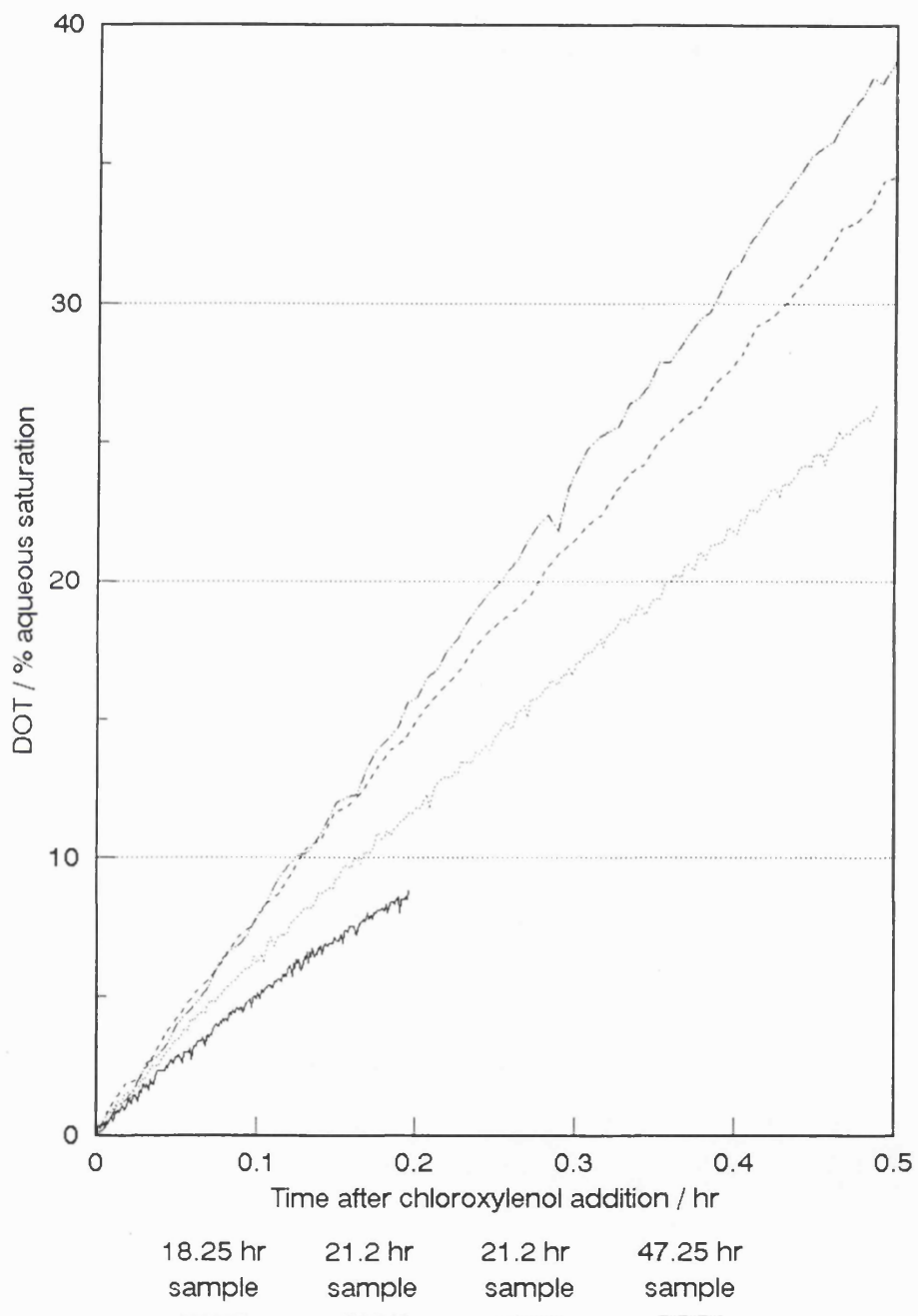


Figure 3.73 - The drop in DOT after the addition of chloroxylenol to the reactor of the bacterium sampled from the fermentation at 34°C.



### 3.3.5 - The Tolerance of P.putida to a variety of solvents

A number of solvents were analysed to determine whether the rate of loss of OUR was similar to that obtained with chloroxylenol and could be described by the equation:-

$$(DOT_0 - DOT_t) = ([OUR_0 - OUR_f] / c) \cdot (1 - \exp^{-ct}) + (OUR_f \cdot t)$$

A long chain alcohol was initially selected as the solvent to compare its toxicity to that of chloroxylenol. The addition of alcohols of carbon length from four to nine at below aqueous saturation to broth had resulted in negligible metabolic activity in *S.cerevisiae* where as slight activity was observed with decanol and undecanol (Bar, 1988). Octanol has been shown to have greater toxicity than other alcohols of shorter chain length (Osborne et al, 1990). Octanol was selected on the basis that it was toxic at low concentrations and also the partitioning of compounds between octanol and water has been used previously as an indicator of hydrophobicity and biocompatibility.

Figure 3.74 demonstrates that an increase in octanol concentration in the reactor results in a more rapid decrease in OUR. The concentrations quoted are the initial value in the electrode, the concentration at the end of the assay was not measured. Four of the profiles were fitted to the above equation to obtain the C value which are plotted against octanol concentration in Figure 3.75. A major difference between these values and that obtained for chloroxylenol was that  $OUR_f$  was always zero for octanol where as  $OUR_f$  was 60 % of  $OUR_0$  for a chloroxylenol concentration of  $0.067 \text{ gl}^{-1}$ . Octanol toxicity would therefore be comparative to a higher chloroxylenol concentration where  $OUR_f$  would be much lower.

The curve fitted to the profile obtained for the bacterium at a octanol concentration of  $0.82 \text{ gl}^{-1}$  indicate that the behaviour of the octanol toxicity is different at higher concentrations (Figure 3.76) in that it did not follow

Figure 3.74 - Octanol toxicity upon the OUR due to ethanol consumption as demonstrated by changes in the DOT profile.

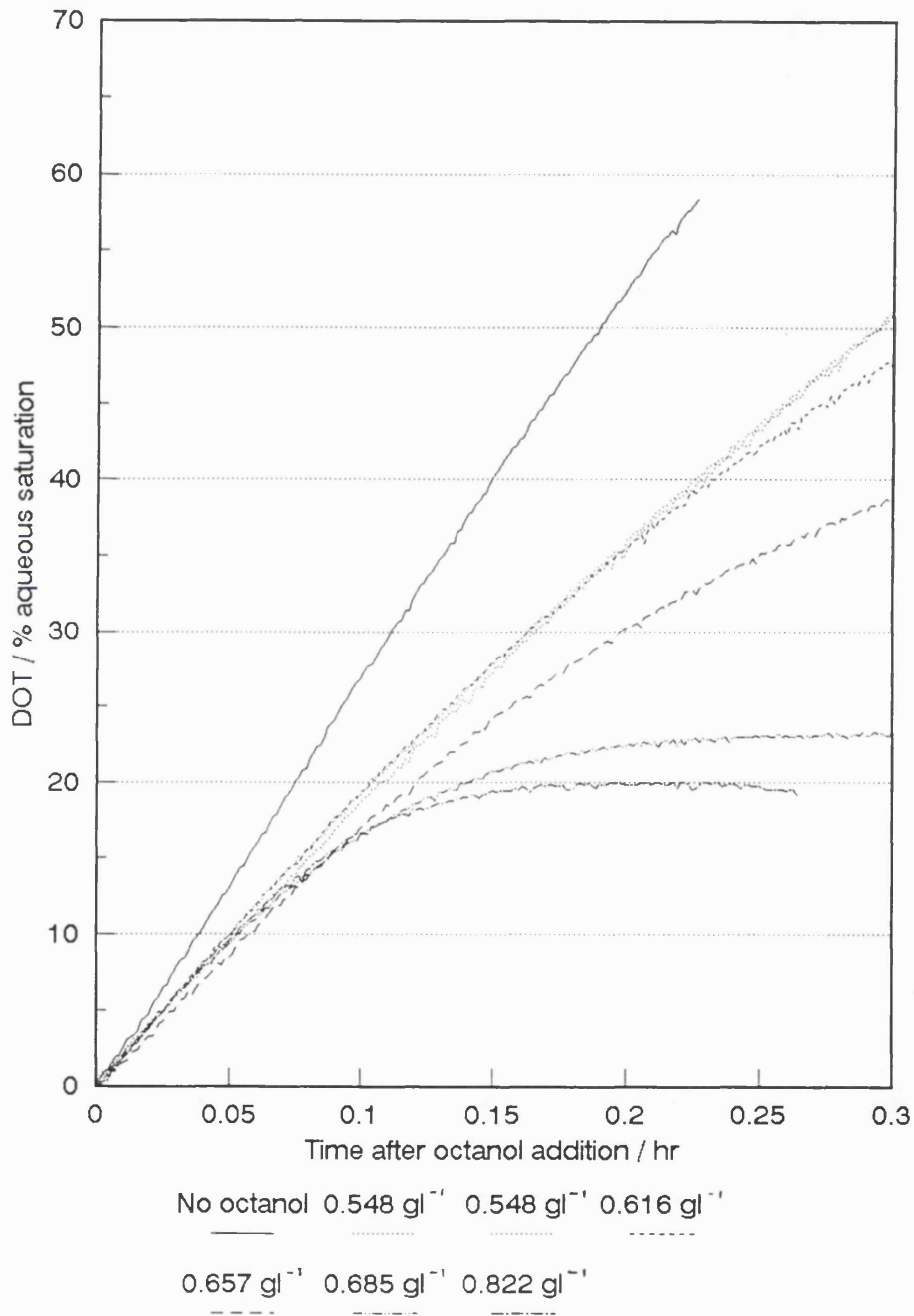


Figure 3.75 - The relationship between the calculated C values obtained from the profiles in Figure 3.74 and octanol concentration.

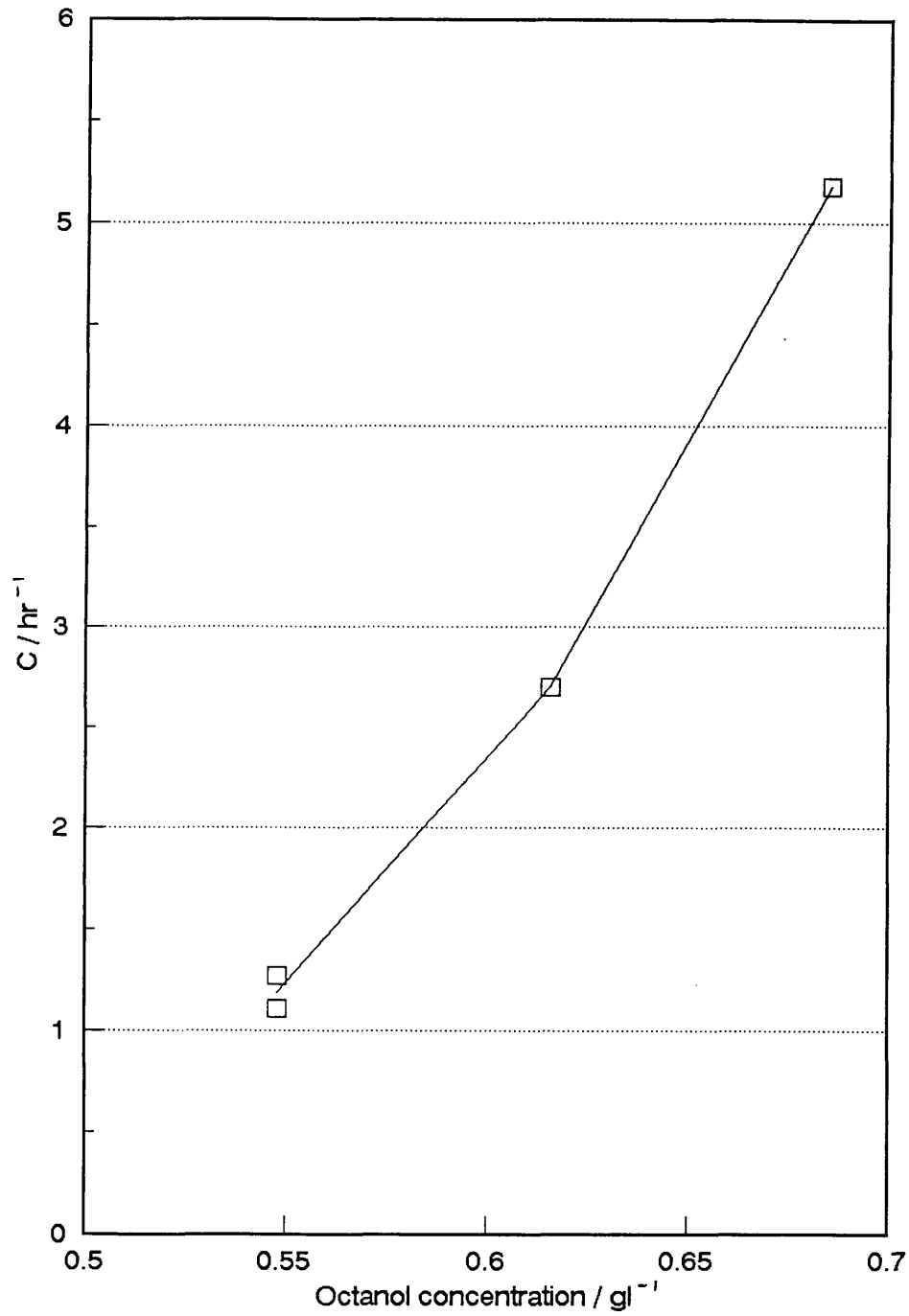
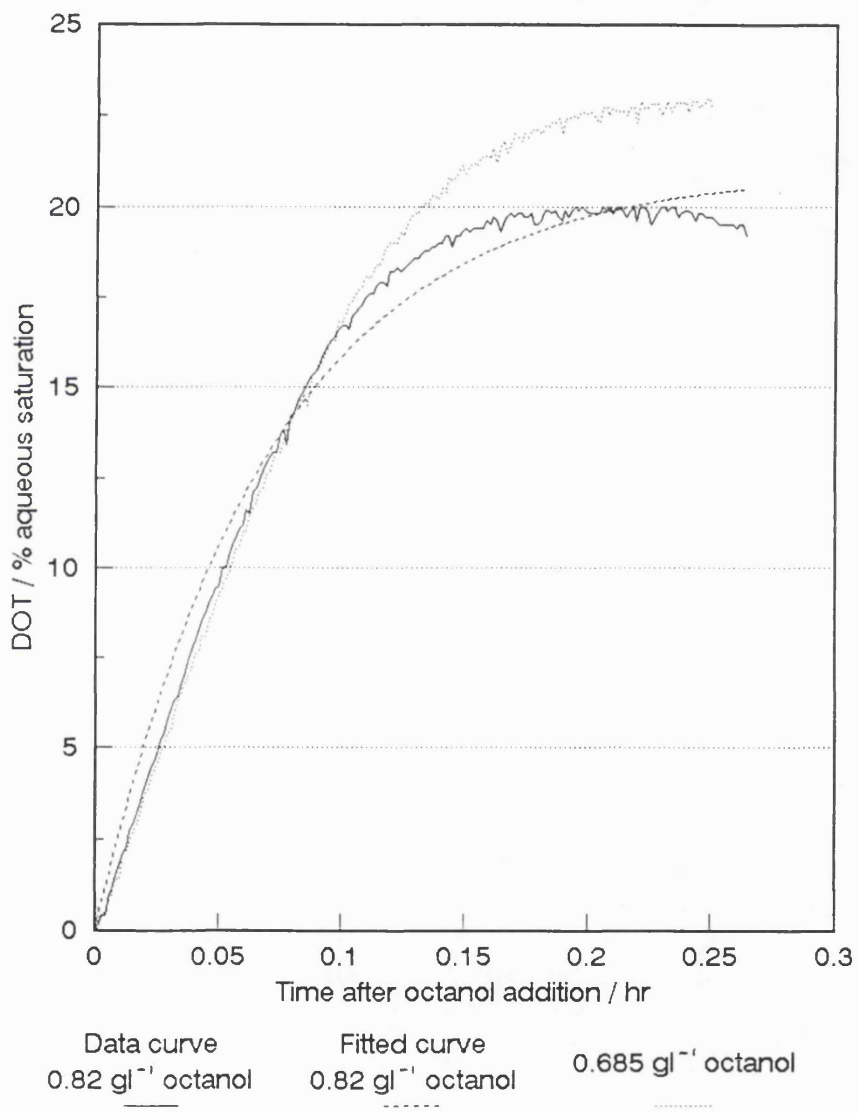


Figure 3.76 - Octanol toxicity at  $0.82 \text{ gl}^{-1}$  indicating non-exponential decay in OUR as compared to fitted curve.



exponential type decrease in activity. The aqueous saturated concentration of octanol at 25°C is 0.54  $\text{gl}^{-1}$  (Riddick et al, 1986), at 30°C it would be higher and could be close to 0.82  $\text{gl}^{-1}$ . Undissolved octanol could therefore be present in the reactor soon after its addition in ethanol. The profile at an octanol concentration of 0.685  $\text{gl}^{-1}$  had a close fit to the exponential decay equation.

A number of other long chain alcohols were analysed to determine their toxicity at low concentrations and were compared to the profile obtained with octanol. All the alcohols and hexane were added at a reactor concentration of 0.0833 % (v/v). The profiles indicate that the toxicity of the solvents increase as the chain length increases (Figure 3.77). The  $\text{OUR}_f$  value in all cases was zero indicating complete inactivation of the bacteria was possible.

Fluorobenzene is the substrate in the biotransformation and has been shown to inhibit the growth and  $\text{OUR}$  of the bacteria at above 40 % aqueous saturation (Section 3.2.1.1). Fluorobenzene toxicity was therefore analysed at 22, 44, 66 and 88 % aqueous saturation (0.341, 0.683, 1.024 and 1.365  $\text{gl}^{-1}$ ) to give the profiles in Figure 3.78 and is compared to chloroxylenol at 0.067  $\text{gl}^{-1}$ . The C values calculated are plotted against fluorobenzene concentration (Figure 3.79).  $\text{OUR}_f$  was always zero which was similar to that obtained with the long chained alcohols and different to chloroxylenol which was 68 % of the  $\text{OUR}_0$  value.

Ethyl, butyl and pentyl acetate were analysed to determine whether the increase in toxicity of the solvents increased upon an increase in the Log P value. Pentyl acetate was shown to be the most toxic solvent of the three, ethyl acetate was not toxic even at a concentration four times greater than pentyl acetate (Figure 3.80). The C values obtained for pentyl acetate (3.34  $\text{gl}^{-1}$ ) and butyl acetate (6.53  $\text{gl}^{-1}$ ) were 7.55 and 25.7  $\text{hr}^{-1}$  with the  $\text{OUR}_f$  value

Figure 3.77 - The effect of 0.083 % (v/v) of decanol, hexane, nonanol, heptanol and octanol upon the OUR due to ethanol consumption.

