Process design for the oxidation of fluorobenzene to fluorocatechol using *Pseudomonas putida* ML2

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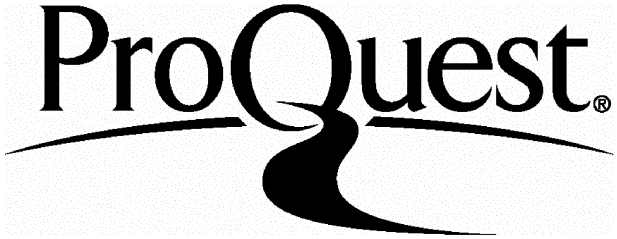

Dedication

To Ciara,

To my mum and dad

This thesis is dedicated to the fond memory of Dr Phil Geary and Dr Margot Kogut

In arching too the pastor own`d his skill
For e`en though vanquished he could argue still,
and words of learn`d length and thundering sound
amazed the gazing rustics ranged round
and still they gazed and still the wonder grew
that one small head could carry all he knew

Oliver Goldsmith

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I finally acknowledge the perseverance shown by my mother and father in forcing me to go to school all those years ago!

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The last word must belong to none other than my friend and uncle Dick Power. Some things you just can't put a price on!

Go raibh maith agaibh go leir.

Abstract

In this thesis, a framework for biotransformation process design has been outlined. A particular biotransformation (the microbial oxidation of fluorobenzene to fluorocatechol) was chosen to illustrate the benefits of adopting such a structured approach.

This reaction system is characterized by the following:

Fluorobenzene (the poorly aqueous soluble, volatile substrate) causes reversible, cellular activity inhibition at 0.8g/ L.

First order kinetics are observed for fluorobenzene conversion up to 0.1g/ L, after which, zero order kinetics apply.

Fluorocatechol (the completely aqueous soluble product) is toxic at 0.2g/ L causing irreversible activity loss.

An assay capable of measuring the intrinsic biocatalytic activity of the cells has been developed.

These characterization data have been used to define a number of process options. One of these has been developed to promote production levels up to seventy times the cellular toxicity limit of the biocatalyst (0.185g fluorocatechol/ g dry wt/ hr) for 11 hours operation.

Ten modes of oxygen supply were evaluated including a novel membrane oxygenator, a perfluorocarbon, a solvent, head pressurisation and oxygen enrichment.

Two modes (membrane oxygenator and pure oxygen) were analysed during continuous biotransformations for their ability to eliminate fluorobenzene volatilization loss.

The membrane oxygenator completely eliminated all fluorobenzene loss from the biotransformation.

13 conclusions have been drawn which are listed on page 120.

By adopting the biotransformation process design framework presented in this thesis, a bioreactor configuration has been developed which has successfully overcome the difficulties associated with oxygen supply to a whole cell catalysed, aromatic oxidation having a volatile, inhibitory, poorly aqueous soluble substrate and a toxic, completely aqueous soluble product.

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Abbreviations

	terminal mixing time
C	concentration difference
L	microlitre
A, B, D	constants
A	area
AFR	air flow rate
a_L	saturation concentration
a_w	activity dependence on water
C*	dissolved oxygen concentration in equilibrium with the gas phase
C	bulk dissolved oxygen concentration
C	degrees centigrade
C_i	column inlet fluid concentration
C_L	equilibrium liquid phase concentration
C_o	column outlet fluid concentration
cSTR	continuous stirred tank reactor
C_x	equilibrium solid phase concentration
DOT	dissolved oxygen tension
g dry cell wt/ L	grams dry cell weight per litre
G.C.	gas chromatography
g	grams
gFC	grams of fluorocatechol
HPLC	high pressure liquid chromatography
hr	hour
K_L	adsorption equilibrium constant
K_La	volumetric oxygen transfer coefficient
L	litre
m	metres
M	molar

min	minute
ml	millilitre
mM	millimolar
mmHg	millimetres of mercury
mmol	millimole
N	agitation speed
Ndi	impeler tip speed
OD	optical density
OTR	oxygen transfer rate
P / V_L	volumetric power input
P	power input
PP9	perfluoromethyldecalin
psi	pounds per square inch
qG	gassing rate
qO_2	microbial oxygen uptake rate
STR	stirred tank reactor
uG	linear gas velocity
UV	ultraviolet
V	volume
V_G / V	volumetric gas flow
V_L	liquid volume
vvm	volume per volume per minute
x	mass of adsorbent

CHAPTER I

INTRODUCTION

1 INTRODUCTION

1.1 Biotransformations

The processing of biological materials into desirable products has been practised throughout the ages beginning with alcohol fermentation and vinegar production (**Rose, 1981**).

In recent decades, reactions carried out with the aid of enzymes or intact micro-organisms have been used with ever increasing frequency and success to catalyse synthetic chemical reactions (**Vandamme, 1980; Tramper *et al*, 1985; Jones, 1986; Crout and Christen, 1989; Davies *et al*, 1989; 1990; Cayen, 1991;Holt, 1993; Roberts, 1993; Leuenberger and Wirz, 1993**). Gradually, a technique called biotransformation has been moulded.

In the course of a biotransformation the selective enzymatic transformation of a well defined substrate is taking place. This is often a single step in a synthetic sequence composed mainly of chemical reactions. Table 1.1 lists the most widely used biocatalyst systems and their scope of application.

In biotransformations a well defined reaction is performed by one or a few enzymes of a microorganism on an added substrate (which may be a natural compound or not). The product does not undergo further change, accumulates in the system and can ultimately be isolated.

Biotransformations can not only be an alternative to well established chemical methods but may also enable the realisation of reactions which are difficult or impossible to induce, by purely chemical means.

Biocatalyst	Whole cell	Cell free preparation
Types:	growing cells	cell free extracts
	resting cells	purified enzymes
	lyophilised cells	treated or modified enzymes
	treated or modified cells	
Forms:	free cells	free form
	micro capsules, immobilised cells	micro capsules, micro emulsions
		immobilised form
Environment:	aqueous solution	aqueous solution
	aqueous solution containing organic cosolvent	aqueous solution with organic cosolvent
	cell containing preparations in organic solvent	water-organic solvent biphasic system
	Water organic solvent biphasic system	water-restricted organic solvent

Table 1.1 Types of biocatalyst systems

Some of the special features of biotransformations are listed in Table 1.2.

Special features of biotransformations
• wide selection of reactions
• efficiency and speed of catalysis
• mild operational conditions (room temperature, atmospheric pressure and nearly neutral pH)
• high selectivity with respect to type of reaction, substrate required and substrate stereo structure
• the capacity to introduce functional groups at positions otherwise unreactive towards common organic reagents
• associated environmental hazards are generally small

Table 1.2 Special features of biotransformations

1.2 Biotransformation Process Design

The aim of biotransformation process design (BPD) is to replace existing energy consuming processes of doubtful specificity with safer, cleaner, less energy intensive biological processes (enzymic or whole organism based) and to develop novel processes and new products with superior properties.

Most commercial biotransformation processes have been designed on a case by case basis. There is no published set of rules or guidelines for process design (which reflects the current embryonic stage of this field of research).

What is needed, therefore, is a formal structured approach which can be used. This introduction will develop a framework for BPD which can be employed to develop such processes.

Historically, one design method has been to adopt criteria for stirred tank bioreactor scale-up as the convention for BPD. Table 1.3 lists the most common of these criteria.

Criteria	Nomenclature
Volumetric oxygen transfer coefficient	$K_L a$
Volumetric power input	P / V_L
Volumetric gas flow	V_G / V
Impeller tip speed	$N d_i$
Agitation speed	N
Mixing time	t

Table 1.3 Common criteria for scale-up of stirred tank bioreactors

While this approach is often adequate and satisfies many requirements, there are quite a number of other challenges and considerations which need to be addressed in parallel with these criteria. These are listed in Table 1.4

Challenges:	Considerations:
Poorly aqueous soluble substrates	Substrate / product properties (Melting point, boiling point, aqueous and organic solubility, pH, stability and volatility)
Substrate / product inhibition / toxicity	Kinetics of reaction

Table 1.4 Challenges and considerations in BPD

BPD must establish a design procedure which will promote optimum operating conditions while addressing the challenges and considerations listed in Table 1.4.

1.2.1 A framework for biotransformation process design

It is imperative to adopt an overall perspective. The biotransformation (and consequently bioreactor design) must not be viewed in isolation, but as part of the overall process design challenge.

It is important to have a procedure which can be adopted (and adapted) to a wide range of processes. A design philosophy which could be adopted is illustrated in Figure 1.1.

The initial challenge lies with biocatalyst selection and production. The next challenge lies with the establishment of an assay capable of independently measuring biocatalytic activity. Characterization must then take place which will lead to the development of a range of process constraints.

These constraints can then form the basis of a process option selection procedure which will, in turn, define a final process specification. The objective of this procedure must be to act as a process of elimination rather than as a means of evaluating all possible considerations (**Woodley and Lilly, 1994**).

1.2.2 Biocatalyst preparation

The protocol involved in biocatalyst production and preparation is illustrated in Figure 1.2.

Where whole cells or fermentation products are to be used as biocatalyst, a standardised fermentation protocol needs to be developed which can consistently produce biocatalyst with the desired attributes and a reproducible fermentation profile with the required final biomass yield. Dissolved oxygen tension (DOT), optical density and cell dry weight can prove to be reliable measures of growth.

Harvesting and preparation of the biocatalyst can be carried out in parallel. This usually involves a centrifugation and resuspension step in the case of whole cell biocatalysts or a disruption step in the case of cell free extracts.