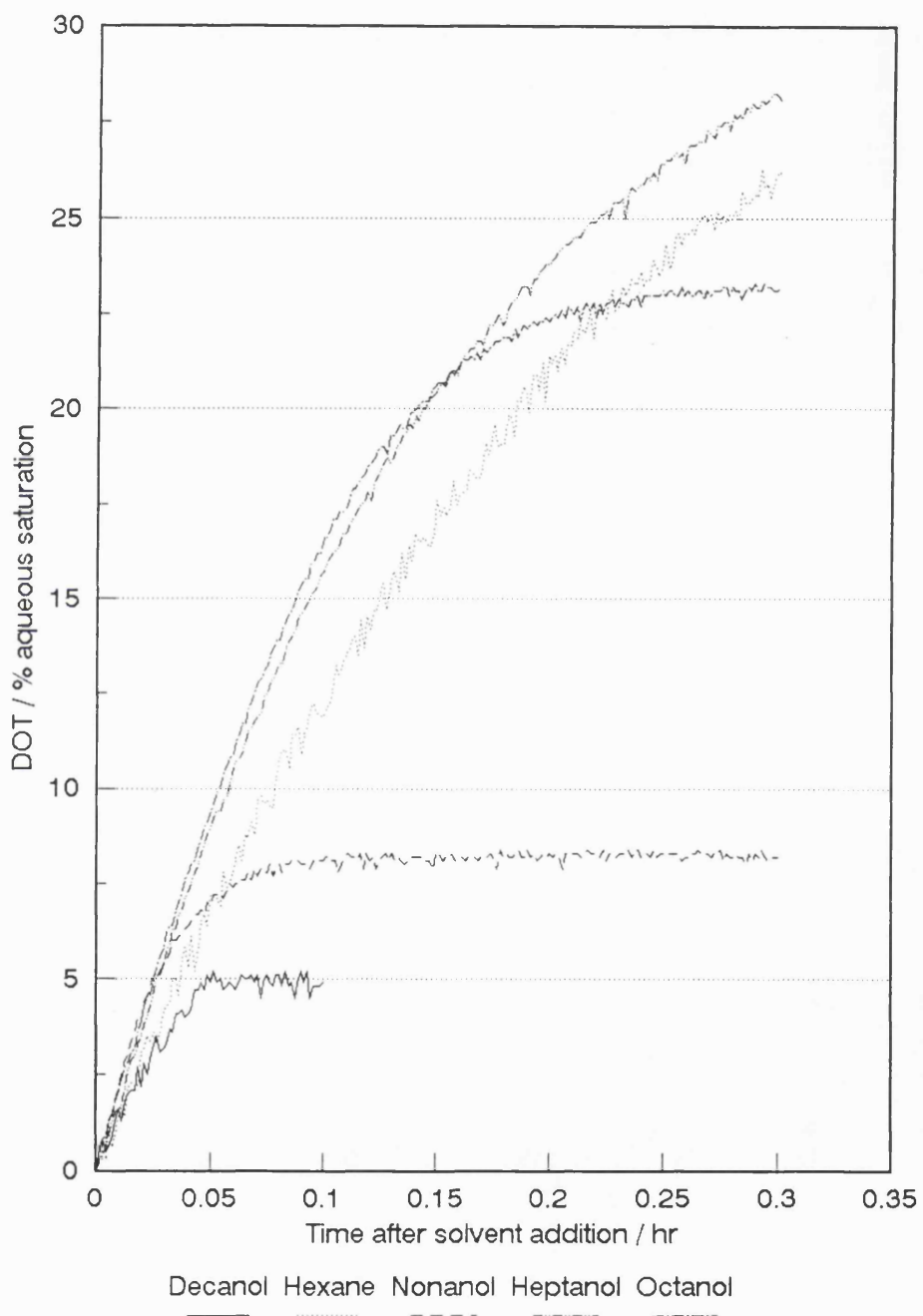




Figure 3.78 - DOT profiles of *P.putida* ML2 against time demonstrating fluorobenzene inhibition of the OUR due to ethanol consumption.

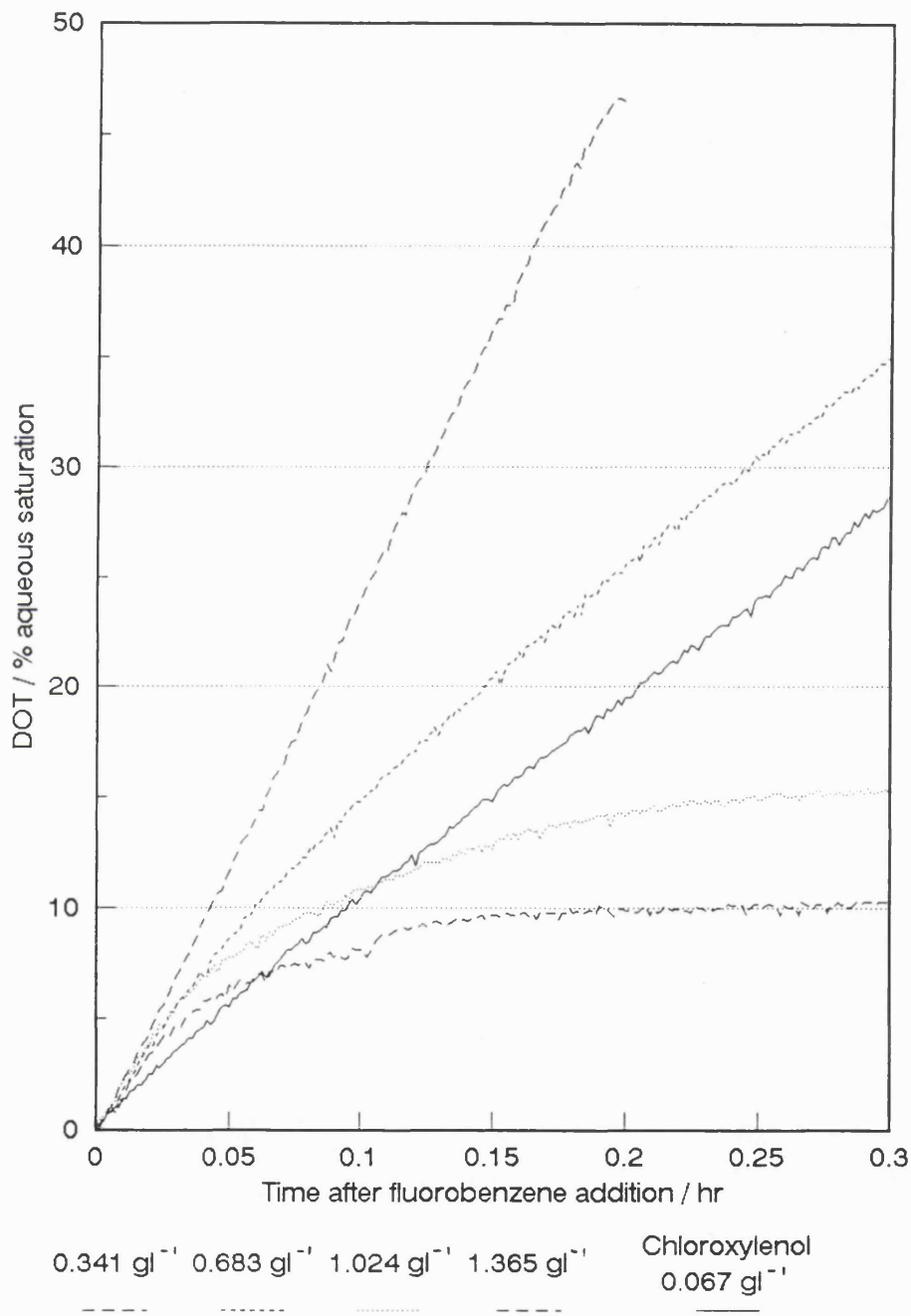


Figure 3.79 - The relationship between the calculated C values obtained from the profiles in Figure 3.78 and fluorobenzene concentration.

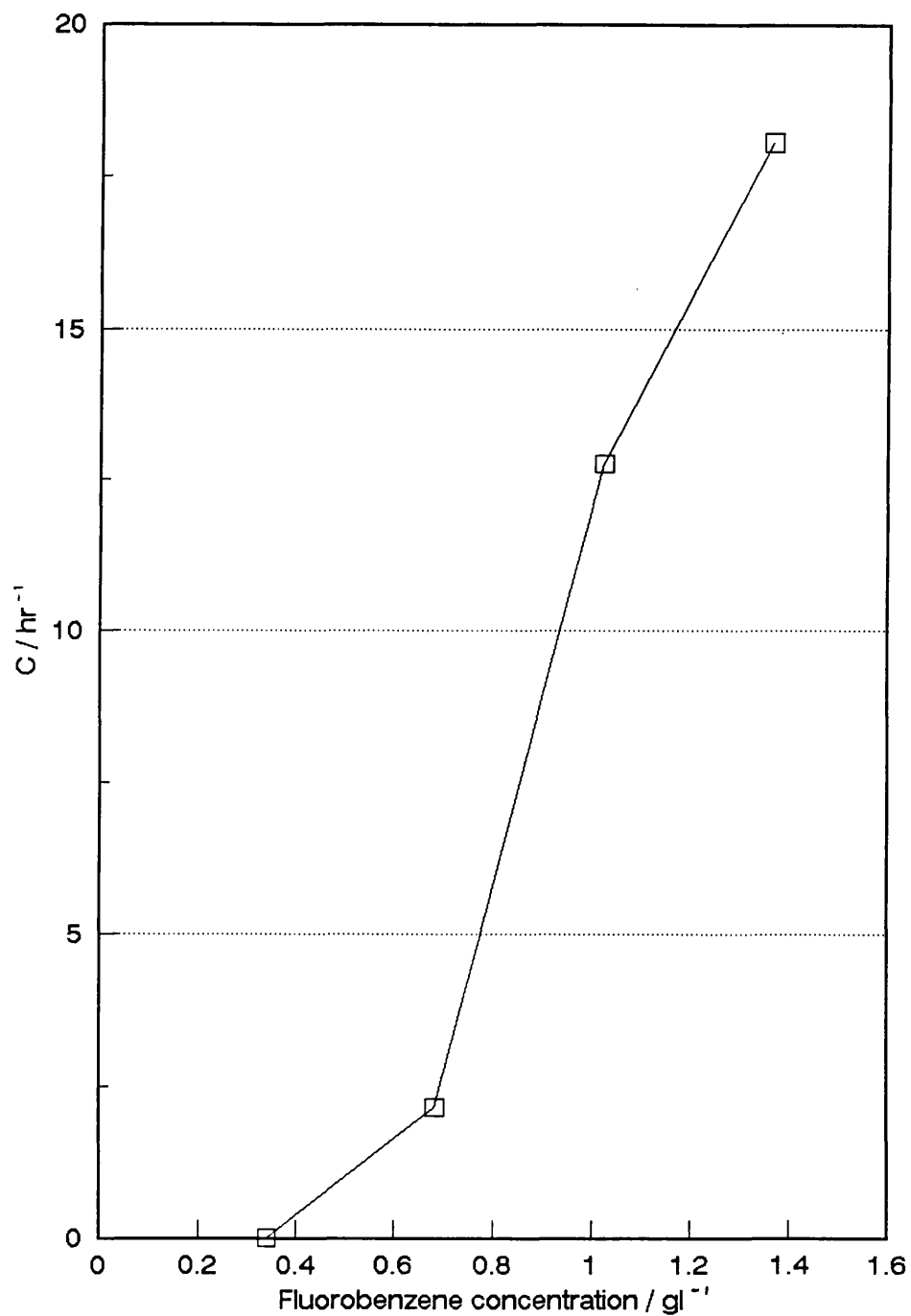
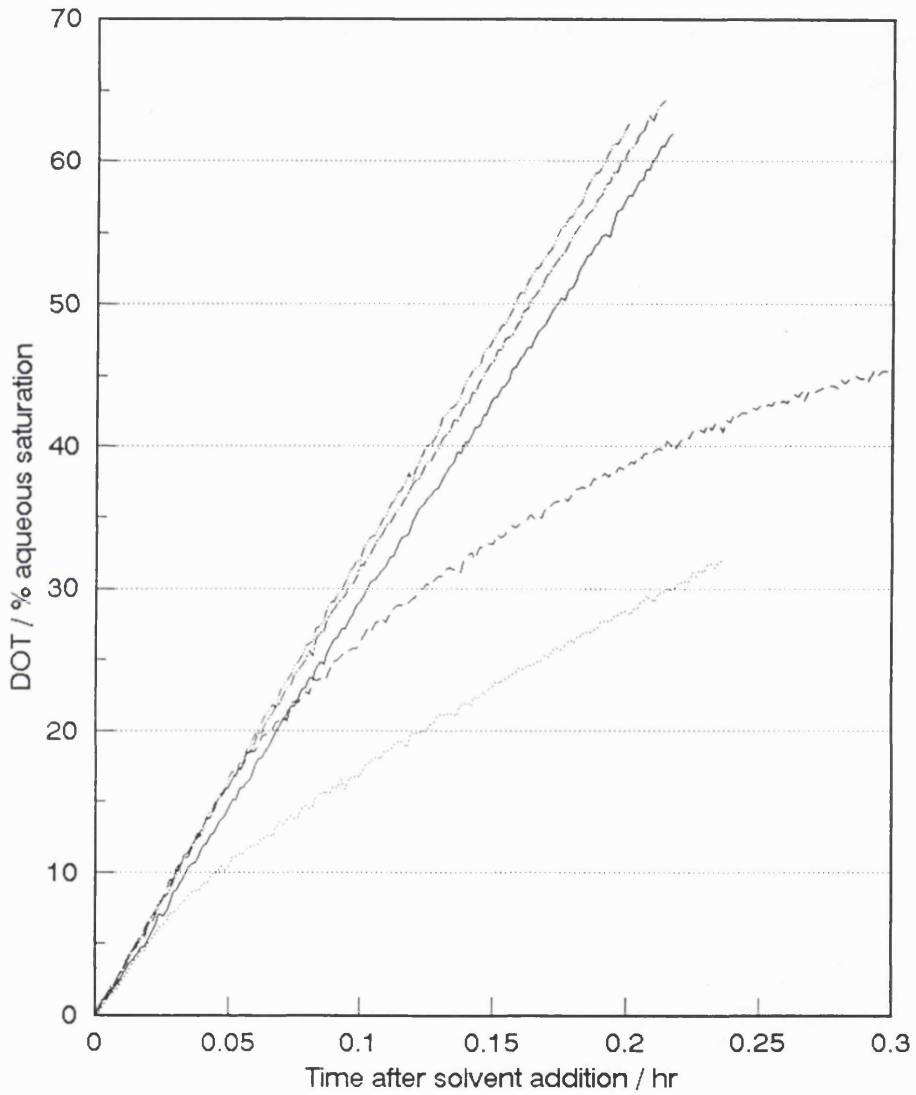


Figure 3.80 - DOT profiles of *P.putida* ML2 against time demonstrating ethyl, butyl and pentyl acetate inhibition of the OUR due to ethanol consumption.



Ethyl acetate 3.26 gl -----	Ethyl acetate 13.05 gl _____	Butyl acetate 3.26 gl -.-.-.-
Butyl acetate 6.53 gl .....	Pentyl acetate 3.34 gl - - - -	

reaching 1.6 and 36 % of  $OUR_0$ . The C value obtained for this culture when chloroxylenol was added at a concentration of  $0.067 \text{ gl}^{-1}$  was  $2.95 \text{ hr}^{-1}$  with the  $OUR_t$  decreasing to 11 % of the  $OUR_0$  value.

### 3.3.6 - The effect of magnesium and EDTA ions upon the solvent tolerance of *P.putida* ML2

The tolerance of *P.putida* ML2 to solvents has been shown to decrease upon addition of EDTA to the biotransformation (Harrop, 1990). This was thought to be due to the destabilisation of the outer membrane by the removal of the magnesium ions which binds the lipopolysaccharide molecules together. The effectivity of chloroxylenol as a disinfectant against Gram - bacteria has been shown to increase upon the addition of EDTA (Russell & Furr, 1977). Chloroxylenol ( $0.05 \text{ gl}^{-1}$ ) has been shown to have greater inhibition of the OUR due to ethanol consumption if  $K_2\text{EDTA}$  ( $2 \text{ gl}^{-1}$ ) had been included as compared to chloroxylenol alone at a concentration of  $0.067 \text{ gl}^{-1}$  (Figure 3.81). An increase in  $K_2\text{EDTA}$  concentration to  $10 \text{ gl}^{-1}$  had not resulted in significant changes in the toxicity of chloroxylenol. Magnesium sulphate added to the electrode to a concentration of  $10 \text{ gl}^{-1}$  had protected the bacterium against the toxicity of chloroxylenol ( $0.067 \text{ gl}^{-1}$ ). The bacteria were also protected by  $\text{MgSO}_4$  ( $2 \text{ gl}^{-1}$ ) to a higher chloroxylenol concentration of  $0.33 \text{ gl}^{-1}$ , further increase in  $\text{MgSO}_4$  concentration had not resulted in greater tolerance (Figure 3.82). The profiles obtained at a chloroxylenol of  $0.13 \text{ gl}^{-1}$  or  $0.33 \text{ gl}^{-1}$  were similar when  $10 \text{ gl}^{-1}$   $\text{MgSO}_4$  had been added indicating similar toxicity, a reduction in chloroxylenol toxicity to  $0.067 \text{ gl}^{-1}$  had resulted in lower toxicity.

$\text{MgSO}_4$  has been shown to protect the bacterium against the toxic fluorobenzene concentration of 80 % aqueous saturation (Figure 3.83), but further increases in  $\text{MgSO}_4$  concentration had not resulted in greater solvent tolerance.

Figure 3.81 - The effect of  $K_2EDTA$  and  $MgSO_4$  upon the tolerance of *P.putida* ML2 towards chloroxylenol.

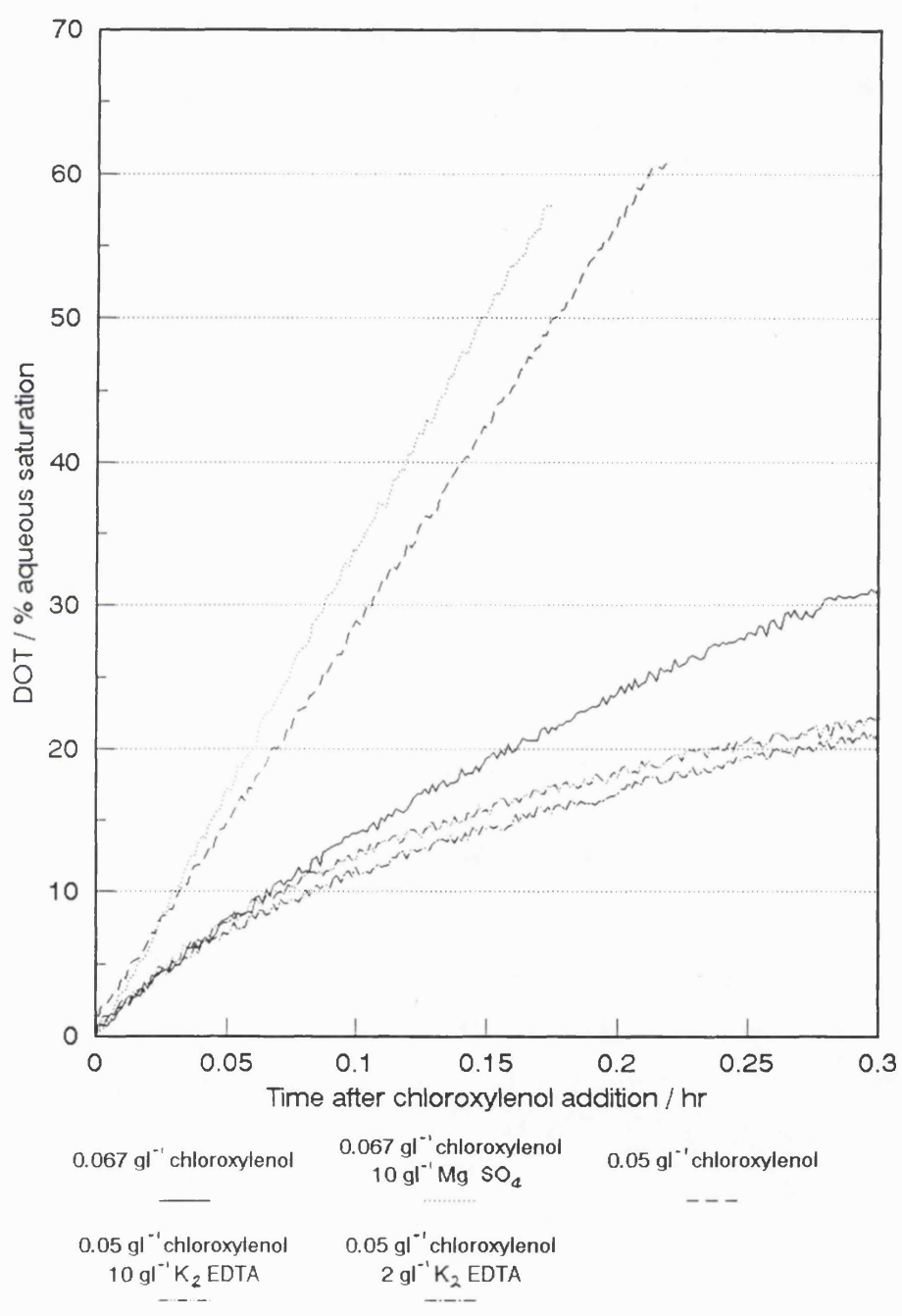


Figure 3.82 - The effect of various concentrations of  $MgSO_4$  upon the tolerance of *P.putida* ML2 towards chloroxylenol.

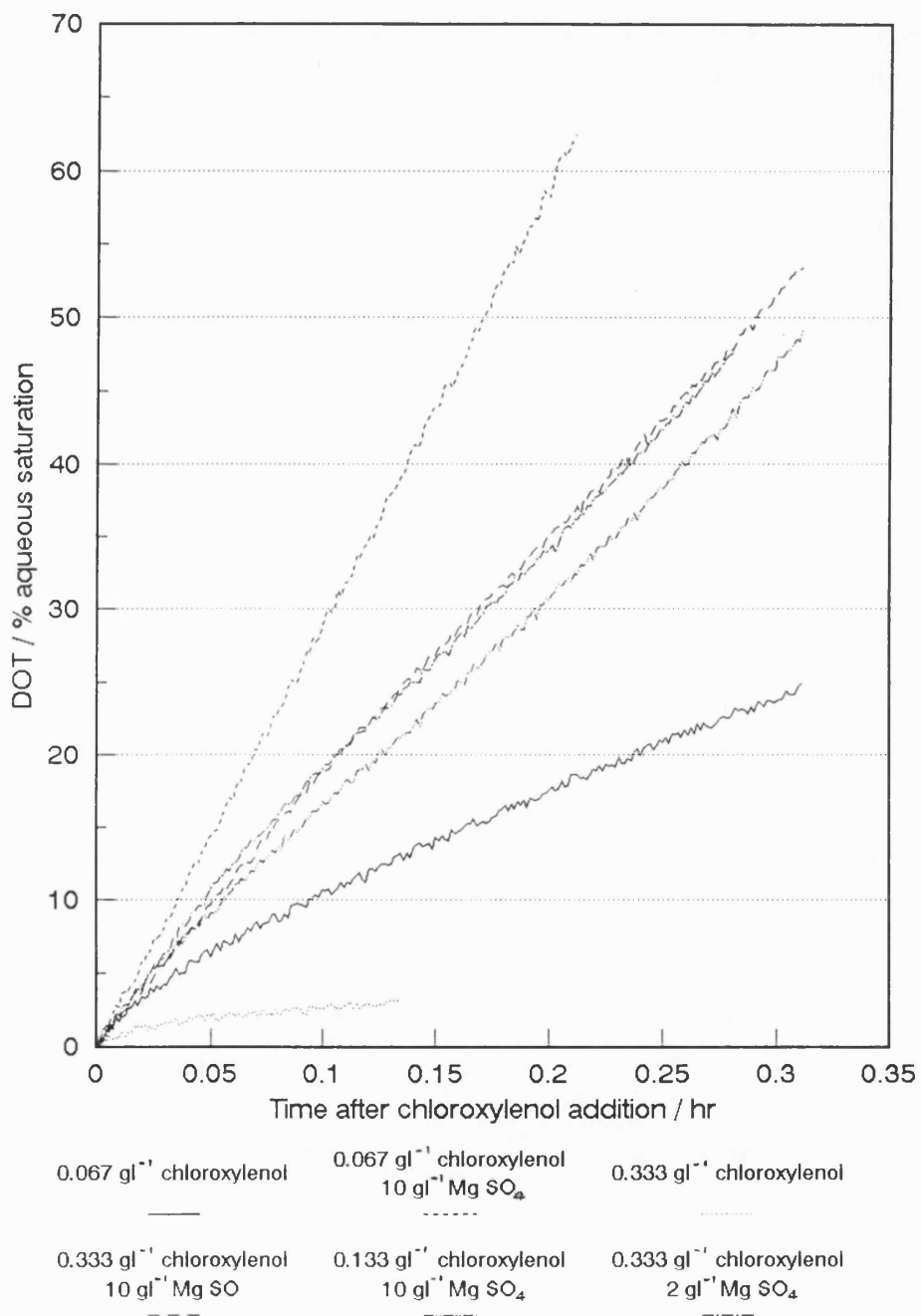
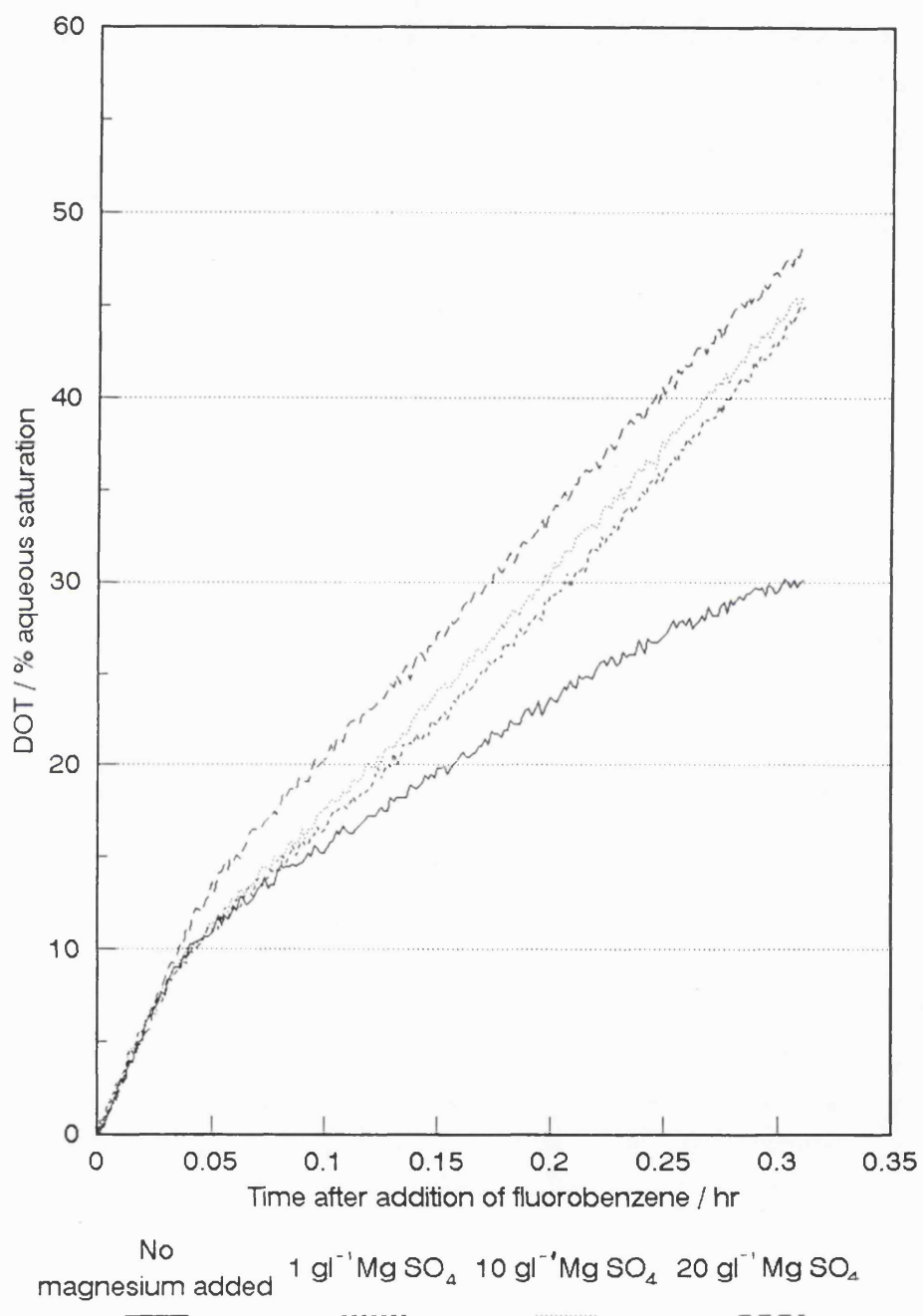


Figure 3.83 - The effect of various concentrations of  $MgSO_4$  upon the tolerance of *P.putida* ML2 towards fluorobenzene.



Dichloroethane toxicity towards the bacterium had also been increased by the addition of  $K_2EDTA$  to the reactor but reduced when  $MgSO_4$  was added (Figure 3.84).

The effect of  $MgSO_4$  was studied further with the assay performed at  $30^\circ C$  and at the lower temperature of  $23^\circ C$ . The bacterial culture was more tolerant to the dichloroethane when the temperature was at  $23^\circ C$  but the addition of  $MgSO_4$  did not increase the tolerance further (Figure 3.85).  $MgSO_4$  did increase the tolerance of the culture towards dichloroethane at  $30^\circ C$  but the profile did not reach linearity within 0.2 hr which was observed for the cells at  $23^\circ C$ . The culture at  $30^\circ C$  reached a linear profile when fluorobenzene was the solvent with  $MgSO_4$  added. This indicates that the protection of the bacterial cells with magnesium ions is dependent on the particular toxic solvent which it is exposed.



Figure 3.84 - The effect of  $K_2EDTA$  and  $MgSO_4$  upon the tolerance of *P.putida* ML2 towards dichloroethane.

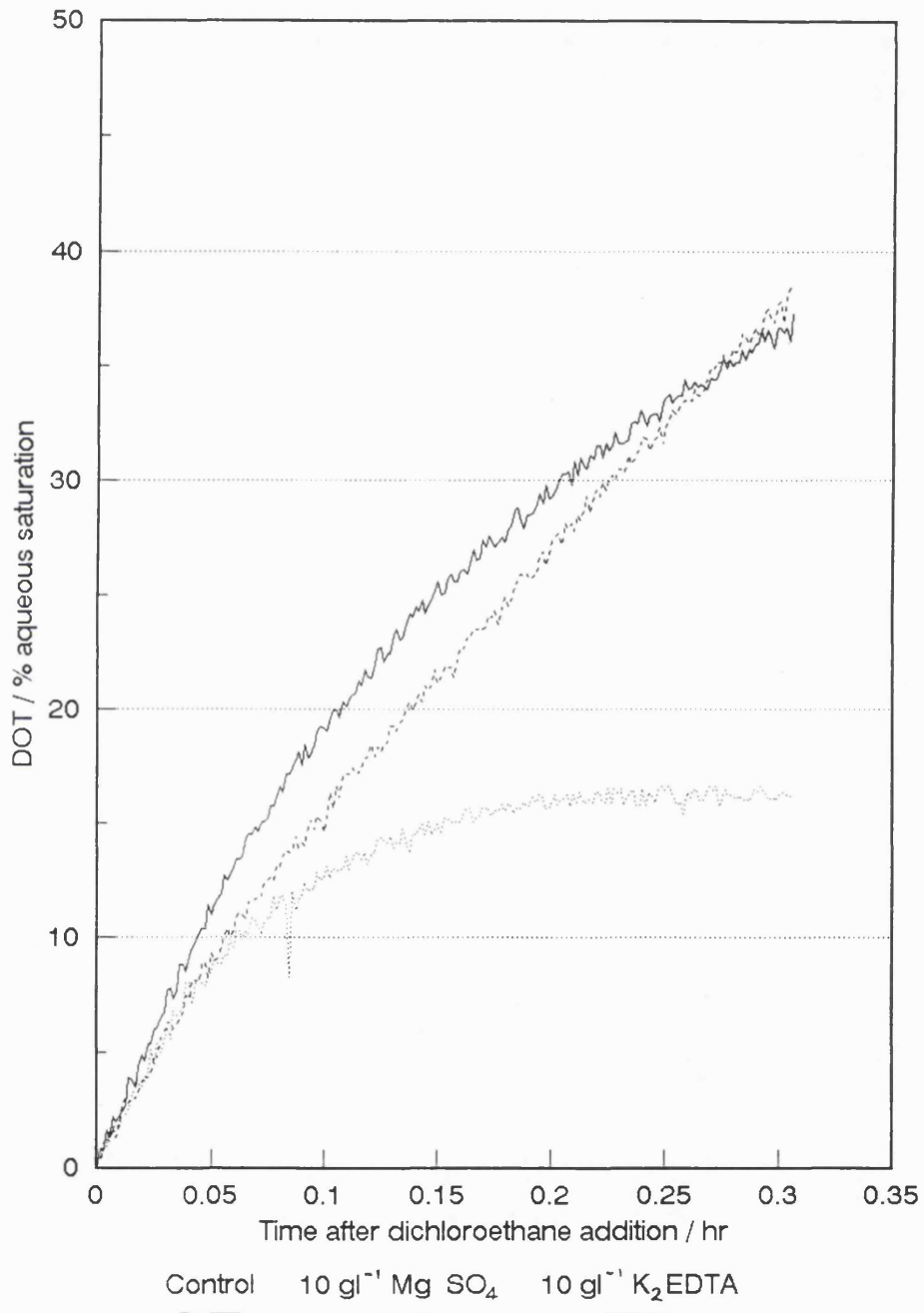
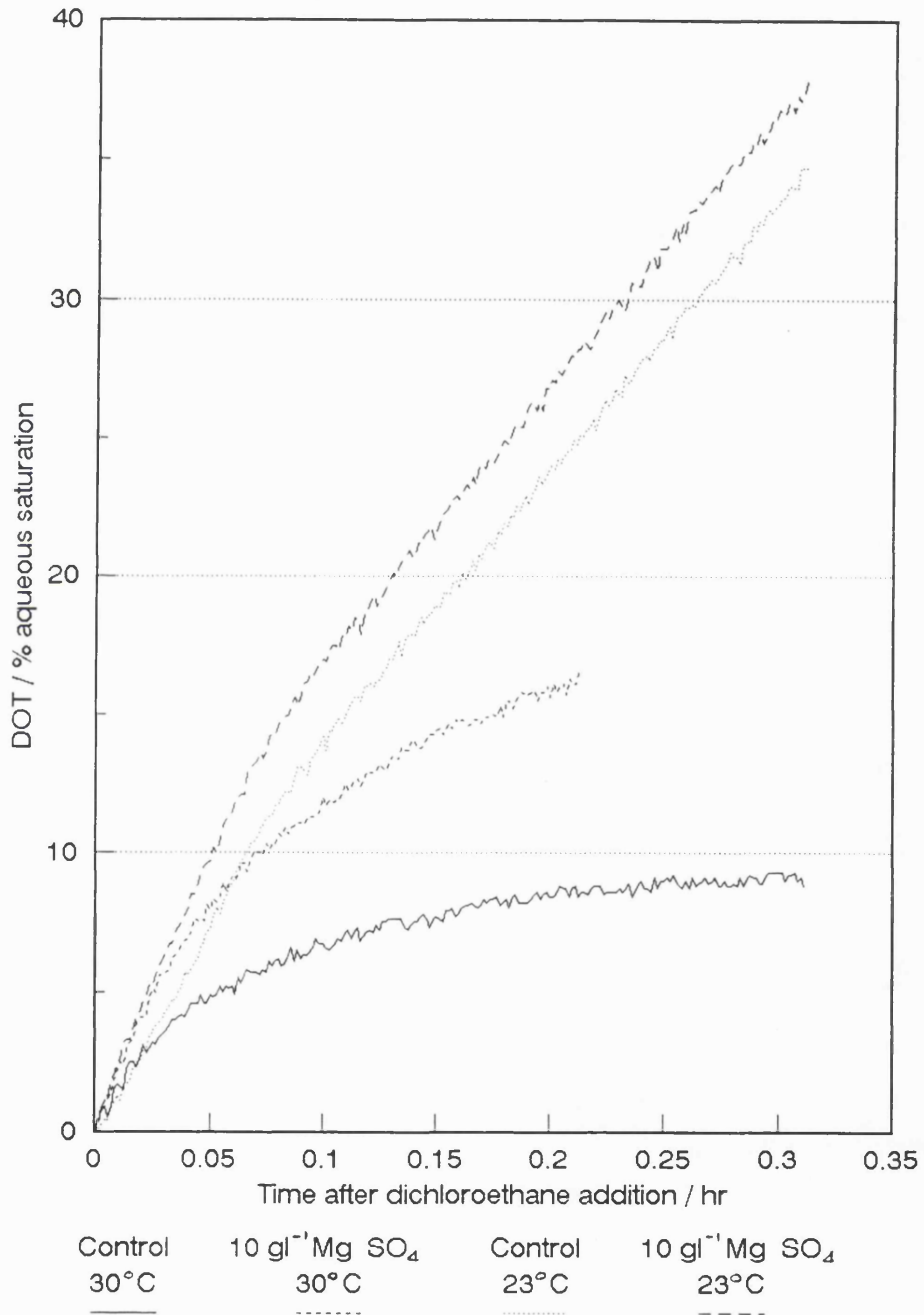


Figure 3.85 - The effect of a decrease in reactor temperature and  $MgSO_4$  upon the tolerance of *P.putida* ML2 towards dichloroethane.