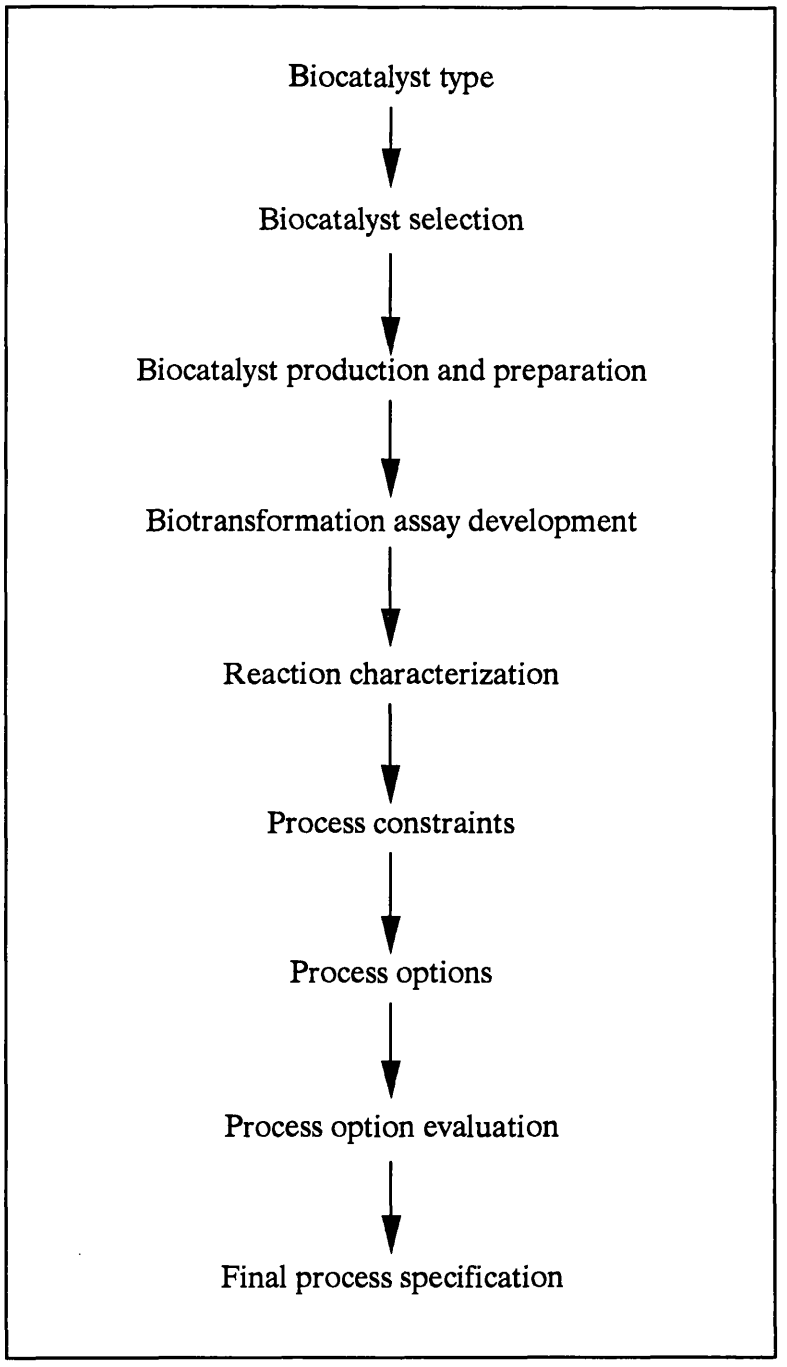

Figure 1.1: Biotransformation process design framework

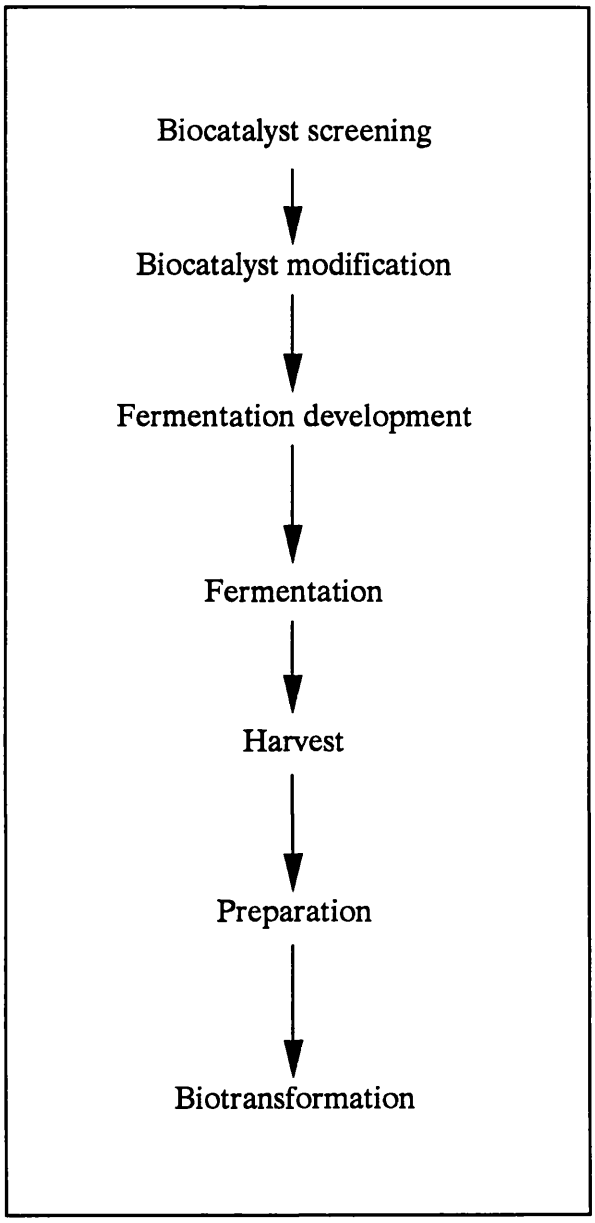

Figure 1.2: Biocatalyst production protocol

Fermentation conditions must be developed to obtain a high biocatalyst yield (with the desired activity). For example, care must be exercised in the choice of fermentation carbon source since some carbon sources result in repression of certain enzymes or enzyme systems (**Axcell and Geary, 1973**). It may also be necessary to establish the influence of DOT, pH, temperature, time of storage and type of downstream processing on the biocatalyst itself.

1.2.3 Biotransformation assay development

It is necessary to develop a biotransformation rate assay capable of accurately and reproducibly measuring activity independently of the biotransformation itself. This assay could be used as a tool to study activity profiles during fermentations, to aid in the evaluation of substrate and product inhibitory/toxicity levels and to evaluate the influence of different BPDs on the biocatalytic activity during biotransformations.

1.2.4 Reaction Characterization

The issues which must be resolved through characterization of the biotransformation reaction system are summarised in Table 1.5. Certain data can be obtained from the literature. However, the majority of the issues require experimental evaluation, emphasising the importance of reaction characterization.

It may be desirable to neglect a detailed characterization of the biotransformation reaction system. Such a decision may prove to be a false economy. A detailed focused characterization will lead to the accumulation of information pertaining to the biotransformation reaction system. These data can prove vital in establishing a BPD where optimum biotransformation operation will be promoted during continuous operation.

The accumulation of such details can be important in assessing the influence of different BPDs on biocatalytic activity.

Reaction component:	Feature:
Substrate and Product	Aqueous and organic solubility
	Melting and boiling point
	Physical state during biotransformation
	Temperature and pH stability
	Stability under biotransformation conditions
	Volatility
Biocatalyst	Activity influence of pH, (Temperature and dissolved oxygen tension)
	Activity influence of medium constituents: elemental composition, acid and alkali
	Cofactor requirement
	Single or multistep biotransformation
	Nutrient requirement
	Influence of operating conditions on biocatalyst (Interface and agitation)
	Activity dependence on water (a_W)
Reaction	Requirement for molecular oxygen
	Acid / alkali requirement
	Gas production
Interaction of substrate, product and biocatalyst	Substrate / product inhibition
	Substrate / product toxicity
	Substrate / product stability
	Reaction kinetics

Table 1.5 Summary of reaction characterization considerations

1.2.5 Process option evaluation

Once production of the biocatalyst and an assay capable of measuring its biocatalytic activity have been developed, reaction characterization will lead to the establishment of a number of process constraints which can be evaluated. These constraints will define the final process specification.

This procedure will have more to do with a process of elimination, than with investigating all conceivable process variations. This approach can be described as biotransformation process option evaluation.

The most important components of this procedure are substrate supply, bioreactor design and product recovery. These elements and a process flow sheet representing their interrelationship are illustrated in Figure 1.3.

1.2.5.1 Bioreactor design

One definition of a bioreactor is a device in which materials are treated to promote biochemical transformation of substrate by the action of cells or cell free enzyme systems.

Virtually all bioreactors deal with heterogeneous systems involving one or more phases. Thus, to achieve the required biochemical change, interphase mass and heat transfer must occur.

The development of a bioreactor is one of the primary challenges in BPD. To aid the design process, Table 1.6 provides criteria which should be addressed in parallel with the issues outlined in Table 1.5.

The literature contains numerous papers dedicated to the development of new and novel bioreactors and reviews of new generation bioreactors. Brauer (**Brauer, 1987**) describes a selected group of high efficiency bioreactors covering the stirred tank bioreactor, bioreactors with internal biosuspension circulation, multistage bioreactors, reciprocating bioreactors and high porosity packed bioreactors. Halling (**Halling, 1987**) and Belfort (**Belfort, 1989**) review membrane reactors. Other papers related to the subject of new and novel bioreactor designs have been published (**Davis and Watson, 1985; Lilly and Woodley, 1985; Tramper *et al*, 1986; Kornfield *et al*, 1986; Grady, 1989; Berovic, 1991; Wall and Hill, 1992; Elezier, 1993;**).

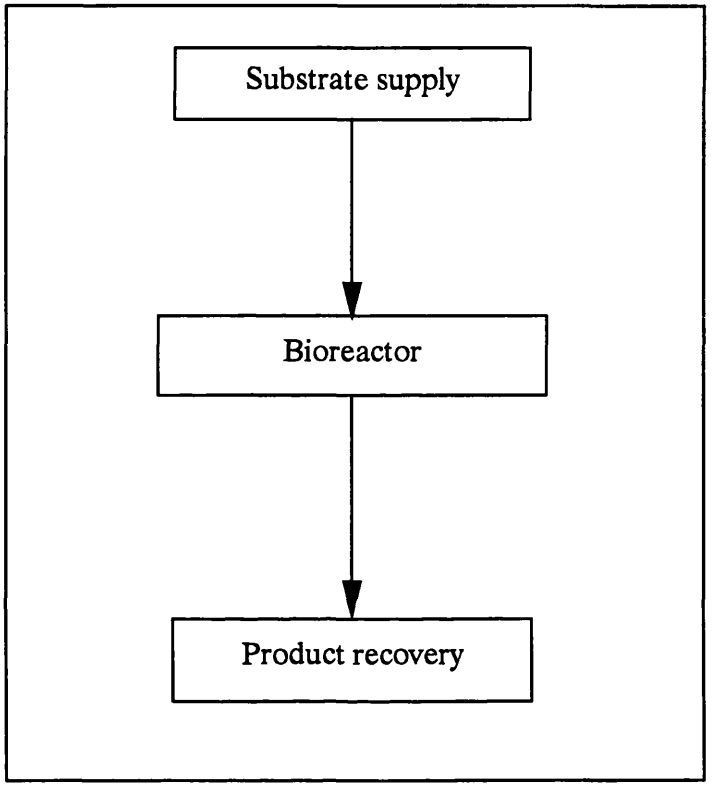

Figure 1.3: Biotransformation process design flow sheet

Criteria:
• High rate of biocatalyst conversion per unit biomass
• When uniform distribution is required, completely well mixed solution requiring distribution of biocatalyst, oxygen, temperature, pH, substrate and product concentration over the complete bioreactor volume
• Low energy requirement
• Match characteristics of the reaction with the reactor (Batch STR, CSTR, Plug flow).
• Effective supply and removal of substrate and product
• Small bioreactor volume and site requirement, closed emission proof design, simplicity of construction

Table 1.6 Criteria for biotransformation bioreactor design

It is noteworthy that the stirred tank is still the preferred bioreactor design. Because of high bioreactor capital costs, industry prefers to modify existing bioreactors (e.g. replace stirrers) rather than introduce completely new bioreactor designs. Furthermore, application of the same general bioreactor design to different microbial processes facilitates a high degree of flexibility in biotransformation process design.

This is the reason why the stirred tank bioreactor is often the first choice when a new process is developed and it is only when the requirements (for, say, gas-liquid mass transfer) cannot be met in this type of bioreactor that other designs are considered.

1.2.5.2 Substrate Supply

The issue of substrate supply to the biotransformation is less challenging if the substrate is aqueous soluble, non toxic (or non inhibitory), non volatile and does not form an explosive mixture with any other biotransformation constituents. Substrate can be dissolved in a second liquid phase or added in batch or continuous mode to facilitate continuous uninhibited biocatalytic activity.

The situation becomes more complex however when substrate solubility, toxicity, volatility, explosive limits or other conditions referred to in Table 1.5 have to be addressed.

If the substrate is inhibitory or toxic to the biocatalyst above a certain concentration then rigorously controlling its feed rate to the biotransformation (exploiting feed back control analytical methods) will prevent deleterious effects during biotransformation.

It may be possible to increase the solubility of low aqueous soluble substrates by the use of water miscible solvents (although this approach may have limited capacity to raise the solubility of certain organic compounds). An alternative approach is to carry out the reaction with the poorly soluble substrate dissolved in an organic phase in the reactor (**Cooney and Heuter, 1974; Buckland *et al*, 1975; Butler, 1977; Antonini *et al*, 1981; Carrera, 1984; Fukui and Tanaka, 1985; Dordick, 1989**).

One documentation of an integrated approach to reactor and downstream product recovery design and operation is outlined by Lilly and Woodley (**Lilly and Woodley, 1985**) for biotransformations involving water insoluble organic substrates leading to a two liquid phase biocatalytic reactor design. They describe a simple classification system for reactions involving both substrates and/or products which have low aqueous solubilities and therefore exploit the benefits of a second liquid phase.

All of the modes of substrate supply discussed above are crucial in forming an assessment of the feasibility of any method of substrate addition and should be assessed along with the alternative process options available.

1.2.5.3 Oxygen supply (a special case of substrate supply)

Some biotransformations of commercial interest involve the incorporation of molecular oxygen. Therefore, providing an adequate oxygen supply for aerobic biotransformations is critical to the maintenance of biocatalytic activity.

Unfortunately, oxygen mass transfer to the biotransformation medium is often a major productivity limitation because of oxygen's low aqueous solubility (approximately 1.26mmol/ L at ambient temperature and pressure). Thus, ensuring an adequate oxygen supply to a biotransformation can pose significant problems.