#### 4 - DISCUSSION

There is an increasing interest in the use of microorganisms to carry out regioselective and stereospecific reactions as part of an overall process to produce useful compounds for the pharmaceutical, food and chemical industries. Large numbers of papers have been published on the biotransformations themselves but little has been reported on the optimum conditions for the production of the biocatalyst. The objective of this present study was to examine the influence of the fermentation conditions on the activity and stability of the aromatic oxidation activity by Pseudomonas putida.

##### 4.1 - Fermentation

Before examining the influence of the growth conditions on the aromatic oxidation activity and the solvent tolerance of the bacterium, it was essential to determine the effects on the rate of growth and the final biomass yield. These will affect the cellular productivity of the batch fermentation which may be important to the economics of the biotransformation process.

In this study four different fermentation conditions were investigated and the parameters necessary to characterise the fermentations are listed in Table 4.1. The data for the three experiments at 30°C and 20% DOT confirm the reproducibility of the fermentation (Figures 3.4 and 3.5). Changing the growth conditions to 5 % DOT and 30°C and 20 % DOT and 26°C had resulted in the growth phase taking 5.9 and 2.5 hr longer to finish. This is a large percentage of the fermentation time but when considered as a percentage of the whole process time which would include inoculum development, fermenter set up, biotransformation reaction and downstream processing the time would be insignificant as long as an added advantage had been gained from changing the growth conditions. The growth of the cells at 5 % DOT and 30°C had resulted in a lower maximum CER at 44 % of the

Table 4.1 - Growth characteristics at four different fermentation conditions.

Growth Conditions	20 % DOT			5 % DOT	20 % DOT	20 % DOT	
	30°C			30°C	26°C	34°C	
Time to finish/ hr*	17.0	16.7	16.9	22.8	19.4	28.8	22.3
Maximum CER /mmoll hr <sup>-1</sup>	170	173	184	78	142	114	115
Maximum A <sub>670</sub> value	17.6	17.8	16.9	15.6	13.7	-	12.0
Maximum acid added / mmol	662	680	672	644	662	687	685
Total carbon evolved as carbon dioxide/gl <sup>-1</sup>	6.66	6.70	6.70	6.51	6.90	6.85	7.27

\* - Time after inoculation for growth phase to finish

fermentation at 20 % DOT and 30°C leading to the fermentation requiring a lower maximum  $K_a$  value. This would lead to a lower power consumption, but the total power consumption could still be higher due to the lengthened fermentation time. More importantly, the lower maximum CER and OUR would be more easily achievable at pilot and production scale.

The two fermentations at 34°C had shown significant variations between batches which was due to differences in inoculum level. Expression of the CER data and the total carbon dioxide evolved data on a logarithmic scale showed that the exponential rate of increase in activity had decreased with time indicating the toxicity of the higher growth temperature (Section 3.1.5.2). The reproducibility of the fermentations at 34°C and 20 % DOT could be a major problem since variations in the inoculum level will lead to variations at the end of the growth phase. The yield of cells at 34°C and 20 % DOT was 69 % of that at 30°C, 20 % DOT; similarly the fermentations at 30°C, 5 % DOT and 26°C, 20 % DOT had lower cell yield at 89 and 79 %. This is surprising as cell yield often increases at lower temperature.

With the exception of the fermentations at 34°C and 20 % DOT, there were a number of similarities between the other fermentations done at 30 and 26°C. These include the total carbon dioxide evolved and the total acid added to control pH, in that the fermentations at 26°C and 20 % DOT, 30°C and 5 % DOT were within 4 % of the average value obtained for the three fermentations at 30°C and 20 % DOT. The main similarity between the fermentations was that the growth phase was split into two sections although succinate was the sole carbon source. The two sections were not separated by a stationary phase and were only obvious in all the fermentations when the data for carbon dioxide evolved were analysed on a logarithmic scale. The fermentations had finished in a similar manner by carbon starvation and although the cell yield was lower and the length of the

growth phase was longer these were not as significant as the aromatic oxidation activity which had varied from 0 to 7.5 mmol g<sup>-1</sup> hr<sup>-1</sup> for the fermentations at 30°C and 20 % DOT. The variations in the aromatic oxidation activity were a result of changes in the growth and carbon metabolism of the bacterium; these had therefore been analysed in detail.

#### 4.2 - Fermentation kinetics

The initial OUR profile (Figure 3.4) and the biomass profile (Figure 3.8) had not indicated significant variations in the metabolism of the bacterium during the fermentation. Analysis of the growth rate and carbon source uptake would allow the modelling of the fermentation so that comparative analysis with other fermentations under different growth conditions could be made. Expression of the CER and total carbon dioxide evolved data on a logarithmic scale had indicated that the metabolic activity was not exponential throughout the growth phase. Changes in the metabolism during the growth phase had also been demonstrated by the changes in RQ values (Figure 3.5) and variations in the aromatic oxidation activity (Figure 3.44). In modelling the fermentation the carbon uptake was calculated by assuming a yield of biomass upon the carbon source which remains constant throughout the growth of the bacteria. This was not true for the fermentation at 30°C and 20 % DOT since the biomass increased exponentially where as the total carbon dioxide evolved did not from 12 hr after inoculation onwards.

Changes in the metabolism were indicated in CER, OUR and biomass data with the specific CER and specific OUR value decreasing during the growth phase rather than remaining the same. The fermentation was further analysed so that the carbon data, biomass and the addition of acid to control pH could be related to each other and would give an indication of the changes in carbon metabolism. The changes in the metabolic activity of the bacterium had indicated a greater

effect upon the aromatic oxidation activity rather than the growth of the cells.

Acid input was analysed as the first indicator of changes in metabolic activity since it could be directly related to the uptake of succinate. Complete consumption of succinate (0.93 mol) would result in the medium requiring replacement with the equivalent amount of acid, in this case 0.93 mol  $\text{H}_2\text{SO}_4$ . Table 2.1 had shown that 0.64 to 0.69 mol of  $\text{H}_2\text{SO}_4$  had been added to the fermentation which is 26 to 31 % lower. A direct relationship between succinate uptake and acid input was not obtained and would suggest that some of the succinate had been taken up as a neutral molecule (e.g. diammonium succinate). The acid input was therefore compared to carbon dioxide evolved so that if a byproduct was produced it may be indicated by variations in carbon dioxide evolved as compared to acid input. A linear correlation had been obtained between them for the fermentation at 20 % DOT (30 and 26°C) which did not indicate the variations in metabolism as discussed previously. Two linear correlations had been obtained for the fermentation at 30°C and 5 % DOT, one at the start and the other at the finish indicating that a byproduct had been produced in this case. The fermentation at 5 % DOT (30°C) had clearly indicated a difference in the metabolism where as the fermentations at 20 % DOT (30 and 26°C) had not.

Acid input had been shown to correlate well with carbon metabolism due to close linear fit obtained for the three fermentation conditions which was better than the fit between acid input and biomass. A linear fit had not been obtained between biomass and carbon dioxide evolved for the fermentation at 20 % DOT and 30°C where as a linear fit was obtained for the fermentation at 5 % DOT and 30°C. This close relationship between biomass and carbon dioxide evolved was also indicated by the fact that the growth rate ( $0.19 \text{ hr}^{-1}$ , 15 - 22.8 hr after inoculation) was similar to the exponential rate of carbon dioxide evolved from the fermenter

( $0.21 \text{ hr}^{-1}$ , 15 - 22.8 hr after inoculation). It suggests strongly that in the second section of the growth phase the bacterial <sup>culture</sup> was consuming a single carbon source which was likely to be a byproduct of the succinate consumed in the first section of the growth phase. The bacteria growing at 20 % DOT and 30°C indicate that during the growth phase a mixture of metabolic activities were indicated which includes full consumption of succinate, succinate consumption with byproduct accumulation and byproduct consumption.

The carbon dioxide evolved could not be related to the biomass since both increased exponentially but at different rates. During the first section of the growth phase of all three different fermentation conditions the growth rate was lower than the exponential rate of increase of total carbon dioxide evolved. The three fermentations at 30°C and 20 % DOT had a growth rate between 55 and 57 % of the exponential rate of total carbon dioxide evolved, this was similar to the fermentation at 26°C (56 %). This would indicate that the metabolic activity of the bacterium had slowed down due to the decrease in temperature but the amount of byproducts formed and the changes in metabolism during the growth phase was similar. The fermentation at 5 % DOT and 30°C had a growth rate 43 % of the exponential rate of total carbon dioxide evolved indicating that the DOT had a greater effect upon the growth of the bacteria than its carbon metabolism. It is clear that DOT can affect the metabolic pathways in different ways, the control of the induction of the hydroxylating enzymes of Pellicularia filamentosa had an optimum of 15 % DOT where as the optimum for expression was 30 % DOT (Clark et al, 1982). Critical DOT values for penicillin production (30 % DOT) and the OUR of the culture (7 % DOT) were two distinct parameters since below these levels the activities decreased rapidly (Vardar & Lilly, 1982).

In all three fermentation conditions the growth of the bacterium was shown to be uncoupled from substrate

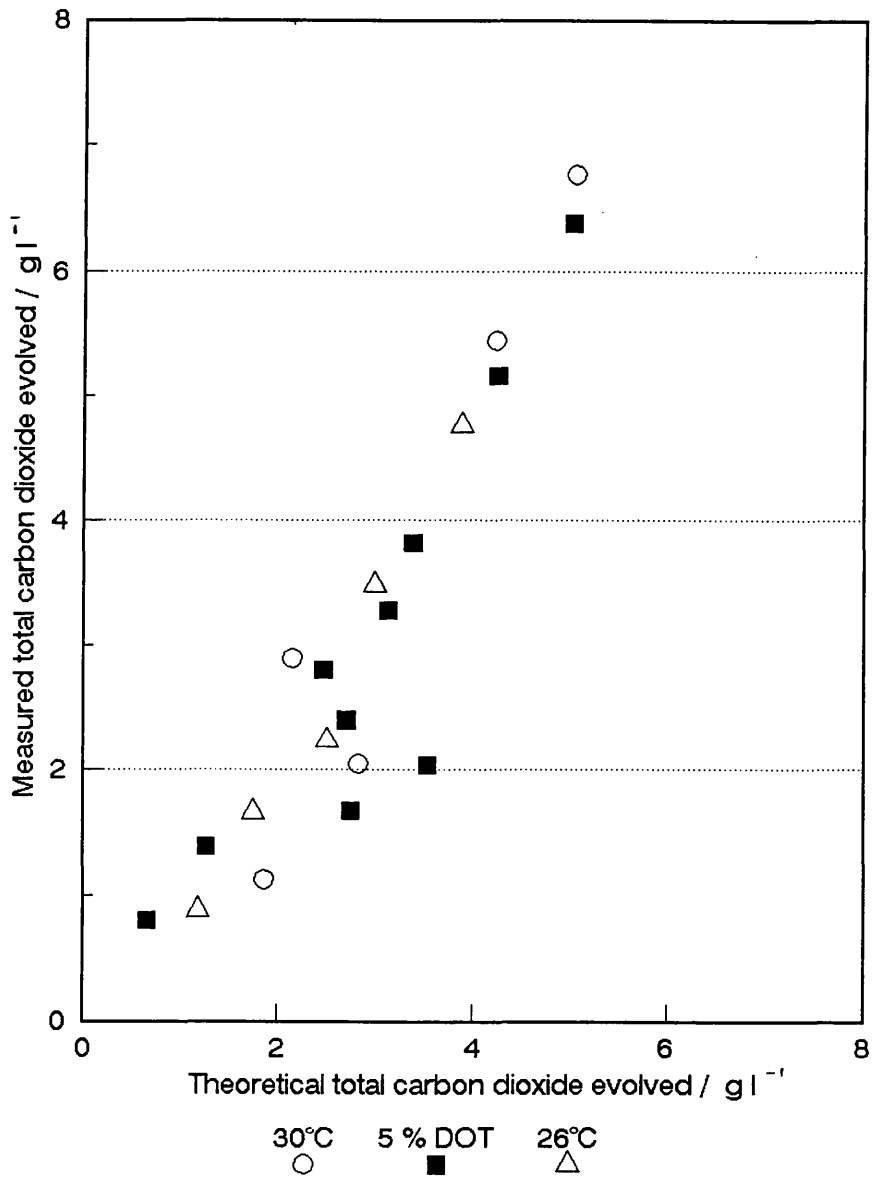


consumption. This type of growth has been observed under various growth conditions (Stouthamer, 1979) but in this case the growth was likely to be uncoupled due to the accumulation of byproducts.

Changes in the metabolic activity of the bacterium had been indicated but had not been clearly defined. The samples taken from the fermentations had been analysed for carbon content so that the actual carbon source concentration could be calculated. The term carbon source concentration was used rather than succinate concentration since it would include any organic byproducts of succinate consumption. The profiles obtained for the three fermentation conditions were linear and did not indicate variations in the metabolic activity. A carbon balance for the fermentation conditions was sought but the balance increased with time rather than remaining at one level. The possible error involved was thought to be the total carbon dioxide evolved data, therefore it was further analysed. The total carbon dioxide evolved was plotted against the theoretical value which was obtained by subtracting the carbon content of the fermentation broth away from the total carbon content of the medium ( $8.95 \text{ g l}^{-1}$ ). The profile obtained (Figure 4.1) indicated that there were two types of error. The first was the large scatter of points at the low theoretical values due to inaccurate values obtained from the total carbon analyser at higher concentrations. The second error was a linear relationship between the theoretical and measured value which would indicate that the calculated exponential increase in carbon dioxide evolved was correct and not due to error in the measurements. Detailed modelling would not be possible due to the error involved with the measurements.

The analysis of the changes in metabolic activity was not followed any further since it had been clearly shown that there were two sections to the growth phase and the growth of the bacterium was uncoupled from carbon metabolism. In this

Figure 4.1 - Comparison of the measured total carbon dioxide evolved data to the theoretical value obtained by subtracting the carbon content of the fermentation broth from the initial carbon content of the medium.



study the effect of growth upon the aromatic oxidation activity and solvent tolerance was the important factor and not the detailed analysis of bacterial physiology. In summary the growth of the bacteria at 5 % DOT as compared to 20 % DOT at 30°C had indicated the accumulation of byproducts due to the lower DOT but the growth at 26°C as compared to 30°C was slower but similar in nature.

#### 4.3 - Aromatic oxidation activity

Before the influence of the fermentation conditions on the aromatic oxidation activity could be assessed it was necessary to develop an appropriate assay. The development of the assay was described in Section 3.2.1 and two main considerations were substrate and cell concentration.

Optimum toluene concentration for hydroxylation by P.putida UV4 had been shown to be between 18 and 22 % aqueous saturation (Hack,1992). In this study fluorobenzene oxidation was shown to be rate limiting at a concentration below 20 % aqueous saturation (Figure 3.35a). Similarities between the concentration of the substituted benzene (toluene or fluorobenzene) and the rate of reaction by P.putida had also included the compounds being toxic at a concentration above 50 % aqueous saturation. The toxicity of fluorobenzene is discussed further in Section 4.6.

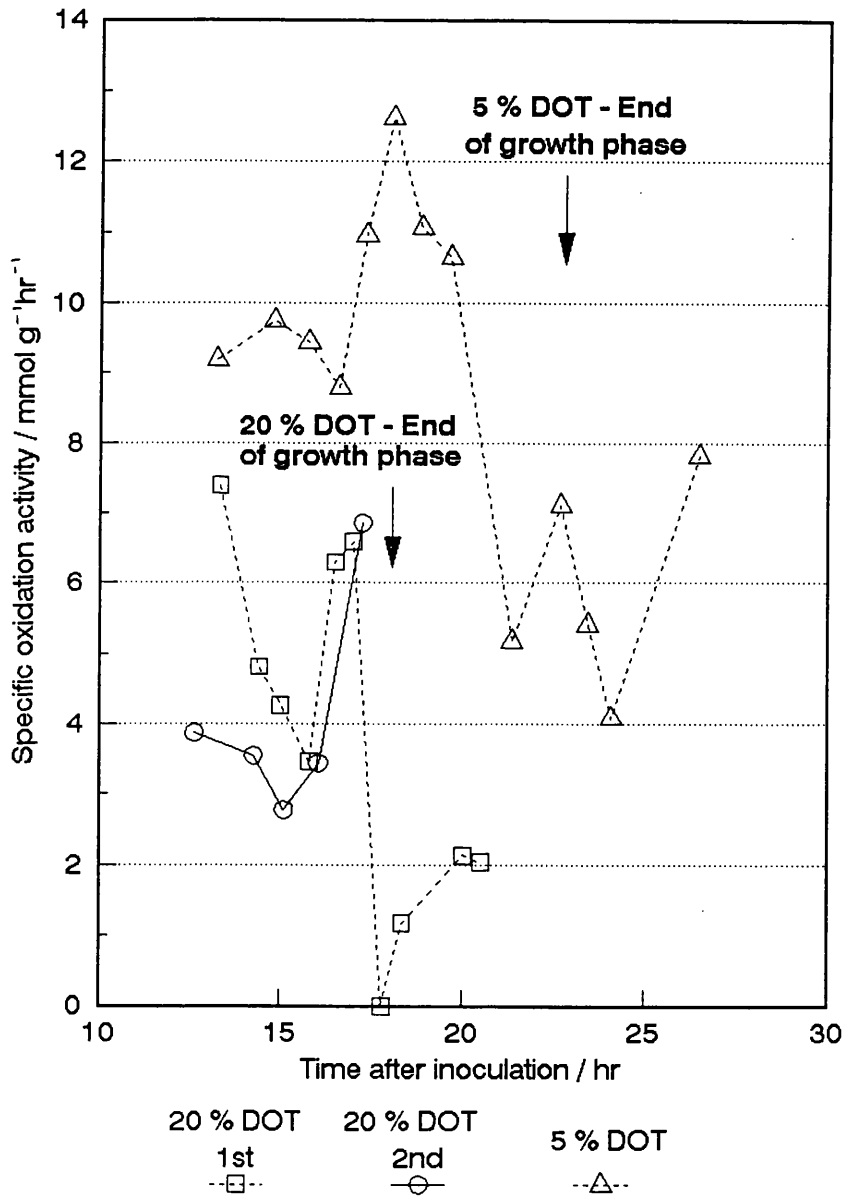
The profile obtained to show the effect of cell concentration upon the specific aromatic oxidation activity (Figure 3.37) was similar to that obtained by Harrop (1990) with naphthalene hydroxylation. Maximum specific activity had been obtained at a cell concentration between 0 and 0.5  $\text{gl}^{-1}$ , once the cell concentration was increased to 2.25  $\text{gl}^{-1}$  the activity had decreased to 50 % of maximum.