Traditionally, oxygen is supplied to a biotransformation by bubbling air through the growth medium accompanied by vigorous agitation to effect a fine bubble dispersion in the STR configuration. Various bioreactor designs and impeller shapes have been proposed to maximise oxygen transfer from the gas to the aqueous phase (**Jansen *et al*, 1984; Ghommidh *et al*, 1986; Hu *et al*, 1986; Reuveny *et al*, 1986; Piehl *et al*, 1988; Swaaij and Versteeg, 1992; Schluter and Deckwer, 1992**)

Attempts at increasing stirrer speed and/or air sparging rate are often constrained by the mechanical operational capacity of particular bioreactors. These actions may increase the process operating cost and simultaneously introduce a new set of problems, e.g. excessive foaming.

The actions that increase oxygen mass transfer, namely agitation and sparging have only limited effectiveness in certain classes of bioreactors where cells are highly susceptible to hydrodynamic forces (**Oh *et al*, 1987; Yang and Wang, 1992**) which has led to the development of a range of non-conventional oxygen supply modes.

Examples of some unconventional methods for enhancing oxygen supply include:

- *In situ* generation of molecular oxygen with hydrogen peroxide and catalase (**Schlegel, 1977; Ibrahim and Schegel, 1980a, 1980b; Seip *et al*, 1993; Wilson, 1994**).
- Co-immobilization or mixed culture of an oxygen producing photosynthetic alga (**Adlercreutz and Mattiasson, 1982; Adlercreutz *et al*, 1982; Khang et al, 1988**).
- The introduction of an immiscible phase of inert hydrocarbons or perfluorocarbons with high oxygen solubilities (**Bruining *et al*, 1986; Mattiasson and Aldercrutz, 1987; Cho and Wang, 1988; King *et al*, 1989; Rols and Goma, 1989; Ho et al, 1990; Ju and Armiger, 1992**).
- The use of membrane systems to enhance oxygen transfer capacity (**Yasuda and Lamaze, 1972; Kornfield *et al*, 1986; Cote *et al*, 1988; Drury *et al*, 1988; Wang *et al*, 1988; Grootjen *et al*, 1990; Mano *et al*, 1990; Ahmed and Semmens, 1992; Henzler and Kauling, 1993**).
- Raising the oxygen partial pressure in the gas phase with oxygen enriched air or by mixing air with oxygen (**Flickinger and Perlman, 1977; Yamada, 1978**) or by increasing the operating pressure in the bioreactor head space.

The biocatalyst could also be genetically manipulated to reduce its biotransformation oxygen requirement. Oxygen incorporated during biotransformations would then be used exclusively for the biotransformation and not for purposes considered as "excess to requirements" such as respiration or metabolism. It may also be possible to replace oxygen with another terminal electron acceptor such as sodium nitrate.

Of the many methods of oxygen supply categorised as surface aeration, membrane aeration, bubble aeration and stirred tank aeration, it is significant that the sparged stirred tank is still the preferred mode of oxygen transfer, for reasons outlined in 1.2.5.1.

The copious literature cited above reflect the intense activity in the area of oxygen supply research. Many bioreactor designs to enhance oxygen transfer rates are still undertaken on a case by case basis with a specific biotransformation process in mind. There is, as yet, no indication that the sparged STR will be abandoned in favour of any new or novel method of oxygen supply.

One of the major disadvantages associated with this bioreactor design (the gas sparged STR) is the significant loss, through volatization of volatile organic components present in the biotransformation medium (**Matter-Muller *et al*, 1981; MacKay and Yeun, 1983; Roberts and Daupliker, 1983; Cho and Wakad, 1987**). The driving force for volatization is the presence of gas bubbles in the biotransformation medium, coupled with the high agitation rates required to maintain adequate oxygen mass transfer levels.

One means of overcoming the environmental problem caused by volatization is to provide oxygen transfer in the absence of gas bubble formation, otherwise known as bubbleless aeration. This developing technique of bubbleless aeration is gaining widespread attention (**Cote *et al*, 1988; Semmens *et al*, 1989; Ahmed *et al*, 1992 a,b; Henzler, 1993**) in order to overcome the environmental problem associated with the loss of volatile organic components in normal sparged stirred tank aeration devices.

There is a wide range of oxygen supply modes to choose from in designing any biotransformation process. Clearly, choice of supply depends on the requirements of the individual biotransformation process itself.

1.2.5.4 Product recovery

The use of continuous product recovery in biocatalytic applications can provide both kinetic and thermodynamic advantages. When a product is continuously removed from solution, a biocatalyst that is subjected to decreased productivity due to end-product inhibition will show an increase in activity (**Freeman *et al*, 1993**). With the correct choice of product recovery systems, it will no longer be necessary to work with dilute solutions, and so product recovery costs can be reduced.

The equilibrium position of biocatalysed equilibrium reactions may also affected by the introduction of a suitable product recovery technique. Many equilibrium reactions are driven, not by providing large excesses of the reactants, but by continuously removing product, the reaction may be "pulled" toward completion and large excesses of reactants are no longer required (**Halling, 1987**).

Separation and purification processes in biotransformations exploit differences in physico-chemical properties of biological molecules including molecular size, charge, solubility, hydrophilic or hydrophobic nature and biological affinity. Some of the methods employed in product recovery are listed in Table 1.7 together with their main principles of operation. Many of the methods described in Table 1.7 are not what could be considered "on-line" product recovery methods. However, operated in combination with a continuous separation device, e.g. a membrane system, they could be.

Mode:	Principle of operation:
Crystallisation	Purified chemical solids produced from impure solutions in a single processing step. Solute is cooled, leading to supersaturation initiating crystal growth. Can be operated continuously.
Membrane processes	Membrane processes are classified on the basis of solute size being separated (**Docksey, 1986**). Pressure is the main driving force although concentration polarisation can be a problem. Reverse osmosis: $10^{-8} \leq 10^{-5}$cm Ultra filtration: $10^{-7} \leq 10^{-3}$cm Microfiltration: $10^{-6} \leq 10^{-2}$cm Conventional filtration: $10^{-3} \leq 10^{-1}$cm
Chromatography	**Adsorption**: Dissolved solute binds to solid adsorbent based on chemical or physical affinity. Major adsorbents or ion exchange supports include carbon based material, synthetic resins, zeolites and gel. **Affinity**: Specific and efficient purification of a high value protein from a dilute solution. Specific ligand may not be available. **Gel filtration:** Seperates biomolecules on basis of hydrodynamic volume or molecular weight limitations with respect to scale of operation. **Large scale HPLC:** Small particle size packings in HPLC facilitate rapid equilibration between stationary and mobile phases, resulting in high resolutions and flow rates.
Liquid-Liquid extraction	Preferential partitioning of a solute between immiscible liquid phases. Constraints dictated in previous substrate supply section apply.
Supercritical fluid extract	Compressed gas acts as extracting solvent to provide superior quality selective removal and thermal stability of e.g. volatile flavour components.
Electrophoresis	Exploits net surface charge of molecules and their size to achieve seperation in an electrical field.
Lyophilization	Method of drying achieved by freezing substance, causing ice to sublime directly to vapour by exposing to low partial pressure of water vapour.
Complex formation	Formation of a reversibly bound complex between product and chelating agent.

Table 1.7 Product recovery methods and mode of operation

Choice of product removal technique should be dictated by criteria such as selectivity, stability, process compatibility, biocompatibility and environmental compatability.

1.3 Whole cell biotransformations

In Section 1.1, some of the special features of biotransformations were discussed and why they would be chosen over traditional chemical synthetic routes. In deciding on the use of biotransformations in a synthetic facility, one is faced with the choice of employing microrganisms or isolated enzymes. The commercial availability and cost of the required enzyme, compared with the availability of a suitable microrganism will often be the deciding factor.

The use of isolated enzymes is advantageous in that undesirable by-product formation, mediated by contaminating enzymes is avoided. Enzymes are specific for selected reactions and can usually be purchased, in small amounts,"off the shelf". However, extraction and purification of the enzyme is costly, enzyme co-factor recycling is sometimes necessary and enzymes are frequently less stable in purified form than in crude preparations or when present in whole cells.

Thus, in many industrial biotransformation processes, for greater cost effectiveness, the biocatalyst used is in the form of whole cells (**Nikolova and Ward, 1992**). Microrganisms (or whole cell biocatalysts) are a desirable choice as biocatalysts since they possess great ability to adapt to environmental change (**Pool, 1990**).

Their use, over isolated enzyme systems, has several advantages in that they provide an internal environment for enzyme catalysis involving labile enzymes which would otherwise be inactivated in an aqueous solution. Examples of these include the dioxygenase enzyme complex found in many *Pseudomonas sp.* and cyanide oxygenase which are inactivated almost immediately on release from the cell. The cell wall protects the enzyme from air-liquid and liquid-liquid interfaces (**Williams et al, 1990**).

Low enzyme production levels and incorrect protein folding contrasted with progress made in cellular genetic engineering has aided in the employment of whole cell biocatalysts in industry (**Ferreira *et al*, 1984; Brookes and Lilly, 1986; Brink and Tramper, 1987; Taylor, 1987; Hudlikey *et al*, 1988 Nikolova and Ward, 1991; Larroche, *et al*, 1992**).

Whole cell biotransformations can manifest problems related to undesirable by-product formation from many different enzymes. In these cases, reaction conditions have to be manipulated to minimize by-product formation or product degradation. Metabolic conditions can often be designed to promote cofactor regeneration, thus avoiding the problems which are encountered in isolated enzyme type biotransformations.

It must be noted that the reasons listed above, which work to the advantage of whole cell biotransformations, can also be a liability in biotransformation process design. These issues need to be addressed in process design because whole cell systems are often more difficult to work with than purified enzymes.

A review of the literature reveals the high level of activity in this field (**Meijer and Shoemaker, 1988; Welsh *et al*, 1989; Mountfort *et al*, 1990; Roberts, 1990; Taylor *et al*, 1990; Meyer, 1991; Nikolova and Ward, 1992; Roberts and Turner, 1992; Leuenberger and Wirz, 1993; Roberts, 1993**).

1.4 Whole cell aromatic oxidations

One class of whole cell biotransformations which is interesting and particularly challenging is the whole cell catalysed oxidation of aromatic compounds. Many of these biotransformations involve the conversion of poorly aqueous-soluble substrates which are inhibitory or toxic to the microbial catalyst (**Ley *et al*, 1987; Yoshikawa, 1990; Collins *et al*, 1993**).

In some cases, such as benzene and toluene oxidation, the organism (*Pseudomonas putida*) is inhibited by substrate concentrations well below the aqueous saturation level (**Jenkins *et al*, 1987; Van den Tweel *et al*; 1987; Brazier *et al*, 1989; Hack *et al*, 1991; Harrop *et al*, 1992**). Product inhibition/toxicity and substrate volatility are issues which must also be addressed in whole cell aromatic oxidation process design (**Lynch *et al*, 1992**).

One example of this class of biotransformation is the conversion of fluorobenzene to fluorocatechol using *Pseudomonas putida* ML2. This reaction involves a volatile, inhibitory, poorly aqueous soluble, substrate and a toxic completely aqueous soluble, product. It was chosen as an exemplary reaction system to illustrate the benefits of approaching biotransformation process design in the structured manner detailed in Section 1.2.

1.4.1 The oxidation of fluorobenzene to fluorocatechol

The bacterium *Pseudomonas putida* ML2 was selected from benzene enriched soil in a Shell oil refinery in Holland (**Axcell and Geary, 1973**). A semi constitutive mutant of *Pseudomonas putida* was obtained by irradiating the bacterium with ultra-violet light.

Fluorobenzene is metabolised via fluorobenzene-*cis* glycol to fluorocatechol by the enzymatic action of *Pseudomonas putida* ML2. It is a two step reaction involving the complex benzene dioxygenase enzyme system (**Ornston and Stanley, 1966; Gibson *et al*, 1970; Axcell and Geary, 1973; Axcell and Geary, 1975; Crutcher and Geary, 1979; Geary *et al*, 1984; Whited *et al*, 1986; Zamanian and Mason, 1987**). The oxidation of fluorobenzene to its corresponding catechol is illustrated in Figure 1.4 along with the important features of the reaction itself.

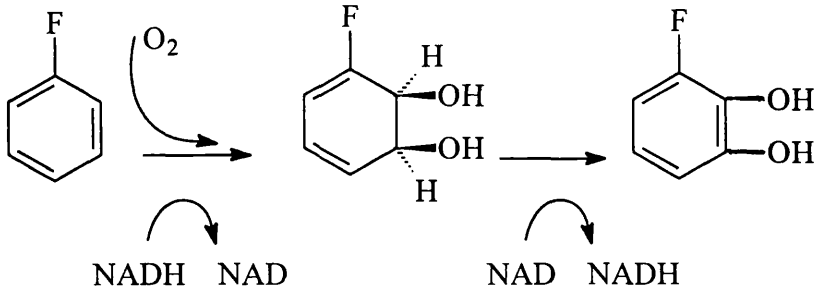

Figure 1.4 The biotransformation of fluorobenzene to fluorocatechol using *Pseudomonas putida* ML2

The first step involves the incorporation of molecular oxygen into the aromatic nucleus resulting in the formation of fluorobenzene *cis*-dihydrodiol. NADH, the energy source to drive this oxidation process is concomitantly reduced.