#### 4.4 - The effect of growth conditions on aromatic activity

Changes in the metabolism of the cells resulted in variations in the aromatic oxidation activity which were due to differences in protein expression and/or protein degradation. Intracellular proteolytic activity is a necessary part of microbial metabolism but careful control of proteolysis is required since low activity can result in the accumulation abnormal proteins (Gottesman & Zipser, 1978) but overproduction of proteases can lead to cessation of growth (Goff & Goldberg, 1987).

The fermentations at 30°C (20 and 5 % DOT) had indicated three sections to the aromatic oxidation activity (Figure 4.2). Detailed analysis of the carbon dioxide evolved had indicated two sections to the growth phase which corresponded to two sections to the aromatic oxidation activity. The aromatic oxidation activity of the bacteria had increased from the first section to the second which was due to a decrease in metabolic activity as indicated by a decrease in the exponential rate of carbon dioxide evolved. Near the end of the growth phase as the carbon source had been finished the aromatic oxidation activity had fallen to zero (30°C and 20 % DOT) and 5.2 mmol g<sup>-1</sup> hr<sup>-1</sup> (30°C and 5 % DOT). Previous research had shown that nutrient limitation leads to rapid loss of protein synthesis (Pine, 1972). This was contrary to when *P. putida* ML2 was grown on benzene as carbon source since high stability of the aromatic oxidation activity was maintained over a number of hours (Zamanian, 1989). The activity had only dropped to approximately 80 % of the initial value 3 hr after the benzene supply had stopped indicating that the enzymes are protected in some way against proteolysis since they are essential for growth. Certain proteins have been shown to be protected against inactivation by the addition of specific compounds to the medium (Pine, 1972). The aromatic oxidation activity had increased at the end of the growth phase indicating the presence of carbon source was not essential for protein synthesis. This

Figure 4.2 - Comparison of the aromatic oxidation activity of the samples taken from the fermentations controlled at 5 and 20 % DOT (30°C).



was further demonstrated with the carbon limited fermentation since at no time was the activity zero.

Large variations in activity have been associated with changes in the growth conditions (Fulks & Stadtman, 1985). The intracellular degradation of aspartate transcarbamylase of Bacillus subtilis with  $\text{NH}_4^+$  as nitrogen source was 10 to 20 times faster than bulk protein turnover but inclusion of a number of amino acids as <sup>the</sup> nitrogen source had resulted in the intracellular degradation rate decreasing to an undetectable level (Bond et al, 1983). Variability in the aromatic oxidation activity had been demonstrated by the carbon limited fermentation (Figure 3.53) and the fermentation at 34°C with oscillating DOT (Figure 3.49) as a result of the oscillatory metabolic activity. Protein inactivation may not be due to proteolysis alone since the glutamine synthetase of Klebsiella aerogenes is inactivated by oxidation of amino acid residues which then makes it more susceptible to degradation by proteases (Fulks & Stadtman, 1985). The rapid changes in activity of the bacterium as observed under oscillatory metabolism could be due to chemical inactivation rather than proteolytic inactivation alone.

The oscillatory metabolism could not be directly related to the aromatic oxidation activity but regulated changes in DOT during growth had resulted in explainable changes in aromatic oxidation activity. Decreasing the DOT to zero during the growth phase had resulted in the activity of the bacteria increasing from 4.5 to 12.0  $\text{mmol g}^{-1} \text{hr}^{-1}$  after 2.0 hr (Figure 3.47), a similar result had been observed with the bacteria initially grown at 20 % DOT which were then taken to low  $K_a$  in that the activity had increased from 0 to 2.3  $\text{mmol g}^{-1} \text{hr}^{-1}$  (Figure 3.48). The result of the decrease in DOT was the cessation of growth and a reduction in carbon metabolism (Section 3.1.4.2 and 3.1.4.3) and directing metabolites towards protein synthesis as indicated by the higher

activity. Increasing the DOT to remove oxygen limitation had resulted in an increase in metabolic activity but a decrease in aromatic oxidation activity (Figure 3.47), in this case an increase in carbon metabolism had resulted with metabolites redirected from enzyme synthesis towards growth. A similar observation had occurred when two fermentations had become iron limited in that the addition of iron sulphate had resulted in a rapid increase in growth of the cells but a decrease in aromatic oxidation activity (Figure 3.54).

High aromatic oxidation activity could be related to a slow down in metabolic activity. The fermentation at 30°C (20 % DOT) had activity between 2.8 to 3.9 mmol g<sup>-1</sup> hr<sup>-1</sup> from 12 to 16 hr after inoculation (Figure 4.2) which was similar to the fermentation at 26°C which had an initial activity of 3.8 mmol g<sup>-1</sup> hr<sup>-1</sup>, 14.0 hr after inoculation (Figure 3.51) although the growth rates were different. The iron limited fermentation which took 25 hr to finish had resulted in higher activities at between 4.2 and 5.2 mmol g<sup>-1</sup> hr<sup>-1</sup> (15 to 20 hr after inoculation, Figure 3.55) which could be due to lower growth rate but was not as high as those obtained when the cells had become oxygen limited. The large increases in aromatic oxidation activity was a result of a transition from high to low growth rate rather than a continuously low growth rate. This had been clearly indicated by the fermentation described in Section 3.2.4.5 in which a short transition to low DOT had resulted in high aromatic oxidation activity (6.9 mmol g<sup>-1</sup> hr<sup>-1</sup>) and low growth rate (0.19 hr<sup>-1</sup>) but 3 hr after returning to 20 % DOT the activity had decreased to between 2.5 and 3.5 mmol g<sup>-1</sup> hr<sup>-1</sup>.

The fermentation at 5 % DOT and 30°C was clearly different since the aromatic oxidation activity was approximately double that obtained at 20 % DOT and 30°C (Figure 4.2). This would suggest that DOT had an effect upon enzyme synthesis independent from growth, a difference between carbon metabolism and growth has been discussed in

Section 4.2. The growth rate of the bacterium at 20 % DOT (26 and 30°C) was shown to be 55 to 57 % lower than the exponential rate of carbon dioxide evolved but a reduction in DOT to 5 % aqueous saturation had resulted in this decreasing to 43 %. This fact of higher enzyme activity at low DOT levels or low oxygen transfer rates has been observed for other systems including pullanase activity of Bacillus stearothermophilus (Emanvolova & Kambourova,1992), uricase activity of Bacillus fastidiosus (Bongaerto et al,1978), salicylate hydroxylase activity in Pseudomonas (Russell,1981) and the expression of recombinant  $\beta$ -lactamase by E.coli (Ryan et al,1989). The specific activity of an enzyme will decrease as a substrate becomes limiting therefore the bacterium could overcome this by having a higher concentration of the enzyme in the cell. This was not true for the catechol 1,2 dioxygenase of P.putida since higher specific activities were observed at higher oxygen concentrations (Viliesid & Lilly,1992).

The cytochrome content of bacterial membranes is dependent upon oxygen concentrations as shown by the cytochrome P<sub>450</sub> content of Candida guilliermondii which was approximately three times greater at below 15 % DOT (Mauersberger et al, 1980). The increase in cytochrome content due to low DOT was thought to be the reason for the optimum DOT for induction of progesterone 11  $\alpha$ -hydroxylase by Rhizopus nigricans to be at 10 % aqueous saturation (Hanisch et al,1980) and the induction of 11  $\beta$ - and 19-hydroxylation of Reichstein's substance S by Pellicularia filamentosa at 15 % aqueous saturation (Clark et al,1982). Toluene hydroxylation by P.putida has been shown to be limited by oxygen uptake (Hack,1992) therefore the growth of P.putida ML2 at 5 % DOT could result in an improvement in oxygen consumption leading to increased activity. This could be plausible except for the fact that the OUR due to ethanol consumption was more than three times greater than the OUR due to fluorobenzene oxidation, oxygen uptake was not the



limiting factor in this case.

The optimum DOT for aromatic oxidation activity of P.putida ML2 was below 20 % aqueous saturation which was not comparable to cholesterol oxidase synthesis by Nocardia rhodochrous since the optimum DOT was higher at 50 % DOT (Buckland,1974). Optimal DOT for expression of recombinant  $\beta$ -lactamase by E.coli was at zero DOT (Xiaoli et al,1992), whereas P.putida ML2 at this DOT had lower aromatic oxidation activity than when grown at 5 and 20 % DOT. The optimal DOT for expression of an enzyme is variable depending on the organisms and the enzymes involved. The higher protein expression by E.coli at zero DOT could be due to the fact that it can metabolise anaerobically.

A general analysis of metabolic activity would indicate whether the bacterium had the ability to synthesise products, in this case the enzymes, which can be independent of the growth requirements. The synthesis of bacterial antibiotics could not be related to enzyme synthesis since DOT had the opposite effect (Heinemann et al,1970). Increase in oxygen transfer rate from oxygen limited growth to non-limiting growth conditions had resulted in a 50 % reduction in enzyme yield but had also resulted in prodigiosin synthesis. This could be due to a requirement for proteases to increase the pool of amino acids since they were a prerequisite for commencement of antibiotic production early in the non growth phase (Rollins & Gaucher,1986). Tentoxin and dihydrotentoxin are biosynthesised via protein turnover by Alternaria alternata (Liebermann et al,1991) which strongly suggests that antibiotic production is dependent on the presence of proteases to supply a pool of intermediates. The enzymes involved in the antibiotic production must themselves be stable against proteolysis.

The rate of the enzyme synthesis is dependent on its rate of expression and degradation. Proteolytic activity

during these fermentations was shown to be high due to the rapid changes observed in aromatic oxidation activity during the growth phase. Careful control of the proteolytic activity of the bacteria would be essential to obtain high levels of aromatic oxidation activity.

#### 4.5 - Aromatic Oxidation Stability

Development of a successful biotransformation depends not only on the production of bacteria with a high aromatic oxidation activity but on maintenance of the activity for enough time to produce sufficient amounts of the product, fluorocatechol. The length of the biotransformation process will be highly dependent upon the stability of the aromatic oxidation activity, which will decline due to enzyme inactivation and toxic effects of the reactant and product.

When cultures from fermentations were stored at 0°C the activity declined over the next few hours indicating that the stability of the aromatic oxidation enzymes was not high (Section 3.2.2.1). This was in contrast to bacteria grown in a shake flask since activity was maintained over a number of days which was thought to be due to the bacteria becoming oxygen limited (Section 3.2.1.3). The bacteria grown at 30°C and 5 % DOT had resulted in a number of changes to the growth as discussed in Section 4.1 and 4.2; the aromatic oxidation activity was also higher (Figure 4.2). Analysis of two of the samples had indicated higher stability than the fermentations at 30°C and 20 % DOT but it was not comparable to that obtained with bacteria grown in a shake flask (Section 3.2.4.1).

The high instability of the aromatic oxidation activity of the cells grown at 20 % DOT (30°C) could be artificially high since previous research had shown that centrifuging and washing cells can generally double or triple proteolytic activity (Pine, 1970). The washing stage was a necessary part

of the aromatic oxidation activity assay so as to reduce the endogenous OUR to an acceptable level.

A linear growth curve was obtained for the bacteria grown in a shake flask indicating that they had become oxygen limited (Figure 3.45). The effect of zero DOT upon the bacteria grown in a fermenter had been analysed but had not resulted in higher stability but unlike shake flask culture it had not resulted in further cellular growth (Section 3.2.4.2). A fermentation had therefore been set up in which the bacteria would become oxygen limited but were allowed to grow since a sufficient supply of oxygen was available. Upon reducing the oxygen transfer rate from high to low value the aromatic oxidation activity had varied considerably (Figure 3.48, 17.0 to 22.75 hr after inoculation) and had not resulted in high stability. The DOT profile consisted of a number of cycles in which the DOT would increase after being at a low value before again returning towards zero. The complex oscillatory changes in the metabolism of the bacterium were still present 6 hr after the  $K_L a$  had been reduced and were not regular in shape indicating the inability of the bacteria to metabolise at a steady rate.

Initially the increase in stability of the aromatic oxidation activity as a result of culturing in a shake flask was thought to be due to growth of the bacteria at zero DOT, after observing oscillating DOT profile at low  $K_L a$  it could include the growth at low DOT between 0 and 5 % aqueous saturation or the oscillations in metabolism.

Oscillatory metabolic activity has been observed previously when Klebsiella aerogenes had been grown upon glucose in continuous culture at low oxygen concentrations (Harrison & Pirt, 1967). There was a range of DOT concentrations termed as the transition state where oscillatory metabolism had occurred; above this level the oxygen was in excess, below the minimum level the metabolic

activity had been reduced dramatically resulting in low activity. The OUR had decreased while the DOT was increasing which had similarly observed in this study (Figure 3.21). This had indicated that the bacteria were unable to adjust their metabolic activity to a steady level. Steady state metabolic activity as obtained in continuous culture could be interrupted and never be regained as shown by the fluorescent studies upon yeast at low dilution rates. The metabolic stress of interruption of aeration for 2 mins had resulted in a self-synchronization fluorescence profile which after 18 hr was stable over several days (Scheper & Schugerl, 1986).

The retention of aromatic oxidation activity by the effect of oscillating metabolism was indicated by the retention of activity overnight by the sample taken at the end of the fermentation at 34°C with oscillating DOT. Oscillatory metabolic activity induced by the cyclic feeding of glucose and monosodium glutamate to Streptomyces fradiae had resulted in higher yields of tylosin than was obtained when feed was at a steady rate (Vu-Trong & Gray, 1984). This higher yield could be due to higher enzyme stability since the enzymes involved in tylosin biosynthesis have been shown to be unstable. The specific  $\beta$ -galactosidase activity of Kluyveromyces lactis in continuous culture was shown to be double when operated in a transient operation rather than in a steady state (Martini et al, 1989). The enzyme was shown to be unstable since after a pulse of lactose the enzyme synthesis was repressed and had then resulted in the specific activity dropping rapidly.

The stability of the aromatic oxidation activity was shown to be dependent on the bacterium becoming oxygen limited or metabolically unstable so that the enzymes involved were not inactivated. Inactivation of the bacterium as a whole by the toxic effect of fluorobenzene and fluorocatechol has not been discussed but high levels of the substrate and product could reduce the length of the biotransformation process.

Careful control of their levels could increase the total production time allowing a larger amount of product to be produced.

Fluorocatechol toxicity was shown to be greater than fluorobenzene since a fluorocatechol concentration of  $3.9 \text{ mmol l}^{-1}$  had resulted in a 35 % reduction in the OUR where as a fluorobenzene concentration of  $3.5 \text{ mmol l}^{-1}$  was not toxic. Fluorocatechol ( $1 \text{ gl}^{-1}$ ) completely inhibited aromatic oxidation activity but had only resulted in the endogenous OUR dropping to 50 % of maximum, this had indicated an inhibition of either benzene dioxygenase or benzene cis glycol dehydrogenase. It has previously been shown that catechol and methyl catechol both inhibit the aromatic oxidation activity of P.putida (Robinson<sup>et al,</sup> 1992), the inhibition was directed towards toluene dioxygenase rather than toluene cis glycol dehydrogenase. This would explain the high inhibition of growth upon benzene minimal medium containing fluorocatechol (Figure 3.65b), but it does not explain the inhibition of growth upon succinate minimal medium (Figure 3.65a) or the inhibition of endogenous OUR (Figure 3.64b). The effect of fluorocatechol was also shown to be inhibitory rather than toxic since the cells which had been shaken for 1.5 hr at a fluorocatechol concentration of  $1 \text{ gl}^{-1}$  in buffer had a reduced initial growth but total loss of viability had not been observed (Figure 3.66). Robinson et al (1992) had reached a methyl catechol concentration of  $2.2 \text{ gl}^{-1}$  and a catechol concentration of  $3 \text{ gl}^{-1}$  in their biotransformation indicating that the catechols are not toxic until a high level had been reached.

The observed recovery of the bacteria when grown in succinate minimal medium containing fluorocatechol (Section 3.2.1.6) was due to the induction of a gene or a number of genes which increased the resistance of the bacteria to fluorocatechol. This could be a method of preventing the uptake of fluorocatechol, production of an enzyme to detoxify

it or the production of a compound to bind to it and make it inert. If the increase in resistance was due to prevention of its uptake this maybe advantageous in the biotransformation process to allow a greater concentration to be reached in the external environment as long as the bacterium had not lost the ability to export it from the cell. The catechol 1,2 dioxygenase of P.putida ML2 cannot metabolise substituted catechols but another Pseudomonas had been found to contain a chlorocatechol 1,2 dioxygenase which can metabolise chlorocatechol and was distantly related to the catechol 1,2 dioxygenase (Van Der Meer et al,1991). The induction of this type of enzyme maybe possible if the bacterium has been exposed to inhibiting levels of fluorocatechol for a long period of time. It is also possible that the catechol has polymerised irreversibly to an insoluble compound which was less toxic to the cells due to its inability to enter the cells. These polymers have been observed previously when working with catechol and methyl catechol (Robinson et al,1992). The degradation of fluorocatechol would be disadvantageous in the biotransformation and therefore continuous removal of fluorocatechol before it became highly inhibitory would be necessary. Fluorocatechol had been recovered from cell free culture broths by continuous extraction with diethyl ether for 18 hrs (Schofield et al, 1987). The addition of a second solvent to the reactor could lead to improved productivity by continuous removal of the toxic product from the aqueous phase.

Initial studies upon the toxicity of fluorobenzene towards P.putida ML2 had indicated significant differences from fluorocatechol. It had included the OUR falling towards zero and not remaining at a lower but constant level after addition of the compounds. The effect of fluorobenzene and other solvents upon the activity of the bacteria is discussed in the next section since careful control of their toxicity could lead to improvement in the productivity of the biotransformation.

#### 4.6 - Solvent tolerance

The biotransformation itself may be done in two ways to minimise the toxic effects of the fluorobenzene. In the first fluorobenzene can be fed under controlled conditions. The second approach is to control the concentration of fluorobenzene in the aqueous phase by dissolving it in an added water-immiscible organic solvent.

Thus when assessing solvent tolerance, the effects of both fluorobenzene and added solvents need to be considered. Many researchers have examined the effects of a wide range of solvents on the activity and stability of microorganisms being used in biotransformations (Section 1.2). However, little is known about the effect of the fermentation conditions on solvent tolerance. As indicated in Section 3.3.1 it was decided to choose one hydrophobic compound to test solvent tolerance. To avoid problems arising from the transitory formation of a second liquid phase during addition of the solvent a disinfectant with a reasonable aqueous solubility was selected. It also eliminated the need to operate solvent tolerance experiments where there was a second liquid phase. This was important because previous work (Hocknull,1989) demonstrated that the agitation conditions in such systems greatly affected the rate of loss of activity.