The benzene dioxygenase enzyme system functions to transfer electrons from NADH to the terminal dioxygenase by the sequential oxidation and reduction of a series of flavoproteins.

This step acts as the rate limiting step in the production of fluorocatechol from fluorobenzene. As soon as fluorobenzene *cis*-glycol is produced it is immediately converted to fluorocatechol by the dehydrogenase enzyme system.

Fluorobenzene *cis*-glycol is dehydrogenated in an NAD^+ dependent reaction mediated by *cis*-fluorobenzene dihydrodiol dehydrogenase. Consequently, there is no requirement for NADH within the reaction.

In the absence of fluorobenzene *cis*-glycol dehydrogenase, consumption of oxygen is equivalent to the amount of NADH added. In the presence of fluorobenzene *cis*-glycol dehydrogenase, there exists a mole per mole equivalent between benzene, oxygen and fluorobenzene *cis*-glycol.

Pseudomonas putida ML2 contains active catechol 1,2 dioxygenase and so will produce *cis-cis* mucconic acid from benzene. However, steric hindrance afforded by the fluorine group on the fluorobenzene molecule prevents degradation of fluorocatechol once it has been formed (**Kirk and Creveling, 1984**). Consequently, fluorocatechol will accumulate in solution when *Pseudomonas putida* ML2 is used to oxidise fluorobenzene.

In addition to using this system to demonstrate the biotransformation process design framework discussed earlier, there are economic benefits. The product of the biotransformation, fluorocatechol, is a valuable precursor in the pharmaceutical industry. The substituted catechol moiety is indigenous to a large family of pharmaceutically active drugs, not least of which are the adrenergic catecholamines along with the biogenic amines (**Kirk and Creveling, 1984**). 3-Fluoroveratrole, a catechol derivative, is a valuable intermediate in drug synthesis and is related to the corresponding fluorodimethoxy-phenyl ethylamines (**Ladd and Weinstock, 1981; Ladd *et al*, 1985**)

Although such pharmaceutical precursors can be synthesised by traditional chemical routes, the syntheses are often lengthy, requiring expensive starting materials and are not amenable to scale-up. These difficulties suggest that a biological process could provide an economic alternative to the initial chemical synthetic steps involved. In fact, the cost of producing 3-fluoroveratrole employing traditional synthetic routes can be as much as five times the cost of using biosynthetic routes (**Johnston *et al*, 1987**).

CHAPTER II

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 Suppliers list

The names and addresses of suppliers are given in Appendix 1.

2.2 Microrganism

2.2.1 Isolation of the organism

A benzene oxidising strain of *Pseudomonas putida* (*Pseudomonas putida* ML2) was kindly supplied by Dr P.J. Geary of Shell Research Limited, Sittingbourne, UK. The organism was isolated from a sample of refinery soil obtained in Holland by elective culture in a simple salts medium containing benzene as the sole carbon source. A constitutive mutant was obtained by irradiating the bacterium with ultra violet (UV) light.

2.2.2 Maintenance of the organism

Nutrient agar plates were prepared using a 28g/ L nutrient agar solution. Stock cultures were maintained on nutrient agar plates at 4 C and sub cultured weekly.

2.3 Growth of the organism

2.3.1 Shake flask and fermenter media

A defined aqueous salts medium was used for both shake flask and fermenter growth. The medium constituents (g/ L) are given in Table 2.1.

2.3.2 Shake flask growth

Pseudomonas putida ML2 colonies taken from a nutrient agar plate were used to aseptically inoculate a 500mL shake flask containing 100mL of sterilised aqueous salts medium (The shake flask was autoclaved at 121 C for 20 minutes prior to innoculation). 100μL benzene was added. The shake flask was sealed using a "Subaseal" and incubated for 24 hours in a New Bruswick incubator/shaker at 28 C at 220 rpm. Benzene was the sole carbon source used in shake flask growth and the use of a Subaseal prevented any loss of substrate.

Anhydrous constituent	g/ L
Ammonium sulphate ($(NH_4)_2SO_4$)	1
Magnesium sulphate ($MgSO_4$)	0.2
Iron chloride ($FeCl_2$)	0.016
Disodium hydrogen orthophosphate (Na_2HPO_4)	3
Potassium dihydrogen orthophosphate (KH_2PO_4)	3
Calcium chloride ($CaCl_2$)	0.015
Fructose	25
Trace element solution (2ml).	**g/ L**
Calcium chloride ($CaCl_2$)	0.66
Zinc sulphate ($ZnSO_4$)	0.18
Copper sulphate ($CuSO_4$)	0.15
Manganese sulphate ($MnSO_4$)	0.15
Cobalt chloride ($CoCl_2$)	0.18
Sodium molybdate (Na_2MoO_4)	0.3

Table 2.1 Shake flask and fermenter media constituents

2.3.3 Fermenter growth

Unless otherwise specified *Pseudomonas putida* ML2 was grown in an MBR 2L (working volume) fermenter using the same shake flask media (Table 2.1). The fermenter carbon source was fructose (25g/ L) which was sterilised separately to prevent caramelisation.

Except where stated, fermentation dissolved oxygen tension (DOT) was maintained above 30% air saturation, temperature was controlled at 30 C and pH maintained at 6.8 by the addition of 1.5M NH_4OH and 1.5M H_2SO_4 as required.

Three fermentations were also carried out with an elevated fructose content (30g/ L) to assess the influence of low DOT (0%) on cellular biocatalytic activity.

Parameters could be controlled on-line and were monitored continuously using Bio-pc software from BCS. These included: agitation rate (0-1500 rpm); DOT (0-100% air saturation); pH (0-14) and temperature (0-130 C)

2.4 Biocatalyst Harvest and Biotransformation

Cells were harvested into four centrifuge "pots" (500mL) from the fermenter. Centrifugation took place in an MSE 21 Hi Spin Centrifuge (20 minutes, 6000 rpm, 4 C). After centrifugation the biomass was resuspended in phosphate buffer (2L, 100mM, pH 6.8) and placed in the MBR bioreactor. Cells were allowed to condition at 30C.

A 50% (V/V) suspension of Ethanol and fluorobenzene was pumped to the bioreactor. The rate of addition was controlled by GC monitoring. During the biotransformation, the product loaded cell suspension was pumped from the bioreactor to an adsorbent column connected in an external loop. Fluorocatechol adsorption took place within the column and the cell suspension was pumped back to the bioreactor. Biotransformations were carried out under different conditions. Details of these conditions are given in Section 3.2.6.

2.5 Analytical methods

2.5.1 Cell growth analysis

Fermentation cell growth was monitored using DOT as an indicator of growth. Cell concentration was estimated by measuring optical density (OD) spectrophotometrically at 670nm using a PYE Unicam PU8600 UV/VIS Spectrophotometer. Readings in the range of 0 to 0.4 were considered accurate.

Fermentation samples (10mL) whose OD was measured were placed in dry weight glass tubes and kept in an oven (110C) until constant mass was recorded. OD measurements could then be correlated with cell dry weights Figure 2.1 illustrates the correlation between OD and dry cell weight.

2.5.2 Gas Chromatography (GC)

Fluorobenzene and fluorocatechol were analysed on a VARIAN 3700 gas chromatograph fitted with a 25m, capillary, non polar column (25QC3/BPX5) with a 1μm film thickness. Sample injections were carried out using a Carlo Erba, A200S, autosampler. The operating conditions developed for the detection and analysis of fluorobenzene and fluorocatechol are given in Table 2.2.

GC operating conditions	
Automated linear temperature ramping programme:	
Initial column temperature	122 C
Holding period	1 min
Programme rate	4 C/ min
Final temperature	132 C
Holding period	2 min
Injection volume	1μL
Splitter ratio	1:10
Carrier gas	Helium
Injection temperature	270 C
Detecter temperature	300 C

Table 2.2 G.C. operating conditions

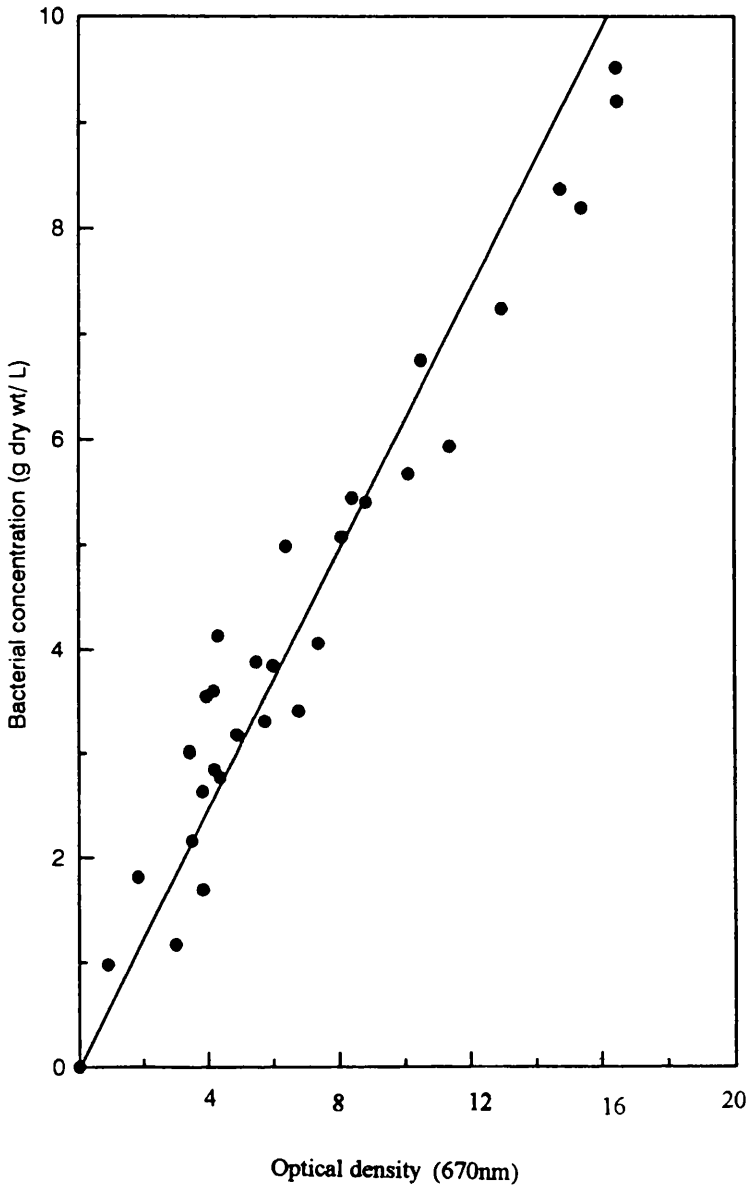

Figure 2.1 Correlation between OD and dry cell weight

Samples were run in duplicate. The pre-column filter was replaced every 50 injections along with the injection septum. This filter was reconditioned by immersing it in concentrated Vortex for 92 hours. It was then dried in an oven for 4 hours (125 C).

Standard curves for the measurement of fluorobenzene and fluorocatechol are illustrated in Figures 2.2 and 2.3 respectively.

2.5.3 Substrate and product standard curves

The standard curves for fluorobenzene and fluorocatechol are illustrated in Figure 2.2 and 2.3. Sensitivity of detection was 0.005g/ L for fluorobenzene and 0.003g/ L for fluorocatechol.

2.5.4 Properties of fluorobenzene and fluorocatechol

The properties of fluorobenzene and fluorocatechol are given in Table 2.3.

Property	Fluorobenzene	Fluorocatechol
Molecular weight	96.11	128.11
Solubility in water	1.52g/ L at 25 C	Complete
Vapour pressure	73mm Hg	7mm Hg
Melting point	-41.09 C	72 C

Table 2.3 Properties of fluorobenzene and fluorocatechol

2.6 Biotransformation rate measurements

2.6.1 Biotransformation rate assay

A cell sample (50mL) was taken and diluted with phosphate buffer (pH 6.8, 100mM) to give a cell concentration of 3.0g dry wt/ L.

The cells were centrifuged at 6000 rpm for 20 minutes at 4 C, the supernatant was removed and the cells were resuspended in phosphate buffer (100mL, pH 6.8, 100mM).

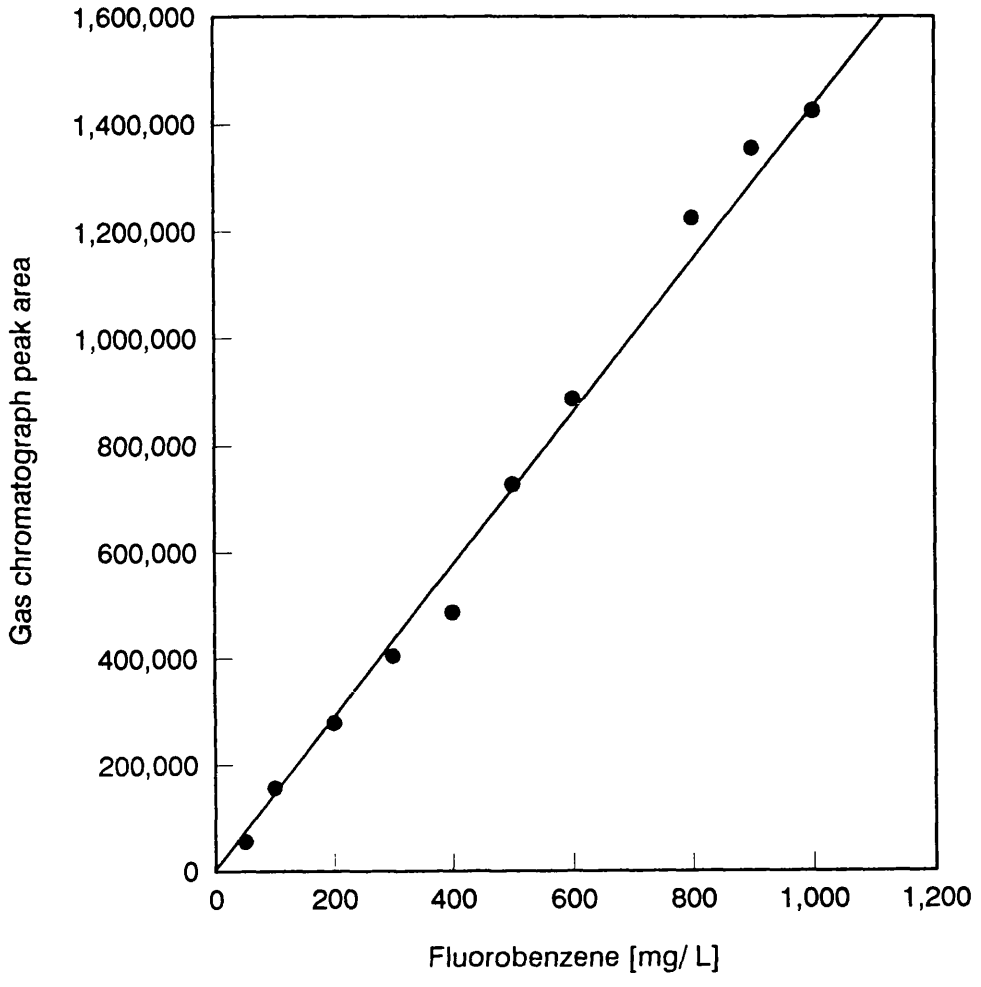

Figure 2.2 Standard curve relating fluorobenzene concentration to G.C. peak area

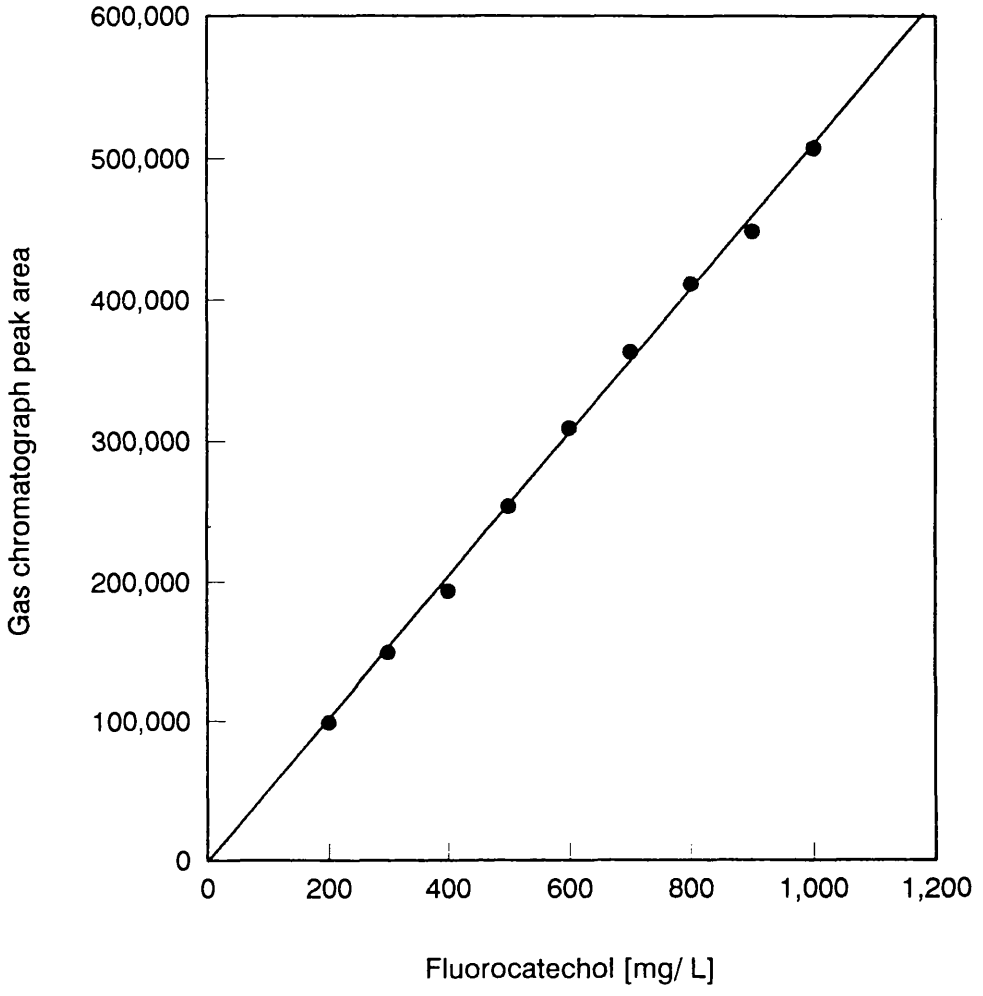

Figure 2.3 Standard curve relating fluorocatechol concentration to G.C. peak area

The resuspended cells (100mL) were placed in a 500mL shake flask with 100μL ethanol/ g dry cell wt. The flask was Subasealed and set to incubate at 28 C at 220 rpm.

Neat fluorobenzene was added to the cell suspension (using a Hamilton microsyringe) resulting in a concentration of 0.5g/ L. This was necessary to produce optimal biocatalytic activity and eliminate any fluorocatechol production lag phase. GC results confirmed that no fluorobenzene was lost using this technique.

A 1ml sample was removed every 15 minutes by inverting the shake flask and using a hypodermic needle and syringe. The sample was analysed by GC. Results were reported as grams of fluorocatechol produced per gram dry cell weight per hour (g FC/ g dry wt/ hr).

2.7 Oxygen transfer rate measurements

2.7.1 Chemicals

Perfluoromethyldecalin (PP9, $C_{11}F_{20}$) was supplied by BNFL Fluorochemicals. Tetradecane ($CH_3(CH_2)_{12}CH_3$) was supplied by Aldrich Chemicals. The properties and structure of PP9 and tetradecane are given in Section 2.7.4 and 2.7.5.

2.7.2 Membrane Oxygenator

The membrane oxygenator was supplied by Membran Corporation, Minneapolis, USA. The module is a hollow fibre membrane aerator, housing 200 sealed hollow gas permeable fibers which can be pressurised with oxygen (0-80psi). The fibers are fixed at one end of the module and free to move at the other. Figure 2.4 is a schematic diagram of the Membran module.

The fluid to be oxygenated is pumped past the fibres (in parallel). As it flows past the surface of the fibres oxygen transfer takes place between the oxygen contained within the fibres and the bulk of the liquid. In effect, the liquid "scrubs" oxygen from the fibre surface. There are 200 fibres with a diameter of 220μm and a length of 1.2m. The housing has a length of 1.4m and a diameter of 12cm.

2.7.3 Oxygen transfer rate determination

2.7.3.1 Probe calibration

Oxygen transfer rate (OTR) measurements were carried out in 6g dry wt/ L of *Pseudomonas putida* ML2 in the same bioreactor used for fermentations (2.3.3) and biotransformations (2.4).

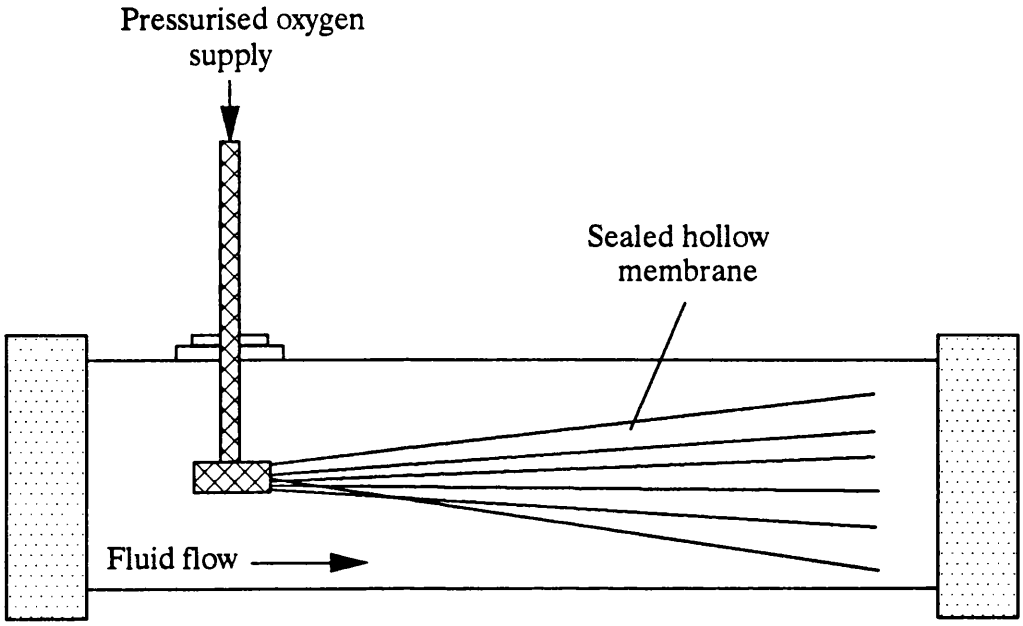

Figure 2.4 Illustration of the membrane oxygenation module

At a predetermined agitation rate (350, 750 and 1100rpm), with the dissolved oxygen probe in the air sparged vessel, DOT reading 100%, the DO probe was removed from the vessel and quickly inserted into a beaker of deionised water, sparged with nitrogen. The chart recorder was concomitantly switched on and switched off when 0% saturation was obtained.

The probe response time was the time taken for the DO to decrease from 100% to 36.8%. It was read from the graph. This procedure was repeated until a consistent probe response time was obtained.

2.7.3.2 OTR determination

The bioreactor was sparged with oxygen free nitrogen resulting in a corresponding 0% DOT reading. The nitrogen supply was shut off and the oxygen supply source under evaluation (at the predetermined gas flow rate: 1.5, 3 or 4.5vvm) was concomitantly switched on along with the chart recorder. The DO was allowed to reach 100% saturation. The chart recorder was turned off. The measured OTR was calculated from the linear region of the DOT graph, as an initial rate.

2.7.4 Properties of Perfluoromethyldecalin (PP9)

The structure of perfluoromethyldecalin PP9, is illustrated in Figure 2.5. Its physical properties are listed in Table 2.4.