The selection of a suitable assay to analyse the solvent tolerance of the bacteria was dependent upon obtaining a simple and quantitative method. For reasons explained elsewhere (Section 3.2.1), the measurement of aromatic oxidation activity was based on measurements of the OUR by the bacteria rather than on the measurement of product formation. Thus when assessing solvent tolerance of the bacteria, it was also sensible to examine the effect on oxygen consumption. This gave a general technique which was not only suitable for this present study but could be used for other biotransformations involving aerobically

metabolising microorganisms.

The assay selected was not affected by changes in the aromatic oxidation activity since it was desirable to keep the two parameters separate from each other. Fluorobenzene the substrate in the aromatic oxidation reaction had been shown to inhibit the growth of the bacteria upon succinate minimal medium at near aqueous saturated concentration. The rate of loss of OUR due to fluorobenzene toxicity was compared to the profiles obtained for chloroxylenol, the test hydrophobic compound. The profiles obtained had shown that fluorobenzene at a concentration of 22 % aqueous saturation was not toxic; this was also the optimum concentration for aromatic oxidation activity. Fluorobenzene was toxic at 44 % aqueous saturation; the profile had shown exponential decrease in activity which could be fitted to the equation developed for chloroxylenol toxicity described below:-

$$\frac{(OUR_t - OUR_f)}{(OUR_0 - OUR_f)} = \exp(-ct)$$

Fluorobenzene had resulted in the OUR reaching zero where as inactivation of the bacterium with chloroxylenol had resulted in the bacteria reaching a steady OUR value. The transmembrane diffusion rate of fluorobenzene into the bacterium could be the limiting factor in the aromatic oxidation activity. The rate at which the hydrophobic compounds, fluorobenzene and chloroxylenol pass into the bacterium will depend on membrane permeability. Increases in chloroxylenol toxicity as a result of an increase in membrane permeability may also result in increases in aromatic oxidation activity. In many cases the opposite was true for example after decreasing the DOT to zero (Section 3.2.4.2 & 3.3.3.2) both the aromatic oxidation activity and the tolerance of the bacteria towards chloroxylenol had increased. The permeability of the bacterium to the hydrophobic substrate fluorobenzene, was not the limiting



factor indicating that changes in the intracellular enzyme concentration due to metabolic changes had resulted in overall change in the aromatic oxidation activity.

The profile obtained for fluorobenzene inactivation could be fitted to the initial equation described by Chick (1908). Chloroxylenol inactivation could not be fitted to this initial equation in that a portion of the OUR remained unaffected, a similar result had occurred with phenol inactivation of Botrytis spores (Henderson Smith, 1921). A phenol concentration of 0.4 % (v/v) had resulted in the viability of 92 to 93 % of the spores being lost exponentially, 7 to 8 % had remained viable throughout the experiment. A lower phenol concentration had resulted in a sigmoid shape which became exponential upon increasing the concentration. Three types of curves had been obtained including a sigmoid profile, exponential decay and an exponential decay with a portion of the population not affected.

The assay was developed with the absence of a second liquid phase so that the number of parameters (e.g. stirrer speed, phase ratio) were reduced to a minimum. Steroid hydroxylation by A.simplex in a second liquid phase had also shown exponential rate of loss of activity due to solvent toxicity which decreased as the logP of the solvent increased (Hocknull, 1989). Octanol toxicity towards S.cerevisiae had resulted in an exponential decrease in membrane integrity as measured by the dielectric properties of the cells (Stoicheva et al, 1989). This would indicate that the equation derived could also be used in other systems and even when a second liquid phase is present. Naphthalene hydroxylation by P.putida had not decreased exponentially after solvent inactivation. A portion of the bacteria became inactive but no further loss of activity had been observed from there onwards (Harrop, 1990). Pretreating the cells with EDTA had resulted in an increase in the susceptibility of the cells

towards the solvents and the profile obtained was exponential. Addition of EDTA had therefore resulted in destabilisation of the outer membrane by removing the magnesium ions binding the lipopolysaccharide molecules together. A new formula for a disinfectant which had EDTA added to disrupt the outer membrane with the chloroxylenol preparation had resulted in an increase in toxicity towards P.aeruginosa (Russell & Furr,1977). Chloroxylenol inactivation of OUR was increased upon addition of 2 gl<sup>-1</sup> K<sub>2</sub>EDTA (Figure 3.81) but addition of 10 gl<sup>-1</sup> K<sub>2</sub>EDTA did not result in further toxicity. This would indicate that the integrity of the bacterial membrane had been maintained by another force such as hydrophobic interactions of the lipid molecules and complete disruption of the outer membrane was not possible.

The profiles obtained for octanol inactivation of the bacteria could also be fitted to the equation for exponential decrease in OUR which had reached zero by the end of the experiment (Figure 3.74). The C value rose as the octanol concentration increased indicating the sensitivity of the assay to changes in the rates of inactivation. At an octanol concentration of 0.82 gl<sup>-1</sup> the decrease in OUR was not exponential and had indicated that another factor was involved in its toxicity at the higher concentration. The aqueous saturated concentration of octanol at 25°C was 0.54 gl<sup>-1</sup> (Riddick et al,1986) and the concentration at 30°C will be higher. The more rapid non-exponential loss of OUR could be due to the appearance of a second-liquid phase which had resulted in a more rapid uptake by the bacterium.

A number of long chained alcohols were analysed to determine their toxicity towards the bacterium. Their toxicity at low concentrations increased with chain length which would correspond to an increase in LogP (Figure 3.77). Osborne et al (1990) had shown that the increase in toxicity of alcohols as their chain length increased could be

correlated with their partition into biological membranes. A similar result had been observed for derivatives of 2,4 dichlorophenol since an increase in the size of the alkyl group in the 6 position had increased its toxicity (Blackman et al,1955).

The OUR profiles after addition of the solvents were shown to be exponential in all cases although with chloroxylenol not all of the OUR was lost. The reason for the OUR reaching a steady value could be due to a number of the bacteria being completely tolerant to the chloroxylenol concentration and had remained unaffected by it or that the chloroxylenol concentration had reached a non-toxic level due to its uptake by the cells. The profile obtained at different cell concentrations (Figure 3.61) indicated that the C value had a steady value for a cell concentration between 2 and 3  $\text{gl}^{-1}$  and it is therefore unlikely that the chloroxylenol had reached a non-toxic level since greater variations would have been expected. The profile of  $\text{OUR}_f$  against chloroxylenol concentration (Figure 3.62) would also have indicated higher  $\text{OUR}_f$  values at the lower chloroxylenol concentration which were non-toxic. The results indicated that a minimum concentration of the solvents required before toxicity occurred; above that level further increase in concentration had resulted in high toxicity.

The assay was selected due to its simplicity which would allow it to be scaled up and be used in as many different systems as possible. By starting from an aqueous system it would be possible to understand the role of the interphase between solvent and aqueous layer in biocatalyst inactivation. The last solvent analysed in Section 3.3.6 was dichloroethane which was selected on the basis that it had a greater density than water and could be added as a second liquid phase.

#### 4.7 - Effect of growth conditions on solvent tolerance

Changes in the growth conditions had major influences upon the metabolism and the aromatic oxidation activity of the bacterium. It was envisaged that a decrease in membrane permeability as a result of a decrease in growth rates would lead to an increase in solvent tolerance. The two fermentations at 30°C and 20 % DOT had shown that the C value (i.e. solvent susceptibility) had decreased from the first sample to the last (Figure 3.64 & 3.65). Completion of the growth phase had resulted in a slow down in the metabolic activity and inability of chloroxylenol to attack the bacterium. The fermentation at 30°C and 5 % DOT showed large variations in C value during the growth phase but overall it decreased. Once the growth phase had stopped the C value (5 hr<sup>-1</sup>) was similar to the fermentation at 30°C and 20 % DOT.

The samples from the fermentation at 30°C and 20 % DOT were taken at the end of the growth phase or afterwards. The samples from the fermentation described in Section 3.3.3.3 in which there was a short transition to zero DOT were taken during the growth phase. The C values again decreased with time after inoculation indicating that as the metabolism was slowing down there was an increase in solvent tolerance. Rapid changes in the aromatic oxidation activity of the fermentation at 26°C and 20 % DOT were observed which were thought to have been induced by poor DOT control (Section 3.2.5.1). Similar rapid changes in C values were not observed (Figure 3.70). In the fermentation at 26°C and 20 % DOT the C values decreased with time during the growth phase. These results have indicated that as the bacteria became less active the solvent tolerance had increased. This may be important when considering biotransformations which have to be metabolically active. It may be best to set up a fed-batch system since the higher the activity the more susceptible the bacteria will be to the solvent.

The fermentation at 26°C and 20 % DOT did not result in

rapid changes in solvent tolerance although major changes in aromatic oxidation activity were observed. The fermentation at 30°C and 5 % DOT resulted in rapid changes in solvent tolerance which were not comparable to either the aromatic oxidation activity or the specific ethanol consumption rate. There was a rapid drop in C value from 29 to 9 hr<sup>-1</sup> (16.6 to 17.3 hr after inoculation) which corresponded to the change in correlation between acid input and carbon dioxide evolved (Figure 3.15b) indicating a change in carbon metabolism possibly from succinate to the byproduct consumption. The second decrease in C value may be due to the fact that the bacteria were growing at a slower rate as they came to the end of the growth phase. The effect of a sudden change in metabolic activity upon the solvent tolerance of the bacteria was demonstrated by the fermentation described in Section 3.3.3.2 in which the DOT was taken to zero at the end of the growth phase. A reduction in metabolic activity due to a decrease in DOT had resulted in an increase in aromatic oxidation activity and a decrease in the susceptibility of the bacteria towards chloroxylenol. At the end of the fermentation the oxygen transfer was increased resulting in an increase in metabolic activity, a decrease in aromatic oxidation activity and an increase in susceptibility to chloroxylenol.

Changes in the metabolic activity of the bacterium resulted in variations in the calculated C values obtained from the OUR profiles. As stated in Section 3.3.1 the assay was not run with the same cell concentration since it was important to obtain an ideal OUR profile for the curve fitting routine. It was therefore important to determine whether the biomass concentration in the electrode and the specific OUR due to ethanol consumption had an effect upon the calculation of the C value and that it was a true indicator of solvent tolerance and not a result of changes in the metabolism. The specific OUR due to ethanol consumption and the biomass concentration were plotted against the C

values for the fermentations at 5 % DOT and 30°C (Figure 3.67) and at 20 % DOT and 26°C (Figure 3.71) and had resulted in a large amount of scatter and no correlation.

The main difference was the change in OUR profile obtained for the bacteria grown at different temperatures. All assays were performed at 30°C. The bacteria from the fermentation at 26°C and 20 % DOT resulted in the OUR going to zero whereas those grown at 30°C had an OUR ( $OUR_f$ ) at the end of the run. Taking the bacteria up from their growth temperature had therefore increased their susceptibility to chloroxylenol. The opposite was true for the bacteria grown at 34°C in that the OUR profiles had lost the distinct exponential decay and become more linear (Figure 3.73). It was difficult to fit the equation to the profiles but in general chloroxylenol was still toxic therefore complete tolerance to the compound could not be obtained. Changes in the trans-membrane diffusion rates of fluorobenzene due to differences in membrane permeability as a result of changes in growth temperature had not resulted in major changes in aromatic oxidation activity. This again indicates that changes in intracellular enzyme concentration is the major contributor to the large variations in activity. Culturing the bacteria at 34°C had led to an increase in solvent tolerance but the fermentation time had been lengthened considerably. A better option would be to reduce the temperature of the biotransformation reaction and growing the bacteria at their optimum temperature. The problem would then be a decrease in aromatic oxidation rate due to the lower than optimum temperature and a greater problem in maintaining reaction temperature.

A biotransformation process can be split into a number of unit operations which could include the fermentation and the biotransformation as two separate units. This work has shown the effect of changes in growth conditions upon the biotransformation, in optimising the process it would be

difficult to consider the fermentation and the biotransformation separately. The factors involved in the design of the process is discussed in the next section.

#### 4.9 - The biotransformation reaction

The purpose of this study was to analyse the effect of growth conditions upon the solvent tolerance and aromatic oxidation activity of P.putida ML2. The production of bacteria which were tolerant to solvents would lead to an increase in options in the development of the biotransformation process. Growing the bacteria at 34°C had resulted in higher solvent tolerance but the longer fermentation time would increase the cost of the process. To determine whether the addition of a second immiscible solvent would result in process improvements we need to consider all aspects of the biotransformation so that the potential advantages and disadvantages are accounted for. The main considerations are the stability of the enzyme, bacterium and product; and the toxicity of the product, substrate and the second additional solvent.

The stability of the enzymes involved in the aromatic oxidation had become a major concern in this work. Retention of activity of the bacteria grown at 30°C and 20 % DOT was low and would limit the options for the process design. The harvesting of these bacteria would not be possible and therefore the biotransformation would follow straight after the fermentation as a fed batch process. If the stability of the bacterium was high it would allow sufficient time to harvest the culture and have them immobilised if required for the biotransformation stage. By immobilising the culture <sup>would</sup> it be possible to prevent them from coming into contact with the second solvent phase, this would allow the selection of solvents with higher toxicity. It is also important to consider the retention of viability after storage since the fermentation and biotransformation could be

performed on different days. Bacteria grown in a fermenter had been shown to retain metabolic activity for at least two days as indicated by the retention of OUR due to ethanol consumption.

Fluorobenzene concentration has been shown to have a narrow optimum range therefore the maximum reaction rate can only be achieved if careful control is maintained. If this cannot be achieved then its concentration must be maintained below the toxic level so that loss of biocatalytic activity is avoided, this lower concentration will automatically result in a slower rate of production.

Control of the aqueous concentration of fluorobenzene so that it does not become toxic could be achieved by supplying it from a second immiscible solvent. From the partition coefficient it is possible to calculate the maximum concentration of fluorobenzene in the solvent so that when it has partitioned to the aqueous phase and reached equilibrium it will be below the toxic aqueous level. The transport of fluorobenzene into the bacterium was improved by its addition in propanol as indicated by the higher aromatic oxidation activity. The optimal fluorobenzene concentration could be lower when supplied from a second solvent due to the possible increase in its transport into the bacterium by the solvent.

Fluorobenzene could be controlled at an optimum concentration whether in a purely aqueous biotransformation or a two liquid phase biotransformation. Fluorocatechol concentration will increase in a normal aqueous biotransformation where as in a two liquid phase biotransformation it could be kept low by extraction from the reaction media into the second solvent phase. The reaction could be completely inhibited by a high fluorocatechol concentration which will lead to a maximum attainable concentration of fluorocatechol irrespective of biocatalyst concentration and specific aromatic oxidation activity. The



biocatalyst concentration and the specific activity will govern the time it will take to reach that maximum fluorocatechol concentration, this level may not be reached if biocatalytic activity is lost due to the instability of the aromatic oxidation activity. A second immiscible solvent phase could lead to an increase in volumetric yield of fluorocatechol by keeping fluorocatechol concentration below inhibitory level but in this case not only will the length of the biotransformation depend on the stability of the aromatic oxidation activity but also on the toxicity of the second immiscible solvent. Optimisation for high aromatic oxidation activity and solvent tolerance could be difficult since the bacteria grown at 5 % DOT and 30°C had high activity but low solvent tolerance. Increasing the solvent tolerance by changes in the biotransformation maybe a better option, one way is to decrease the temperature so that the permeability of the bacteria is decreased. This has the disadvantage of also decreasing the aromatic oxidation activity resulting in a longer reaction time and therefore making it more susceptible to loss of biocatalytic activity.

Removal of fluorocatechol from the aqueous phase maybe essential in the biotransformation since it could be unstable. The instability of fluorocatechol was indicated by the recovery of the bacteria when growing upon succinate minimal medium with fluorocatechol added at a concentration of 0.25 gl<sup>-1</sup>. If the recovery is due to the degradation of fluorocatechol then it must be removed from the aqueous phase so that it does not come into contact with the bacteria. The addition of a second liquid phase will allow the extraction of fluorocatechol from the aqueous phase.

Solvent selection for biocompatibility was initially based on the Log P parameter but analysis of the OUR profile had indicated greater variations between the solvents. The results obtained in Section 3.3.5 and 3.3.6 had indicated that the action of each solvent could be different and that

protection of the bacteria with magnesium ions will only work with certain solvents. This was indicated with dichloroethane in that although there was an increase in tolerance of the bacteria upon addition of magnesium the OUR had not become linear. The profiles obtained with fluorobenzene and chloroxylenol had become linear indicating that a portion of the OUR may remain active throughout the biotransformation. The data obtained had indicated that a significant improvement in solvent tolerance would be obtained by the addition of magnesium ions or the reduction in temperature. Growing the bacteria at 34°C was deemed unsuitable due to the variations in the two batches and also the lengthened fermentation time. Overall it would seem that the effect of growth conditions upon solvent tolerance as a main consideration for the process design would not be as significant as solvent selection and the method of performing the biotransformation. It is important though to consider the effect of the solvent upon growing bacteria in that a rapid increase in metabolic activity had resulted in a reduction in solvent tolerance. This could be important in the biotransformation if the activity of the bacteria was not steady but oscillatory since the solvent tolerance will be low at its most active stage. The carbon limited fermentation had resulted in oscillatory metabolism therefore careful control of the substrate input would be required to avoid these oscillations.

Two main options in the biotransformation became apparent and that is whether the bacteria should be harvested with the biotransformation performed separately or whether it should follow directly after the fermentation as a fed batch process. The fed batch process would be essential if aromatic oxidation activity was low since the growth of the bacteria would maintain the expression of the enzyme. Due to the presence of all growth components in the fed batch stage the presence of fluorocatechol may induce its degradation since it could act as a possible carbon source.

Fluorocatechol will also be inhibitory therefore its degradation will also allow a more rapid growth of the bacteria. Length of the biotransformation in this case will be dependent upon the time it takes to induce an enzyme responsible for the degradation of fluorocatechol.

Immobilisation of the bacteria could result in the protection of the bacteria against the toxic effects of the solvents. It is first important to consider whether immobilisation could be carried out since it can result in problems in the mass transfer of substrate into the particle. Oxygen is a substrate in the biotransformation therefore the rate of reaction will be reduced due to mass transfer problems and would require higher oxygen transfer rates resulting in higher expense. If high metabolism occurred within the immobilised particle problems will occur with the accumulation of gaseous carbon dioxide in the particle leading to poor reactor performance as the particles float to the top of the reactor. Increase in carbonic acid concentration as a result of an increase in carbon dioxide concentration will lead to problems with the decrease of the pH. This problem could be overcome with P.putida by using a highly porous hydrophobic support since the bacteria will adhere to the hydrophobic surfaces and will not require encapsulation. A porous material maybe essential since fluorocatechol will accumulate within the support if mass transfer is low resulting in high inhibition of the activity. These are some of the problems that need to be considered and could result in significantly lowering of the specific rates of reaction.