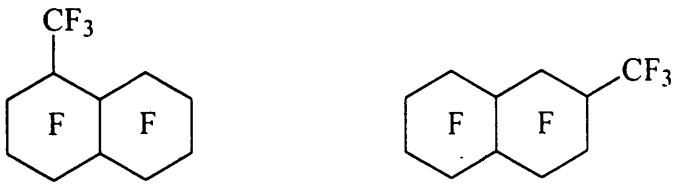

Figure 2.5 Perfluoromethyldecalin; a mixture of two isomers

Properties of Perfluoromethyldecalin, PP9		
Classification	Chemical name:	Perfluoromethyldecalin
	Chemical formula:	$C_{11}F_{20}$
	Hazard Classification:	Non-Hazardous
	CAS Number:	306-92-3
Physical properties:	Appearance:	Clear colourless high density liquid
	Odour:	Odourless
	Boiling Point:	155 C
	Freezing point:	-70 C
	Vapour pressure:	2.9 mbar @ 25 C
	Density:	1.972 Kg/ L @ 25 C
	Solubility in water:	Insoluble
	Solubility in organic solvents:	Sparingly soluble in most common solvents, miscible with CFC's
Reactivity data:	Stability:	Extremely stable
	Flash point:	None
	Incompatibility:	Lithium, sodium, potassium, calcium and barium
	Corrosion:	non-corrosive

Table 2.4 Properties of perfluoromethyldecalin PP9

2.7.5 Properties of Tetradecane

Tetradecane is a straight chain aliphatic hydrocarbon. Its properties are listed in Table 2.5

Properties of tetradecane	
Structure:	$CH_3(CH_2)_{12}CH_3$
Oxygen solubility:	282mg/ L
Molecular weight:	214.39g
Boiling point:	253 C
Density:	0.763g/ L

Table 2.5 Properties of tetradecane

2.8 Studies on the use of adsorbents

2.8.1 Adsorbent selection

A number of adsorbents were short-listed on the basis of their potential to selectively remove fluorocatechol from solution. They included: Spherosil QMA (hydrophilic) XAD 2, XAD 4, XAD 8, XAD 16, XAD 1180, Duolite S861, Spherosil C, Rohm & Hass A568, Amberlite IA35 (all hydrophobic with cationic and anionic surfaces) and Activated carbon (Norit, pK 1-3). Adsorbent particles diameter range was 0.2 to 0.6 mm. A majority of hydrophobic structures was chosen since the aqueous phase was water, a hydrophobic structure was required to preferentially remove fluorocatechol from solution.

Adsorbent (1.5g) was placed in contact with fluorocatechol (1.5g/ L) and separately, a mixture of fluorocatechol and fluorobenzene, to determine the adsorbent with the greatest affinity for fluorocatechol. The two phases were contacted in 6mL eppendorf tubes, sealed and shaken (180 minutes, 30 C, 220 rpm). GC was used to establish liquid phase fluorobenzene and fluorocatechol concentrations, from which the amounts adsorbed onto the solid adsorbent could be ascertained.

2.8.2 Preparation of the activated carbon adsorbent

Extruded activated carbon (Norit pK13; 200g) was washed thoroughly in distilled water (a sieve prevented loss of material) until all the fine carbon particles were removed.

The washed carbon was immersed in deionised water for two hours. This procedure ensured that the activated carbon was properly "wetted". The activated carbon was boiled and allowed to cool, ensuring that it was properly "degassed".

The distilled water was substituted for phosphate buffer (100mM, pH 6.8) and autoclaved (121 C, 15 minutes). The cooled activated carbon was packed into a glass column (0.58 x 0.04cm (h x i.d.)) giving a bed volume of 0.35L. The column inlet and outlet were sealed using metal sieves.

The column was washed by pumping 30 bed volumes (8-10L) of buffer through the column (alternately reversing the flow direction) to displace any remaining residual "fines". At no time should the column be allowed to dry out. The column containing the activated carbon was autoclaved again (121 C, 15 minutes).

2.8.3 Changing the activated carbon column

To change the activated carbon column, during the biotransformation, the fluorobenzene supply was switched off while maintaining aeration. Any product remaining in solution was removed by continuing circulation through the column for a maximum of 1.5 hours (or until the influent fluorocatechol concentration was the same as the effluent).

Any cell suspension remaining in the column was first returned to the fermenter by reversing the recycle pump flow. The recycle pump to the column was switched off and the column changed. The new column was reconnected and the residual buffer displaced by the cell suspension before returning the effluent to the fermenter. The fluorobenzene supply to the bioreactor could then be restarted.

2.8.4 Extraction into butyl acetate

Activated carbon was removed from the column and 0.3L of deionised water was added. The suspension was mixed 220rpm, 30minutes, filtered, and was acidified using 2.5M H_2SO_4 to pH2 following a procedure adopted from SHELL (**Geary, personal communication**).

Butyl acetate was added (10% by volume) and mixing was continued for a further 4 hours. The two phases were separated and the fluorocatechol content of the organic phase established using GC.

CHAPTER III

BIOTRANSFORMATION CHARACTERIZATION LEADING TO BIOREACTOR DESIGN

3 BIOTRANSFORMATION CHARACTERIZATION LEADING TO BIOREACTOR DESIGN

3.1 Introduction

The microbial oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida* ML2 was chosen as a test reaction system to illustrate the benefits of approaching biotransformation process design in the structured manner detailed in Section 1.2.

The biotransformation process design framework adopted in this thesis is illustrated in Figure 1.1. An optimised biocatalyst production protocol would enable the characterization of the biotransformation to take place. This would lead to the identification of a number of process constraints such as substrate / product toxic or inhibitory levels, reaction kinetics and biocatalyst stability when exposed to different environmental conditions (e.g. temperature, pH and DOT).

These process constraints would then define process options which could be evaluated, leading to a final process specification.

3.2 Results

3.2.1 Biocatalyst production and preparation

3.2.1.1 Fermentation and harvest

A typical fermentation (DOT) profile is illustrated in Figure 3.1. Maintenance, growth and harvest details of the organism are described in Section 2.2, 2.3 and 2.4.

Fructose was chosen as the fermentation carbon source as it did not result in repression of the complex dioxygenase enzyme system responsible for the biotransformation of fluorobenzene to fluorocatechol. The growth medium was designed based on the elemental compostion of *Pseudomonas putida* ML2.

A standardised reproducible fermentation protocol was developed which produced a final biomass concentration of 10g dry wt/ L. This equated to a cell yield of 0.4g dry wt/ g fructose.

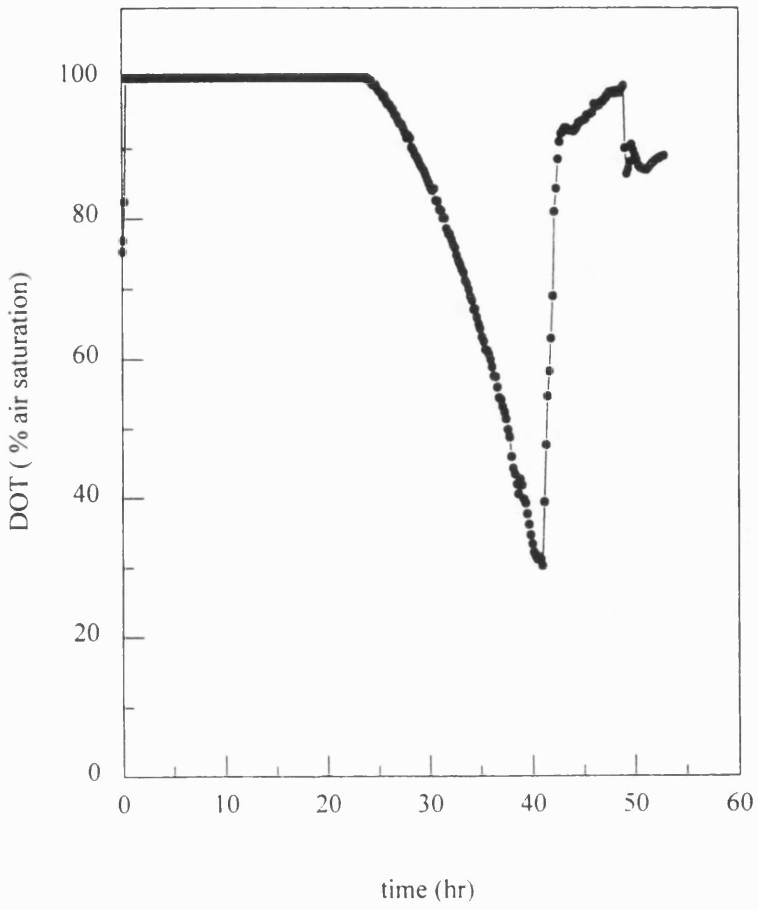

Figure 3.1 Fermentation DOT profile

3.2.2 Biotransformation assay development

3.2.2.1 Biotransformation rate assay

Shake flask biotransformations were carried out (500mL shake flask, 100mL working volume) to develop an activity assay. Biotransformations were carried out at a particular fluorobenzene concentration for a range of cell concentrations. Subsequently, biotransformations were carried out over a range of fluorobenzene concentrations for a particular cell concentration.

The purpose of this work was to develop an assay free from substrate and product inhibition capable of accurately measuring biocatalyst activity levels. Figure 3.2 illustrates a typical biotransformation profile using 3g dry wt/ L and a fluorobenzene concentration of 5mM.

When fluorobenzene was introduced to the cell suspension, fluorocatechol was produced. Samples taken every fifteen minutes were analysed using GC under conditions described in Section 2.5.2.

The total time for the biotransformation was 60 minutes. The maximum activity obtained was 0.2g FC/ g cell dry wt/ hr. Biotransformations with activities less than this implied that the cells were operating at a sub optimal level and were probably exposed to inhibitory or toxic influences within the reaction.

Figure 3.3 shows the results of a series of biotransformation rate assays at a range of cell concentrations from 1.5 to 6g dry cell wt/ L when no substrate or product toxicity or inhibition was observed.. The purpose of this work was to establish if the biotransformation rate assay was operating optimally, free from any inhibitory or toxic reaction conditions and provide a base for future characterization work.

Fluorocatechol produced after sixty minutes (g/ L) and the associated cellular activity (g FC/ g dry wt/ L) are both represented on this graph. The amount of fluorocatechol produced was proportional to cell concentration and the specific activity remained at 0.2g FC/ g dry wt/ hr. This profile suggests that the biotransformation rate assay was operating optimally and was capable of reproducibly reporting biocatalyst activity levels..

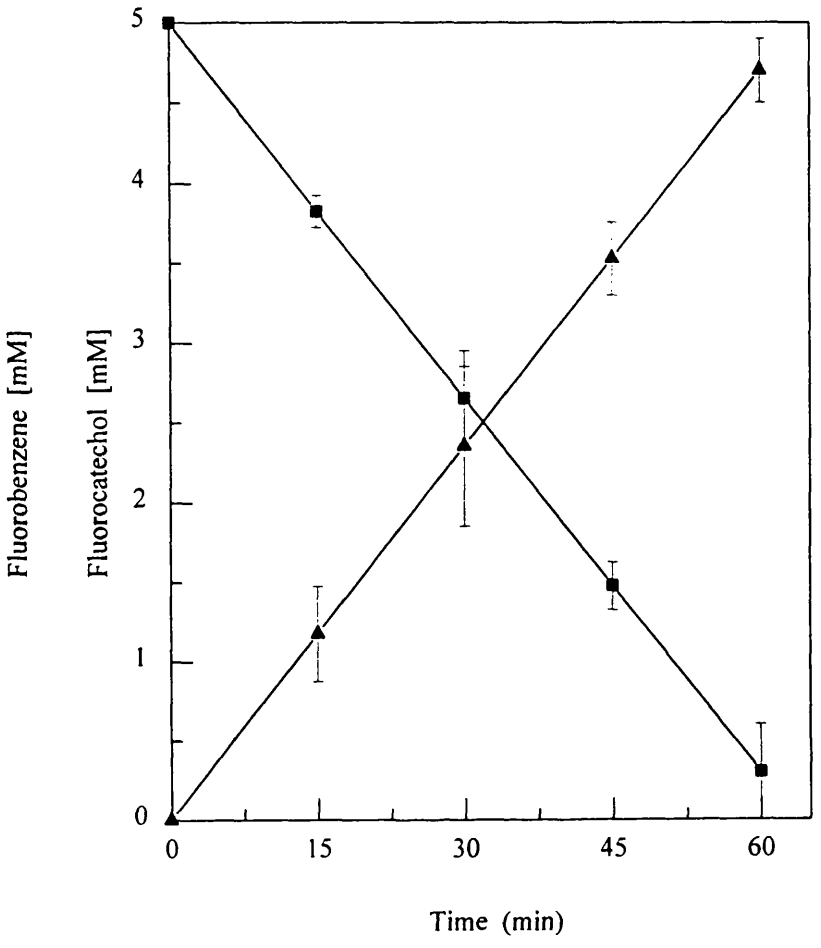

Figure 3.2 Biotransformation profile for 3g dry wt/ L

■ Fluorobenzene concentration

▲ Fluorocatechol concentration

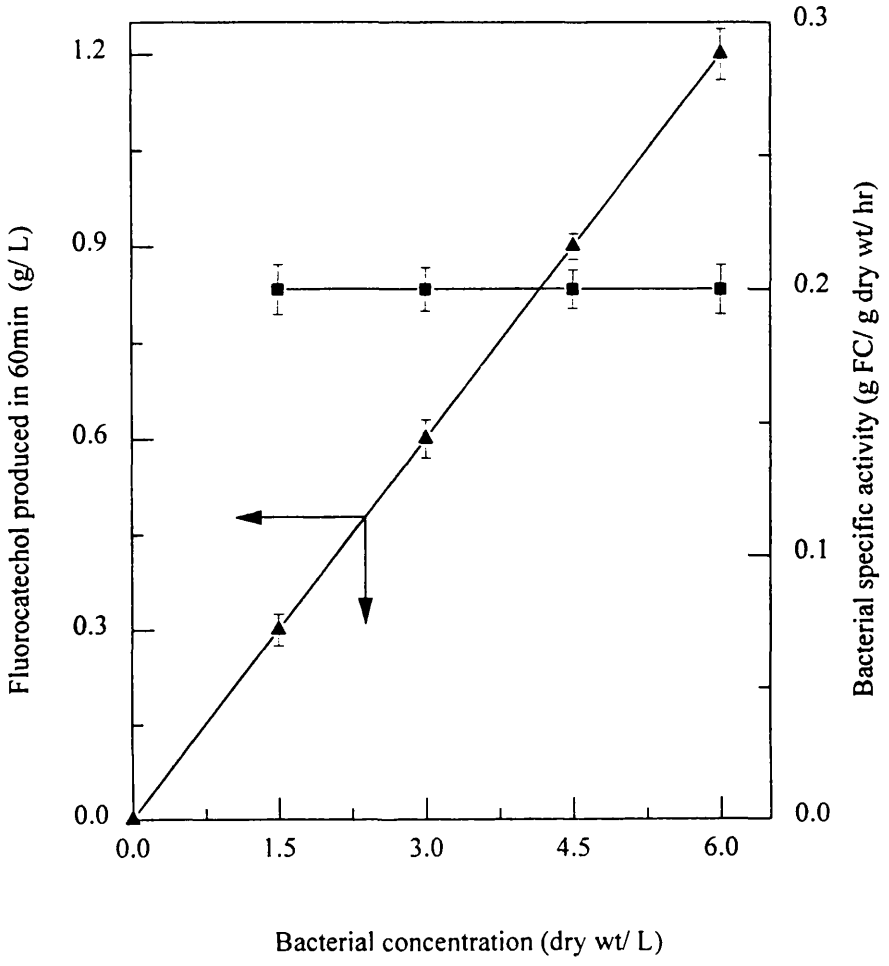

Figure 3.3 Biotransformation rate assays for 1.5 to 6g dry wt/ L

▲ Fluorocatechol produced

■ Specific activity

3.2.2.2 Fermentation biocatalytic activity profile

The fluorobenzene oxidation activity of *Pseudomonas putida* ML2 was measured, using the biotransformation rate assay, throughout the fermentation. A typical fermentation DOT, dry weight and specific activity profile is illustrated in Figure 3.4. After a 24 hour lag phase followed by a period of exponential growth the DOT progressively decreased and then immediately increased upon carbon source depletion.

Figure 3.5 illustrates the biotransformation rate assay profile throughout the growth phase of each of nine fermentations under conditions detailed in Section 2.3. Following the lag phase, the activity of the cells remained constant as the DOT decreased from 100%, (i.e. as growth continued) until 20% DOT. (Activity levels recorded below 30% DOT were achieved by increasing the fructose content from 25 to 30g/L, as detailed in Section 2.3)

The activity then rapidly and irreversibly decreased as growth continued, i.e. as the DOT further decreased to zero. To produce cells, therefore, with optimum biocatalytic activity (0.2 g FC/ g dry wt/ hr) it was necessary to ensure that the DOT did not fall below 20% during the fermentation.

Consequently, the carbon source content was designed so that growth in a "standard" fermentation was complete at 30% DOT.

3.2.2.3 Storage biocatalytic activity

Cells were harvested in the manner outlined in Section 2.4. Cell suspensions at 3g dry wt/ L were stored at 4 C and assayed for biocatalytic activity every 6 hours (Figure 3.6).

Pseudomonas putida ML2 retained 100% activity up to 30 hours after harvesting There was then a rapid progressive loss in biocatalytic activity. Cells could therefore be stored and used for a period of up to 30 hours after harvesting without any loss in biocatalyst activity.

3.2.3 Reaction characterization

3.2.3.1 Reactant / biocatalyst interaction characterization

Shake flask biotransformations were carried out (500ml, 100ml working volume) to establish the influence of a range of fluorobenzene concentrations on the biocatalytic activity of *Pseudomonas putida* ML2.

The biocatalytic activity of 3g dry wt/ L was measured at different fluorobenzene concentrations (0.05 to 1.4g/ L: Figure 3.7). Each point in Figure 3.7 represents an average specific activity over a sixty minute period.