Complete solvent protection of the bacteria by immobilisation was not possible since it was shown that concentrations of octanol below saturation were still toxic. Octanol at high concentration had indicated that an interphase may result in greater toxicity (Section 3.3.5), immobilisation will avoid this problem. Selection of calcium

alginate as the immobilising material could increase solvent tolerance further by stabilising the outer membrane of the bacteria with the divalent calcium ions. The protective effect would be similar to that observed in this work with magnesium ions. The slower mass transfer could actually help the process by allowing a higher concentration of fluorobenzene in the bulk media which would be easier to control. One main advantage of an immobilised system is that the metabolic activity of the bacteria can be recovered by replacing the biotransformation media with growth media, continual rejuvenation of the bacteria could lead to a lengthened biotransformation reaction. The use of solvents at toxic levels in a stirred tank reactor is prohibitive since all the bacteria will be in the presence of the solvent all the time whereas the bacteria at the centre of an immobilised particle will be protected from the solvents by the bacteria near the edge of the particle. This again leads to a process in which the bacterial activity is recovered by providing fresh growth media. Mass transfer problems is the major hindrance in using immobilised reactors, if these can be overcome this could lead to greater usage of a second liquid phase for whole cell biocatalysis.

There are three main options for this biotransformation which are the single aqueous phase biotransformation, two liquid phase biotransformation and the two liquid phase biotransformation with the biocatalyst immobilised. The first two options are similar where as the immobilised reaction is significantly different due to the problems of mass transfer and reactor design. In this biotransformation immobilising the bacteria would be carried out so that a solvent could be added to extract the fluorocatechol from the medium. The solvent selected is likely to be more hydrophilic than the one selected for the normal two liquid phase biotransformation since inactivation of the biocatalyst by the solvent has been reduced. Ideal situation for the use of immobilised biocatalyst will be in a biotransformation in

which the product is toxic but can be extracted by fairly toxic solvents.

The major influence of changes in the fermentation conditions was upon the aromatic oxidation activity of the bacteria and not solvent tolerance. Changes in solvent tolerance had been observed but addition of magnesium ions to the reactor had resulted in greater tolerance. Optimising of the fermentation should be towards obtaining high and stable aromatic oxidation activity because without the activity even with high solvent tolerance there will be no biotransformation. Bacteria with low solvent tolerance but high aromatic oxidation activity will lead to a purely aqueous biotransformation. Solvent tolerance could be improved by optimisation of the biotransformation reaction. The scale of the process will also be important in that at a small scale the fermentation can be easily controlled due to the faster mixing time and harvesting of the biocatalyst will be quicker. Overall control of a large scale reaction will be more difficult due to slow response to changes in the control variables. Downstream processing of large volumes will take longer and again lead to possible degradation.

Optimisation of the process could be carried out by considering the fermentation and the biotransformation as two separate units. The biotransformation could be optimised so that the solvent tolerance is increased whereas the fermentation will be optimised to give the highest total aromatic oxidation. In this study the effect of changes in the fermentation and biotransformation was analysed but at no stage was the implications of these changes upon downstream processing considered. In the end the changes made could result in major problems with the downstream processing, complete process optimisation can only be achieved when all the factors have been considered.

#### 4.9 - Conclusions

- 1) Chloroxylenol, used as a test of solvent tolerance, inactivated the bacterium exponentially with OUR reaching a steady value at the end of the assay. The equation derived to follow the inactivation is shown below and could also be fitted to the data obtained for octanol and other solvents analysed.

$$\frac{(OUR_t - OUR_f)}{(OUR_0 - OUR_f)} = \exp^{-ct}$$

- 2) The specific death rate constant of the bacterium decreased as the fermentation proceeded, this correlates to an increase in solvent tolerance. This was observed for fermentations done under various conditions.
- 3) Cultures grown at 26, 30 and 34°C were tested at 30°C for solvent tolerance. The higher growth temperature resulted in an increase in tolerance to chloroxylenol. However bacterial growth at 34°C was slow and not reproducible which would be undesirable for this biotransformation process.
- 4) Chloroxylenol, fluorobenzene and octanol toxicities, as indicated by the specific death rate constant, were plotted against solvent concentration. A minimum solvent concentration was required before inactivation was observed; above this level solvent toxicity increased rapidly.
- 5) Fluorobenzene and fluorocatechol inhibited bacterial growth and oxygen uptake. A strain of the bacterium was isolated which overcame fluorocatechol toxicity during growth on succinate.

- 6) Addition of magnesium sulphate to the solvent tolerance assay increased the tolerance of the bacterium to chloroxyleneol, fluorobenzene and dichloroethane. A reduction in temperature of the solvent tolerance assay from 30 to 23°C increased tolerance to dichloroethane.
- 7) Two factors can increase the total aromatic oxidation activity. Firstly the yield of biomass and secondly the specific aromatic oxidation activity. Growth at 5 % DOT instead of 20 % DOT resulted in approximately double the specific aromatic oxidation activity. Changes in growth conditions to increase specific aromatic activity had greater effect upon the overall aromatic oxidation activity than an increase in biomass concentration.
- 8) Aromatic oxidation activity of the bacterium grown in the fermenter was lost rapidly after harvesting. The oxygen limited shake flask cultures resulted in high stability of the aromatic oxidation activity over a number of days.
- 9) During the fermentation limited by iron or oxygen the specific aromatic oxidation activity of the bacterium increased dramatically. Bacterial growth upon succinate minimal medium does not require the activity of benzene dioxygenase or benzene cis glycol dehydrogenase. Biocatalytic activity increased during oxygen limitation, enzyme expression was not proportional to substrate concentration. Iron is a component of benzene dioxygenase; the expected reduction in activity following iron limitation was not observed. The bacterium is incapable of controlling the rate of expression of non-essential enzymes for growth limited by oxygen or iron, metabolic energy was directed away from growth. This indicates an overall metabolic control rather than genetic control of a particular biocatalytic enzyme.

- 10) The fermentations at 5 and 20 % DOT had two phases in the growth profile, detected by changes in the rate of carbon dioxide evolution. In the first the rate was exponential whereas in the second either the rate was no longer exponential or as in the fermentation at 30°C and 5 % DOT the rate was lower. On-line gas analysis provided a powerful tool to detect the changes in the rate of carbon metabolism not observed in the biomass data.
  
- 11) Optimisation of the aromatic oxidation activity should be obtainable by changing the growth conditions whereas solvent tolerance could be increased by changing the biotransformation medium.



## REFERENCES

- ALIFIMOFF, J.K., FIRESTONE, L.L. & MILLER, K.W. (1989).  
Anaesthetic potencies of primary alkanols : implications for  
the molecular dimensions of the anaesthetic site. Br. J.  
Pharmacol. 96, 9-16.
- ANDERSON, W.A., LEGGE, R.L. & MOO-YOUNG, M. (1987). Optimisation  
of growth conditions for the induction of tyrosine  
decarboxylase in Streptococcus faecalis. Biot Lett 9, 685-  
690.
- ANLIKER, R. & MOSER, P. (1987). The limits of bioaccumulation of  
organic pigments in fish: Their relation to the partition  
coefficient and the solubility in water and octanol.  
Ecotoxicology and Environmental Safety 13, 43-52.
- ATKINSON, B. & MAVITUNA, F. (1983). Biochemical Engineering  
Handbook, The Nature Press.
- AXCELL, B.C. & GEARY, P.J. (1973). The metabolism of benzene by  
bacteria- Purification and some properties of the enzyme cis-  
1,2- dihydroxycyclohexa-3,5-diene (Nicotinamide adenosine  
dinucleotide) oxidoreductase (cis benzene glycol  
dehydrogenase). Bioch. J. 136, 927-934.
- BALLARD, D.G.H., COURTIS, A., SHIRLEY, I.M. & TAYLOR, S.C. (1983).  
A biotech route to polyphenylene. J. Chem. Soc. Chem. Comm.  
954-955.
- BAR, R. (1988). Effect of interphase mixing on a water-organic  
solvent two-liquid phase microbial system : Ethanol-  
fermentation. J. Chem. Tech. Biotechnol. 43, 49-62.

- BARTL, K., CAVALAR, C., KREBS, T., RIPP, E., RETEY, J., HULL, W.E., GUNTHER, H. & SIMON, H. (1977). Synthesis of stereospecific deuterated phenylalanines and determination of their configuration. *Eur. J. Bioch.* 72, 247-250.
- BECKER, W. & PFEIL, E. (1966). Continuous synthesis of optically active hydroxynitriles. *J. Am. Chem. Soc.* 88, 4299-4300.
- BELLEAU, J. & BURBA, J. (1960). The stereochemistry of the enzymatic decarboxylation of amino acids. *J. Am. Chem. Soc.* 82, 5751-5752.
- BERGER, B., CARTY, C.E. & INGRAM, L.O. (1980). Alcohol-induced changes in the phospholipid molecular species in Escherichia coli. *J. Bact.* 142, 1040-1044.
- BERNADI, R., CARDILLO, R. & GHIRINGHELLI, D. (1984). Formation of the (R) - and (S) - enantiomers of ethyl-3-hydroxybutanoate and of 1-(1,3-dithian-2-yl)-2-hydroxypropane by Microbial reduction. *Journal Chemical Society, Chemical Communications*, 460-461.
- BLACKMAN, G.E., PARKE, M.H. & GARTON, G. (1955). The physiological activity of substituted phenols. II. Relationship between physical properties and physiological activity. *Arch. Bioch.* 54, 55-71.
- BONGAERTS, G.P.A., UITZETTER J., BROUNS, R. & VOGELS, G.D. (1978). Uricase of Bacillus fastidiosus. Properties and regulation of synthesis. *Bioch. Bioph. Acta* 527, 348-358.
- BOND, R.W., FIELD, A.S. & SWITZER, R.L. (1983). Nutritional regulation of degradation of aspartate transcarbamylase and of bulk protein in exponentially growing Bacillus subtilis cells. *J. Bact.* 153, 253-258.

BOYD, D. R., SHARMA, N. D., DORRITY, M. R. J., HAND, M. V., MCMORDIE, R. A. S., MALONE, J. F., PORTER, H. P., DALTON, H., CHIMA, J. & SHELDRAKE, G. N. (1993). Structure and stereochemistry of cis dihydro diol and phenol metabolites of bicyclic azarenes from Pseudomonas putida UV4. J.Chem.Soc.Perkin Trans. 1065-1071.

BRAZIER, A. (1989). PhD Thesis, UCL, University of London.

BRAZIER, A. J., LILLY, M. D. & HERBERT, A. B. (1990). Toluene cis glycol synthesis by Pseudomonas putida; kinetic data for reactor evaluation. Enz. Micro. Tech. 12, 90-94.

BRINK, L. E. S. & TRAMPER, J. (1985). Optimization of organic solvent in multiphase biocatalysis. Biot. Bioeng. 27, 1258-1269.

BUCKLAND, B. C. I. (1974). PhD thesis, UCL, University of London.

BUCKLAND, B., BRIX, T., FASTERT, H., GBEWONYO, K., HUNT, G. & JAIN, D. (1985). Fermentation exhaust gas analysis using mass spectrometry. Bio/Technology 3, 982-988.

CARIDIS, K. A., CHRISTAKOPOULUS, P. & MACRIS, B. J. (1991) Simultaneous production of glucose oxidase and catalase by Alternaria alternata. Appl Micro Biot 34, 794-797.

CEEN, E. G., HERMANN, J. P. R. & DUNNILL, P. (1987). Solvent damage during free cell catalysis and its avoidance: studies of 11 $\alpha$ -hydroxylation of progesterone by Aspergillus ochraceus. Enz. Micro. Tech. 9, 365-368.

CHERRY, P. C., BORTHWICK, A. D., COLES, R. J. & BURN D. (1985). Glaxo Group Ltd, European Patent, E.P. 0,162,592.

- CHIBATA, I., TOSA, T., SATO, T. & MORI, T. (1976). Production of L-amino acids by aminoacylose adsorbed on DEAE-Sephadex. Meth. in Enzymology 44, 746-759 .
- CHICK, H. (1908). An investigation of the laws of disinfection. J. Hyg 8, 92-158.
- CLARK, T.A., CHONG, R. & MADDOX, I.S. (1982). The effect of dissolved oxygen tensions on 11 $\beta$ - and 19-hydroxylation of Reichstein's substance S by Pellicularia filamentosa. Eur. J. Appl. Micro. Biot. 14, 131-135.
- COWAN, D.A. & PLANT, A.R. (1992). Biocatalysis in organic media, Chapter 7, p86-107 in 'Biocatalysis at extreme temperatures - Enzyme systems near and above 100°C', ed. by Adams, M.W.W. & Kelly, R.M., American Chemical Society.
- CRUTCHER, S.E. & GEARY, P.J. (1979). Properties of the iron-sulphur protein of the benzene dioxygenase system from Pseudomonas putida. Biochem. J. 177, 393-400.
- DAGLEY, S. (1986). Biochemistry of aromatic hydrocarbon degradation in Pseudomonas in the Bacteria, Vol 10, Chapter 15. p527-555 ed. Sokatch, J.R., Academic Press.
- DAUGULIS, A.J., AXFORD, D.B. & MCLELLAN, P.J. (1991). The economics of ethanol production by extractive fermentations. Canad. J. Chem. Eng. 69, 488-497.
- DESMET, M.J., KINGMA, J. & WITHOLT, B. (1978). The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of Escherichia coli. Biochimica Biophysica Acta 506, 64-80.

- DODDS, K.L., PERRY, M.B. & McDONALD, I.J. (1987). Alterations in lipopolysaccharide produced by chemostat-grown Escherichia coli 0157 : H7 as a function of growth rate and growth-limiting nutrient. *Can. J. Micro.* 33, 452-458.
- EHRESMANN, B., IMBAULT, P. & WEIL, J.H. (1973). Spectrophotometric determination of protein concentration in cell extracts containing tRNA and rRNA. *Anal. Bioch.* 54, 454-463.
- ELLWOOD, D.C. & TEMPEST, D.W. (1972). Effects of environment on bacterial wall content and composition. *Advances in Microbial Physiology* 7, 83-117.
- EMANVOLOVA, E.I. & KAMBOUROVA, M.S. (1992). Effect of carbon source and dissolved oxygen level on cell growth and pullulanase production by Bacillus stearothermophilus G-82. *World Journal of Microbiology and Biotechnology* 8, 21-23.
- FABER, K. & FRANSSEN, M.C.R. (1993). Prospects of the increased application of biocatalysts in organic transformations. *Tibtech* 11, 461-470.
- FAVRE-BULLE, O., SCHOUTEN, T., KINGMA, J. & WITHOLT, B. (1991). Bioconversion of n-octane to octanoic acid by a recombinant Escherichia coli cultured in a two-liquid phase bioreactor. *Bio/Technology* 9, 367-371.
- FRANTZ, B. & CHAKRABARTY, (1986). Degradative plasmids in Pseudomonas, in the *Bacteria*, Vol 10, Chapter 9, p295-323, ed. Sokatch, J.R., Academic Press.
- FREEMAN, A., WOODLEY, J.M., & LILLY, M.D. (1993). In situ product as a tool for bioprocessing. *Bio/Technology* 11, 1007-1012.

- FRY, J.C. & ZIA, T. (1982). A method for estimating viability of aquatic bacteria by slide culture. *J. Appl. Bact.* 53, 189-198.
- FULKS, R.M. & STADTMAN, E.R. (1985). Regulation of glutamine synthetase, aspartokinase, and total protein turnover in Klebsiella aerogenes. *Biochimica et Biophysica Acta* 843, 214-229.
- GEARY, P.J., SABOOWALLA, F., PATIL, D. & CAMMACK, R. (1984). An investigation of the iron-sulphur protein of benzene dioxygenase from Pseudomonas putida by electron spin resonance spectroscopy. *Biochem J.* 217, 667-673.
- GEARY, P.J., PRYCE, R.J., ROBERTS, S.M., RYBACK, G. & WINDERS, J.A. (1990). The oxidation of norbornadiene and some derivatives using Pseudomonas sp. *J. Chem. Soc., Chem. Comm.* 204-205.
- GILBERT, P. & BROWN, M.R.W. (1978). Influence of growth rate and nutrient limitation on the gross cellular composition of Pseudomonas aeruginosa and its resistance to 3- and 4-chlorophenol. *J. Bact.* 133, 1066-1072.
- GILLELAND, H.E., SINNETT, J.D. & EAGON, R.G. (1974). Ultrastructural and chemical alteration of the cell envelope of Pseudomonas aeruginosa, associated with resistance to ethylenediaminetetraacetate resulting from growth in a  $Mg^{2+}$ -deficient medium. *J. Bact.* 117, 302-311.
- GILLIES, B., YAMAOKI, H. AND ARMSTRONG, D.W. (1987). Natural flavour esters: Production by Candida cylindracea lipase adsorbed to silica gel in 'Biocatalysis in organic media', p227-232 ed by Laane, C., Tramper, J. and Lilly, M.D., Elsevier Science Publishers.

- GODFREY, T. & REICHEL, J. (1983). Industrial enzymology, The application of enzymes in industry. The Nature Press.
- GOFF, S.A. & GOLDBERG, A.L. (1987). An increased content of protease La, the lon gene product, increases protein degradation and blocks growth in Escherichia coli. J. Biol. Chem. 262. 4508-4515.
- GOMEZ, A. & CASTILLO, F.J. (1983). Production of biomass and  $\beta$ -D-galactosidase by Candida pseudotropicalis grown in continuous culture on whey. Biot Bioeng 25, 1341-1357.
- GOTTESMAN, S. & ZIPSER, D. (1978). Deg phenotypes of Escherichia coli lon mutants. J. Bact. 133, 844-851.
- HACK, C.J. (1992). PhD Thesis, U.C.L., University of London
- HANISCH, W.H., DUNNILL, P. & LILLY, M.D. (1980). Optimization of the production of progesterone 11 $\alpha$ -hydroxylase by Rhizopus nigricans. Biot. Bioeng. 22, 555-570.
- HANSSON, L. & HAGGSTROM, M.H. (1983). Effects of growth conditions on superoxide dismutase and catalase activities in Saccharomyces cerevisiae var ellipsoideus. Curr Micro 9, 19-23.
- HARRISON, D.E.F. & PIRT, S.J. (1967). The influence of dissolved oxygen concentration on the respiration and glucose metabolism of Klebsiella aerogenes during growth. J. Gen Micro 46, 193-211.
- HARROP, A.J., HOCKNULL, M.D. & LILLY, M.D. (1989). Biotransformations in organic solvents: A difference between Gram positive and Gram negative bacteria. Biot Lett 11, 807-810.

- HARROP,A.J.(1990). PhD Thesis, U.C.L., University of London.
- HAYMAN,D.F., BRODIE,A.C. & PROCOPIOU,P.A.(1987). Glaxo Group Ltd. European patent 0,221,725. Process for preparing heterocyclic compounds.
- HEINEMANN,B.,HOWARD,A.J. & PALOCZ, H.J.(1970). Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosis by Serratia marcescens. Appl. Microbiol. 19, 800-804.
- HENDERSON SMITH,J. (1921). The killing of Botrytis spores by phenol. Annals Appl Biol 8, 27-50.
- HOCKNULL,M.D.(1989). PhD Thesis. UCL, University of London.
- HOFER,B.,ELTIS,L.D.,DOWLING,D.N. & TIMMIS,K.D.(1993). Genetic analysis of a Pseudomonas locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene 130, 47-55.
- INGRAM,L.O.(1976). Adaption of membrane lipids to alcohols. J. Bact. 125, 670-678.
- ISENSCHMID,A.,MARISON,I.W. & VON STOCKAR,U.(1992). Effect of a near-critical and supercritical fluid on the viability ratio of microbial cells, p407-416 in 'Biocatalysis in Non-Conventional Media', ed. Tramper,J.,Beefink,H.H. & Von Stockar,U., Elsevier Science Publishers B.V.
- JACKSON,R.W. & DEMOSS,J.A.(1965). Effects of toluene on Escherichia coli. Journal of Bacteriology 90, 1420-1425.



- JAKOVAC, I.J., GOODBRAND, H.B., LOK, K.P. & JONES, J.B. (1982). Enzymes in organic synthesis 24. Preparations of enantiomerically pure chiral lactones via stereospecific Horse Liver alcohol dehydrogenase catalysed oxidation of monocyclic meso diols. *J. Am. Chem. Soc.* 104, 4659-4665.
- JOHNSTON, J.B. WINICOV, H. & CUNNINGHAM, V.L. (1987). Microbial catabolites as chemical synthesis intermediates- A cost-benefit analysis. *Biot Prog.* 3, 127-130.
- JOHNSTON, J.B. & RENGANATHAN, V. (1987). Production of substituted catechols from substituted benzenes by a Pseudomonas sp. *Enz Micro Tech* 9, 706-708.
- KATO, K., KAWAHARA, K., TAKAHASHI, T. & IGARASI, S. (1980). Enzymatic synthesis of amoxicillin by cell-bound amino acid ester hydrolase of Xanthamonas citri. *Agric. Biol. Chem.* 44, 821-825.
- KAWAKAMI, K. (1990). Characterisation of production of 1,2-epoxyoctane from 1-octene by non-growing cells of Nocardia corallina B-276 in aqueous-organic media. *Annals N.Y. Acad. Sci.* 613, 707-711.
- KAWAKAMI, K. & NAKAHARA, T. (1994). Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilised whole cells of Pichia pastoris. *Biot. Bioeng.* 43, 918-924.
- KONIGSBERGER, K. & HUDLICKY, T. (1993). Microbial oxidation of 2-bromostyrene by Pseudomonas putida 39/D. Isolation and identification of metabolites. *Tetrahedron:Asymmetry* 4, 2469-2474.

- KUROZUMI, S., TORU, T. & ISHIMOTO, S. (1973). Preparation of 2-(4-hydroxy-1-oxocyclopent-2-ene) heptanoic acid an important prostaglandin synthon. *Tetrahedron Letters* 49, 4959-4960.
- LAANE, C., BOEREN, S. & VOS, K. (1985). On optimizing organic solvents in multi-liquid phase biocatalysis. *Trends in Biotechnology* 3, 251-252.
- LADD, O.L. & WEINSTOCK, J. (1981). Improved synthesis of fluoroveratroles and fluorophenethylamines via organolithium reagents. *J. Org. Chem.* 46, 203-206.
- LEY, S.V., STERNFIELD, F. AND TAYLOR, S. (1987). Microbial oxidation in synthesis: A six step preparation of ( ) pinitol from benzene. *Tetrahedron Letters* 28, 225-226.
- LIERBERMANN, B., SCHUFFENHAUER, S. & IHN, W. (1991). Biosynthesis of tentoxin and dihydrotentoxin via protein turnover by Alternaria alternata. *J. Basic Micro.* 31. 51-57.
- LILLY, M.D., BRAZIER, A.J., HOCKNULL, M.D., WILLIAMS, A.C. & WOODLEY, J.M. (1987). Biological conversions involving water-insoluble organic compounds, p3-17 in 'Biocatalysis in Organic Media', ed. by Laane, C., Tramper, J. & Lilly, M.D., Elsevier Science Publishers B.V., Amsterdam.
- LUDERITZ, O., FREUDENBERG, M.A., GALANOS, C., LEHMANN, V., RIETSCHHEL, E.T. & SHAW, D.H. (1982). Lipopolysaccharide of Gram negative bacteria, p79-151 in 'Current topics in membrane and transport', Volume 17, ed by Bronner, F., Kleinzeller, A., Razin, S & Rottem, S.
- MARTINI, G., MIGNONE, C. & ERTOLA, R. (1989). Studies on beta-galactosidase production in transient operation cultures. *Biot. Lett.* 11, 545-550.

- MATYASHOVA, R.N. (1976). The influence of the concentration of dissolved oxygen on the growth of bacteria of the genus Pseudomonas. Microbiology (Russian) 45, 680-682
- MAUERSBERGER, S., MATYASHOVA, R.N., MULLER, H.G. & LOSINOV, A.B. (1980). Influence of the growth substrate and the oxygen concentration in the medium on the cytochrome P-450 content in Candida guilliermondii. Eur. J. Appl. Micro. 9, 285-294.
- MINNIKIN, D.E., ABDOLRAHIMZADEH, H. & BADDILEY, J. (1972). Variation of polar lipid composition of Bacillus subtilis (Marburg) with different growth conditions. FEBS Lett. 27, 16-18.
- MOTOSUGI, K., ESAKI, N. & SODA, K. (1984). Enzymic preparation of D and L lactic acid from racemic 2-chloropropionic acid. Biot. Bioeng. 26, 805-806.
- NICAS, T.I. & HANCOCK, R.E.W. (1980). Outer membrane proteins H1 of Pseudomonas aeruginosa: Involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, Polymixin B and Gentamycin. J. Bact. 143, 872-878.
- NIKAIDO, H. (1976). Outer membrane of Salmonella typhimurium transmembrane diffusion of some hydrophobic substances. Bioch. Bioph Acta 433, 118-132.
- ORNSTON, L.N. & STANIER, R.Y. (1966). The conversion of catechol and protocatechuate to B-ketoadipate by Pseudomonas putida. J. Biol. Chem. 241, 3776-3786.
- OSBORNE, S.J., LEAVER, J., TURNER, M.K. & DUNNILL, P. (1990). Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb. Tech. 12, 281-291.

OYAMA, K. (1987). Enzymic synthesis of aspartame in organic solvents in 'Biocatalysis in Organic Media', p 209-224, ed by Laane, C., Tramper, J. and Lilly, M.D., Elsevier Science Publishers.

PHILIPPI, M. CHR., JONGEJAN, J. A. & DUINE, J. A. (1987). Enantioselective hydrolysis and transesterification of glycidyl butyrate by lipase preparations from porcine pancreas in 'Biocatalysis in Organic Media', p 279-284, ed by Laane, C., Tramper, J. & Lilly, M.D., Elsevier Science Publishers.

POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. J. Gen. Micro. 24, 15-24.

PINE, M. J. (1970). Steady-state measurement of the turnover of amino acid in the cellular proteins of growing Escherichia coli: Existence of two kinetically distinct reactions. J. Bact. 103, 207-215.

PINE, M. J. (1972). Turnover of intracellular proteins. Annual review of microbiology. 26, 103-126

PINHEIRO, H. M. & CABRAL, J. M. S. (1991). Effects of solvent molecular toxicity and microenvironment composition on the dehydrogenation activity of Athrobacter simplex cells. Biot Bioeng 37, 97-102.

RESNICK, S. M., TOROK, D. S. & GIBSON, D. T. (1993). Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2 dioxygenase and biphenyl 2,3 dioxygenase. FEMS Micro Lett 113, 297-302.

- RIBEIRO, M.H., CABRAL, J.M.S. & FONSECA, M.M.R. (1987).  
Production of L-tryptophan in a two liquid phase system in  
'Biocatalysis in Organic Media', p323-329, ed by Laane, C.,  
Tramper, J. and Lilly, M.D., Elsevier Science Publishers.
- RIDDICK, J.A., BUNGER, W.B. & SAKANO, T.K. (1986). Techniques of  
Chemistry, Volume 2, Organic Solvents, Physical properties  
and methods of purification, 4th edition, John Wiley &  
Sons.
- ROBINSON, G.K., STEPHENS, G.M., DALTON, H. & GEARY, P.J. (1992).  
The production of catechols from benzene and toluene by  
Pseudomonas putida in glucose-fed batch culture.  
Biocatalysis 6, 81-100.
- ROFFLER, R.S., RANDOLPH, T.W., MILLER, D.A., BLANCH, H.W. &  
PRAUSNITZ, J.M. (1991). Extractive bioconversions with  
nonaqueous solvents, p133-172, In 'Extractive  
bioconversions', Eds Mattiasson, B. & Holst, O; Marcel Dekker  
Inc.
- ROLLINS, M.J. & GAUCHER, G.M. (1986). An analysis of  
intracellular proteinase activity during antibiotic  
biosynthesis. Abstr. Can. Soc. Microbiol. 36th Meeting, 67.
- ROSE, A.H. (1961). Synthesis of D-ephedrine, 264-265 in  
'Industrial Microbiology', Butterworths.
- RUSSELL, A.J. (1981). PhD thesis, U.C.L., London University.
- RUSSELL, A.D. & FURR, J.R. (1977). The antibacterial activity of  
a new chloroxylenol preparation containing ethylenediamine  
tetraacetic acid. J. Appl. Bact. 43, 253-260.

- RYAN, W., PARULEKARR, J. & STARK, B.C. (1989). Expression of  $\beta$ -lactamase by recombinant Escherichia coli strains containing plasmids of different sizes - Effects of pH, phosphate and dissolved oxygen. *Biot. Bioeng.* 34, 309-319.
- SANCHEZ-FERRER, A., PEREZ-GILABERT, M. & GARCIA-CARMONA, F. (1992). Protein-interface interactions in reverse micelles, p181 in 'Biocatalysis in Non-Conventional Media', ed. Tramper, J., Beeftink, H.H. & Von Stockar, U., Elsevier Science Publishers B.V.
- SCHEPER, T.L. & SCHUGERL, K. (1986). Culture fluorescence studies on aerobic continuous cultures of Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 23, 440-444.
- SCHOFIELD, J.A. (1987). Shell International Research Maatschappij B.V. European patent 0,252,567. New organofluorine compounds and a biochemical process for their preparation.
- SCHOFIELD, J.A., GEARY, P.J., BETTERIDGE, P.R. & RYBACK, G. (1987). Shell International Research Maatschappij B.V. European patent 0,253,438. New strains of Pseudomonas and their use.
- SCHOFIELD, J.A. (1988). Shell International Research Maatschappij B.V., European patent 0,252,567. Biochemical Process.
- SCHWARTZ, R.D. & HUTCHINSON, D.B. (1981). Microbial and enzymatic production of 4,4-dihydroxybiphenyl via phenol coupling. *Enz Micro Tech* 3, 361-363.
- SHIRAI, K. (1987). Catechol production from benzene through reaction with resting and immobilised cells of a mutant strain of Pseudomonas. *Agric Biol Chem* 51, 121-128.

SIERRA-ALVAREZ,R. & LETTINGA,G.(1991). The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl. Microbiol. Biot. 34, 544-550.

SINENSKY,M.(1971). Temperature control of phospholipid biosynthesis in Escherichia coli. J. Bact. 106, 449-455.

SJURSNES,B.,KVITTINGEN,L. & ANTHONSEN,T.(1992). Controls of water activity by using salt hydrates in enzyme catalysed esterifications in organic media, p451-457 in 'Biocatalysis in Non-Conventional Media', ed. Tramper,J.,Beefink,H.H. & Von Stockar,U., Elsevier Science Publishers B.V.

STEINERT,H.J.,VORLOP,K.D. & KLEIN,J. (1987). Steroid side chain cleavage with immobilised living cells in organic solvents in 'Biocatalysis in Organic Media', p51-63, ed by Laane,C., Tramper,J. and Lilly,M.D., Elsevier Science Publishers.

STOICHEVA,N.G.,DAVEY,C.L.,MARKX,G.H. & KELL,D.B.(1989). Dielectric spectroscopy : A rapid method for the determination of solvent biocompatibility during biotransformations. Biocatalysis 2, 245-253.

STOUTHAMER,A.H.(1979). 'The Search for correlation between theoretical and experimental growth yields' in International Review of Biochemistry, Microbial Biochemistry, Volume 21, J.R. Quale, University Park Press.

SUGAI,T. & MORI.K.(1984). Preparation of (R) -Ibuprofen and related carboxylic acids using microbial oxidation of an aromatic hydrocarbon by Rhodococcus sp BPM 1613. Agric. Biol. Chem. 48, 2501-2504.

SUPHANTHARIKA,M.(1992). PhD Thesis, U.C.L., University of London.

- TAN, H. & MASON, J.R. (1990). Cloning and expression of the plasmid-encoded benzene dioxygenase genes from Pseudomonas putida ML2. FEMS Microbiology Letters 72, 259-264.
- TAN, H.M., TANG, H.Y., JOANNOV, C.L., ABDEL-WAHAB, N.H. & MASON, J.R. (1993). The Pseudomonas putida ML2 plasmid-encoded genes for benzene dioxygenase are unusual in codon usage and low in G+C content. Gene 130, 33-39.
- TAN, H.M. & FONG, P.Y. (1993). Molecular analysis of the plasmid-borne bed gene cluster from Pseudomonas putida ML2 and cloning of the cis-benzene dihydrodiol dehydrogenase gene. Can. J. Micro. 39, 357-362.
- TAYLOR, S.C. (1987). Imperial Chemical Industry PLC. European patent 0,253,485. Cyclic dihydroxy compounds.
- TOSA, T., SATO, T., MORI, T., MATUO, Y., & CHIBATA, I. (1973). Continuous production of L-aspartic acid by immobilised aspartase. Biotechnology and Bioengineering 15, 69-84 .
- TRAMPER, J. & VERMUE, M.H. (1993). Tailoring the medium and bioreactor for biocatalysis. Chimia 47, 110-115.
- VAN DEN TWEEL, W.J.J., MARSMAN, E.H., VORAGE, M.J.A.W., TRAMPER, J. & DEBONT, J.A.M. (1987). The application of organic solvents for the bioconversion of benzene to cis benzeneglycol, p231-241 in 'Bioreactor and Biotransformations', ed by Moody, G.W. and Baker, P.B.



- VAN DER MEER, J.R., EGGEN, R.I.L., ZEHNDER, A.J.B. & DE VOS, W.M. (1991). Sequence analysis of the Pseudomonas sp. strain p51 tcb gene cluster, which encodes metabolism of chlorinated catechols : evidence for specialisation of catechol 1,2-dioxygenase for chlorinated substrates. J. Bact. 173, 2425-2434.
- VARDAR, F. & LILLY, M.D. (1982). Effect of cycling dissolved oxygen concentrations on product formation in penicillin production. Eur. J. Appl. Micro. Biot. 14, 203-211.
- VILIESID, F. & LILLY, M.D. (1992). Influence of dissolved oxygen tension on the synthesis of catechol 1,2-dioxygenase by Pseudomonas putida. Enz Micro Tech 14, 561.
- VLASUK, G.P., GHRAYEB, J. & INOUYE, M. (1985). The major outer membrane lipoprotein of Escherichia coli : Secretion, Modification and processing, p309-329 in The enzymes of biological systems, 2nd edition, volume 2, Biosynthesis and Metabolism edited by Martonosi, A.N.
- VU-TRONG, K. & GRAY, R.P. (1984). Stimulation of enzymes involved in tylosin biosynthesis by cyclic feeding profiles in fed batch cultures. Biot. Lett. 6, 435-440.
- WESTERMAN, P.W., POPE, J.M., PHONPHOK, N., DOANE, J.W. & DUBRO, D.W. (1988). The interaction of n-alkanols with lipid bilayer membranes: a <sup>2</sup>H-NMR study. Bioch. Bioph. Acta 939, 64-78.
- WILLIAMS, A.C., WOODLEY, J.M., ELLIS, P.A. & LILLY, M.D. (1987). Denaturation and inhibition studies in a two-liquid phase biocatalytic reaction: the hydrolysis of menthyl acetate by pig liver esterase, p399-404 in 'Biocatalysis in Organic Media', ed by Laane, C., Tramper, J. and Lilly, M.D., Elsevier Science Publishers.

- WINDHOLZ, M. (1983). Merck Index, 10th Edition, Merck & Co Ltd.
- WITHOLT, B., DE SMET, M.J., KINGMA, J., VAN BEILEN, J.B., KOK, M., LAGEVEEN, R.G. & EGGINK, G. (1990). Bioconversions of aliphatic compounds by Pseudomonas oleovorans in multiphase bioreactors: background and economic potential. TibTech 8, 46-52.
- WOODLEY, J.M. & LILLY, M.D. (1992). Process engineering of two-liquid phase biocatalysis, p147-154 in 'Biocatalysis in non-conventional media, Progress in biotechnology 8', Eds Tramper, J., Vermue, M.H., Beeftink, H.H. & Von Stockar, U., Elsevier Science Publishers B.V.
- WRIGHT, N.E. & GILBERT, P. (1987)a. Antimicrobial activity of n-alkyl trimethylammonium bromides : influence of specific growth rate and nutrient limitation. J. Pharm. Pharmacol 39, 685-690.
- WRIGHT, N.E. & GILBERT, P. (1987)b. Influence of specific growth rate and nutrient limitation upon the sensitivity of Escherichia coli towards chlorhexidine diacetate. J. Appl. Bact. 62, 309-314.
- XIAOLI, L., ROBBINS, J.W. & TAYLOR, K.B. (1992). Effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant Escherichia coli strains. J. Ind. Micro. 9, 1-10.
- YOUNG, J.M., LUKETRIA, R.C. & MARSHALL, A.M. (1977). The effects on temperature on growth in vitro of Pseudomonas syringae and Xanthomonas pruni. J. Appl. Bact. 42, 345-354.
- ZAMANIAN, M. (1989). PhD Thesis. KQC, University of London.

## NOMENCLATURE

$C^*$	- saturation concentration in the aqueous phase
$C_L$	- current concentration in the aqueous phase
CER	- carbon dioxide evolution rate
DOT	- dissolved oxygen tension
g	- gramme
hr	- hour
$K_2EDTA$	- dipotassium ethylenediamine tetraacetic acid
$K_La$	- overall mass transfer coefficient
l	- litre
LPS	- lipopolysaccharide
m	- metre
M	- molar
min	- minute
mol	- mole
Mw	- molecular weight
OUR	- oxygen uptake rate
ppm	- parts per million
rpm	- revolutions per minute
RQ	- respiratory quotient
TCER	- total carbon dioxide evolution rate
TOUR	- total oxygen consumption rate
V	- voltage

n nano,  $\mu$  micro, m milli, c centi.