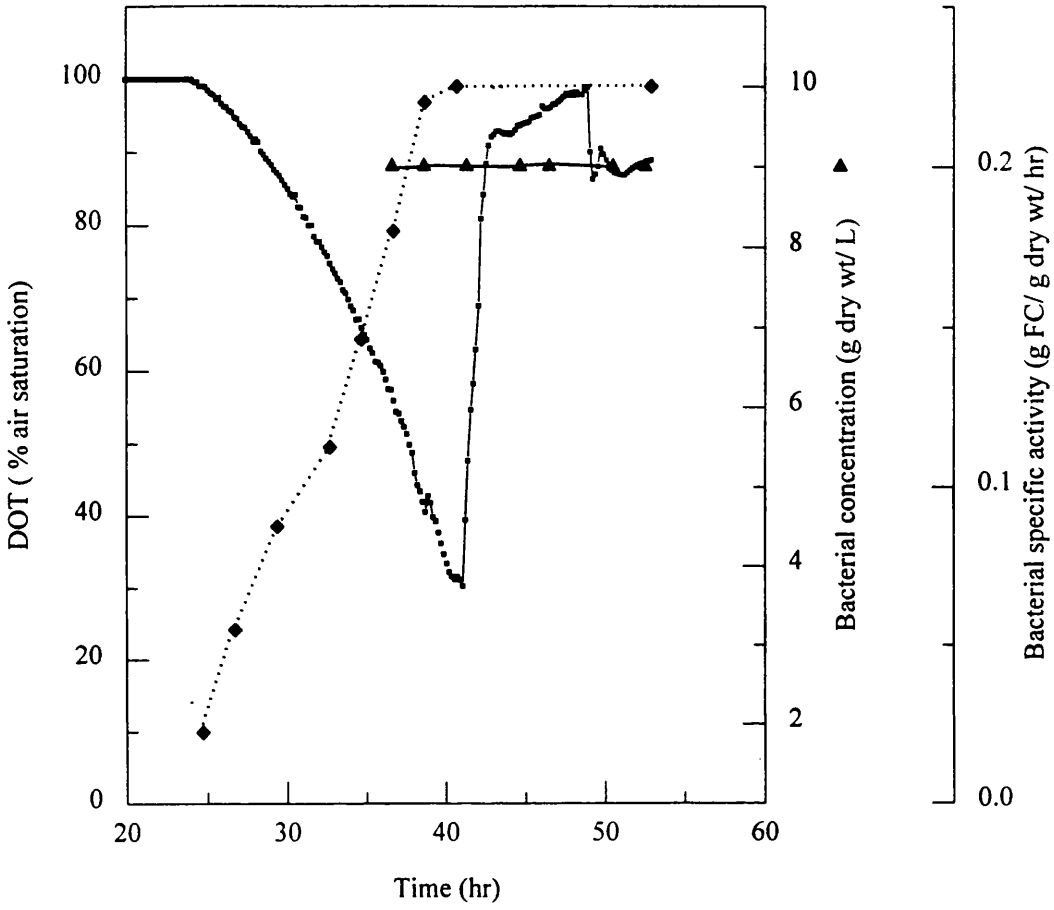

Figure 3.4 Cultivation of *Pseudomonas putida* ML2

■ DOT

♦ Bacterial concentration

▲ Specific activity

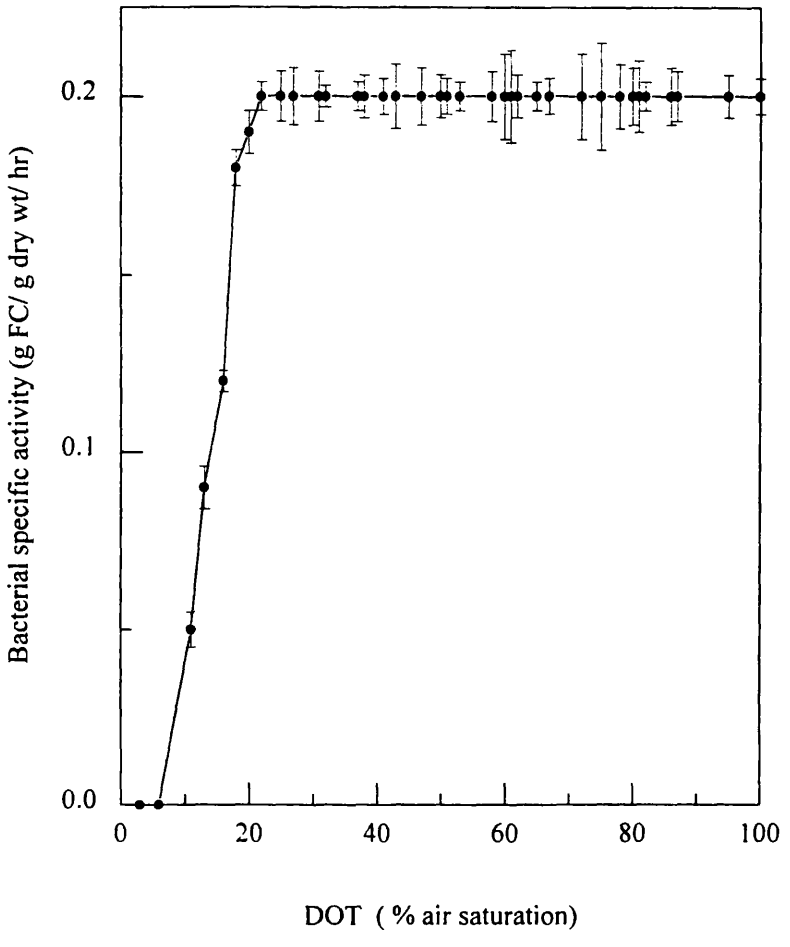

Figure 3.5 Fermentation specific activity profile

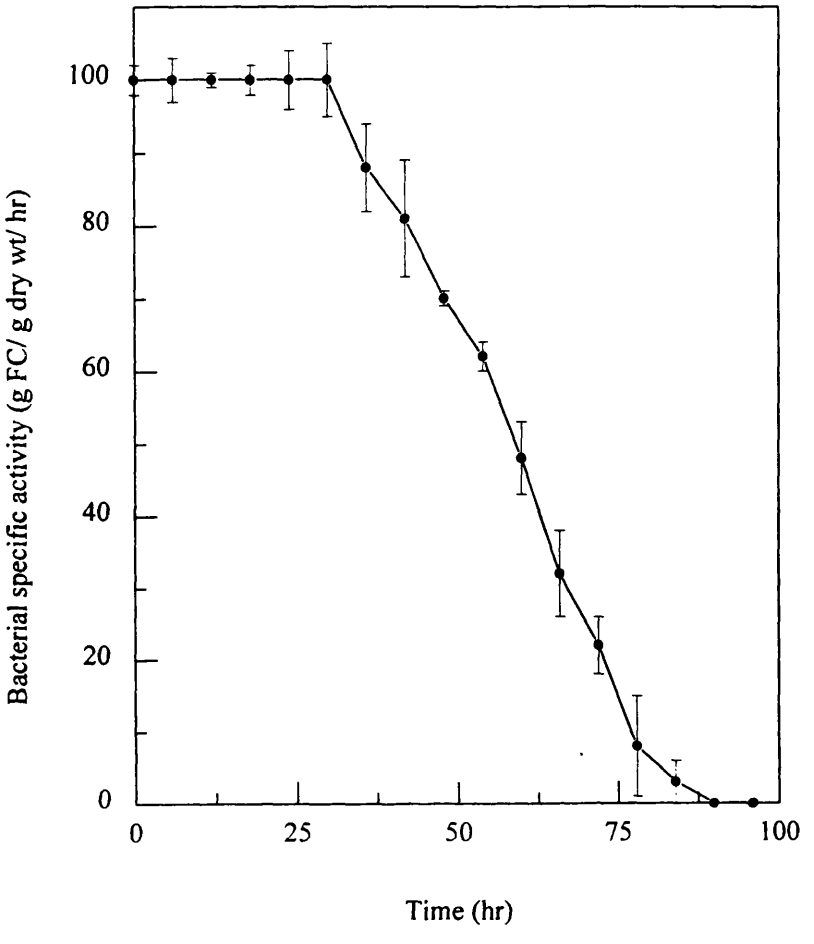

Figure 3.6 Storage specific activity profile

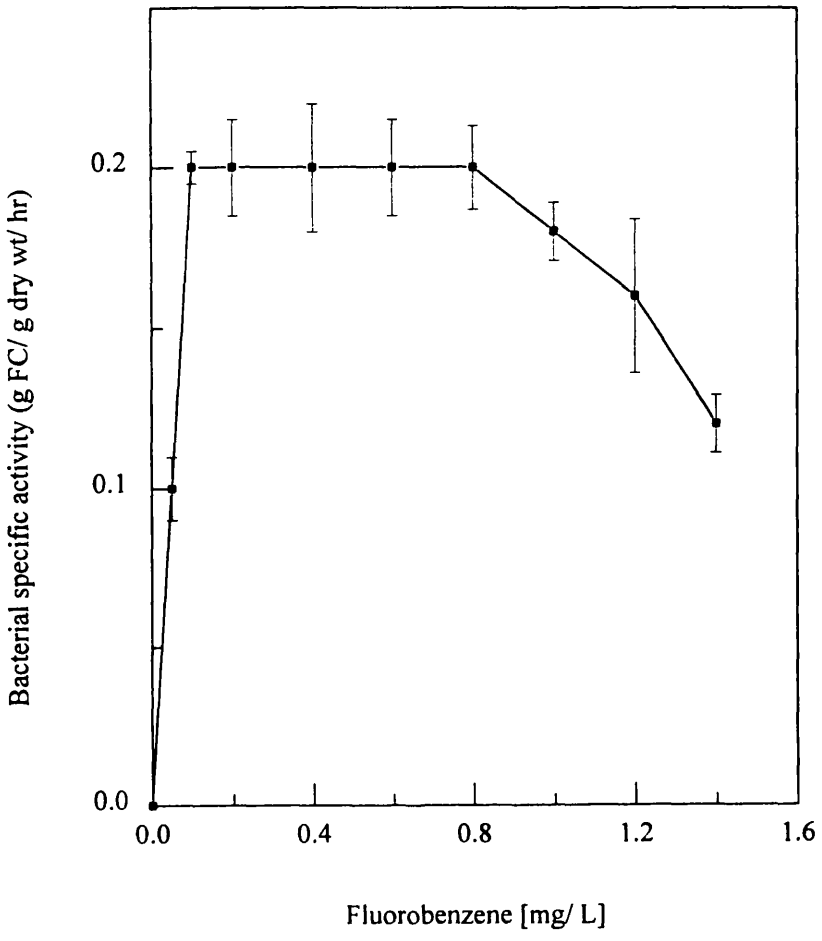

Figure 3.7 Effect of fluorobenzene on bacterial activity

As the fluorobenzene concentration was increased from 0.05 to 0.1g/ L there was a concomittant doubling in biocatalyst activity. This profile is an example of first order kinetics where the rate of reaction is directly proportional to the substrate concentration. As the fluorobenzene concentration was increased above 0.1g/ L there was no further change in biocatalyst activity, or rate of reaction, up to 0.8g/ L. Zero order kinetics, where the rate of reaction was independent of substrate concentration, was observed over this concentration range. Fluorobenzene concentrations at and above 0.8g/ L resulted in a reduction in biocatalytic activity implying that such fluorobenzene concentrations were either toxic or inhibitory to the biocatalyst.

A 3g dry wt/ L sample was then exposed to an initial fluorobenzene concentration of 0.4g/ L (Figure 3.8). The fluorobenzene concentration was gradually increased (up to 1.6g/ L) by introducing fluorobenzene through the Subaseal using a Hamilton syringe. Samples were analysed, *at line*, using the biotransformation rate assay.

The fluorobenzene concentration was subsequently decreased by removing the Subaseal and allowing the volatile fluorobenzene to vapourize, while continuing to assess biocatalytic activity using the *at line* biotransformation rate assay.

Biocatalyst activity remained constant at 0.2g FC/ g dry wt/ hr up to a fluorobenzene concentration of 0.8g/ L. Further progressive increases in fluorobenzene concentration resulted in a reduction in biocatalytic activity. A reduction in fluorobenzene concentration to below 0.8g/L resulted in a concommittant recovery in activity levels to 0.2g FC/ g cell dry wt/ hr.

This work demonstrated that fluorobenzene was inhibitory to *Pseudomonas putida* ML2, causing reversible activity loss, above 0.8g/ L. If fluorobenzene concentrations increase above the inhibitory level, complete activity loss can be recovered by reducing the fluorobenzene concentration below 0.8g/ L again.

3.2.3.2 Product / biocatalyst interaction characterization

Biotransformations (3g dry wt/ L) were carried out with artificially increased fluorocatechol concentrations (up to 0.8g/ L).

After the biotransformations the cells were centrifuged and resuspended in phosphate buffer. Biotransformation rate assays established the residual activity levels (Figure 3.9).

At a fluorocatechol concentration of 0.2g/ L, activity decreased by 10% after 16 hours. As the fluorocatechol concentration was increased, biocatalyst activities decreased until at 0.8g/ L, cellular activity was reduced by 85% after 16 hours.

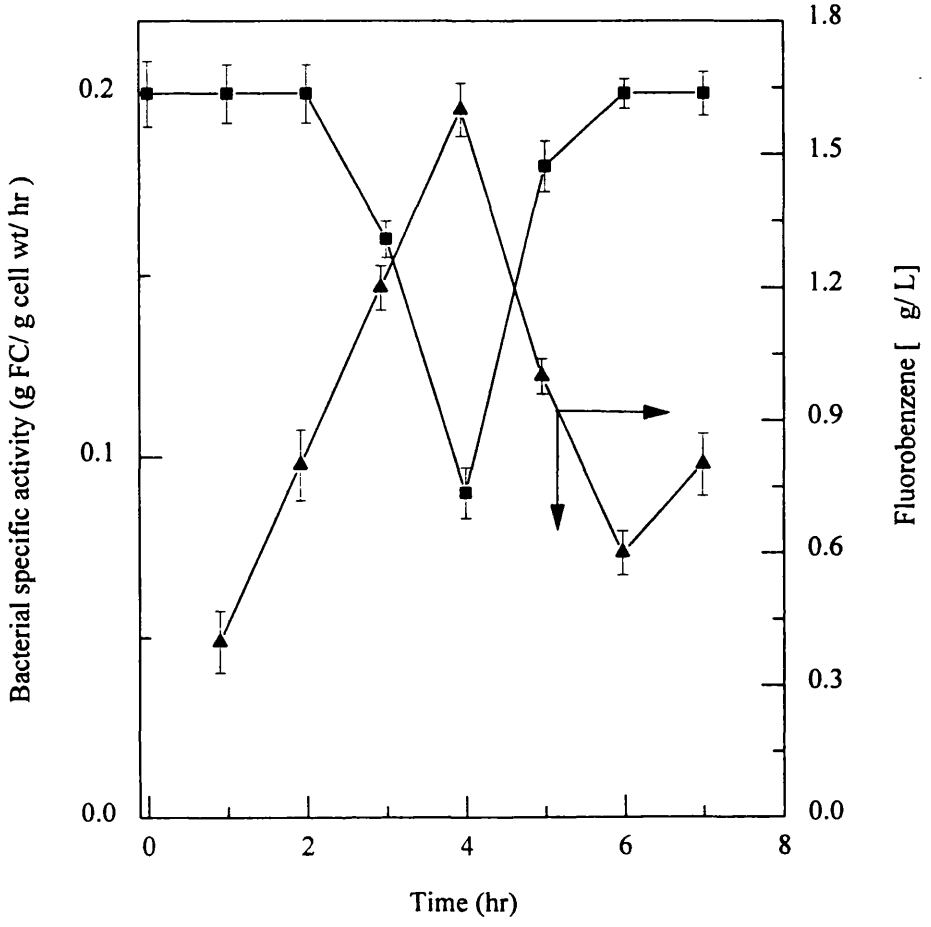

Figure 3.8 Substrate / Biocatalyst characterization

▲ Fluorobenzene concentration

■ Specific activity

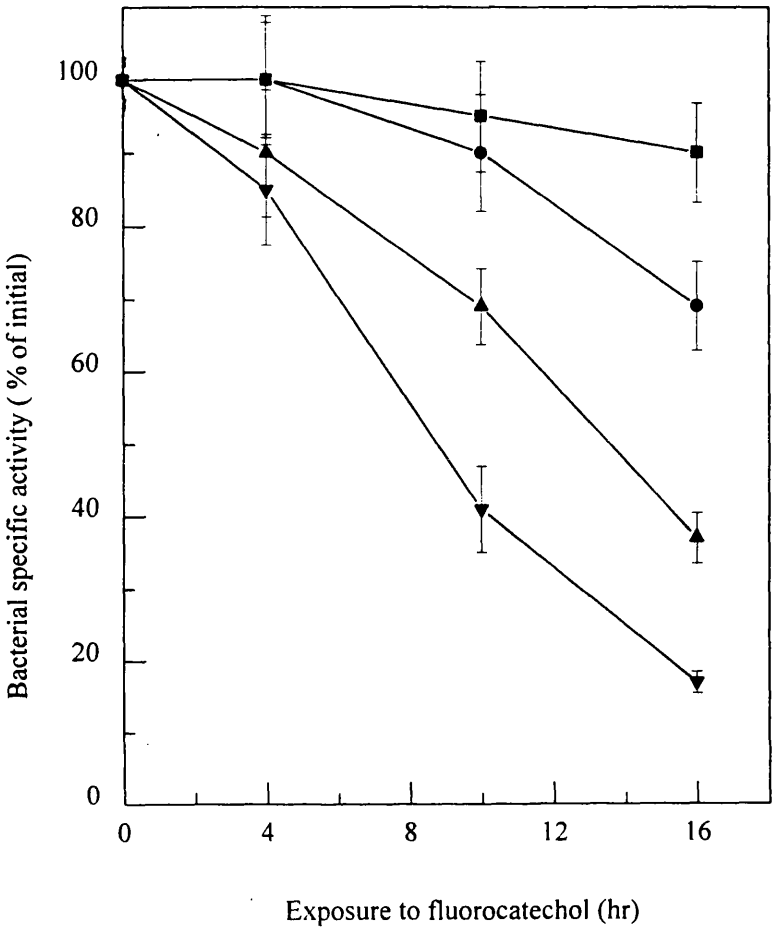

Figure 3.9 Product / Biocatalyst characterization

■ 0.2g/ L fluorocatechol

● 0.4g/ L fluorocatechol

▲ 0.6g/ L fluorocatechol

▼ 0.8g/ L fluorocatechol

This work demonstrated that fluorocatechol was toxic to *Pseudomonas putida* ML2, causing irreversible activity loss at levels of 0.2g/ L and above

3.2.4 Process constraints

The reaction characterization and associated biotransformation assay development work, described in Section 3.2.3, is summarised in Table 3.1.

Reaction component:	**Feature:**
Substrate:	Poorly aqueous soluble (1.52g/ L) Volatile (72mm Hg vapour pressure) Liquid at 25 C
Product:	Completely aqueous soluble Solid at 25 C
Biocatalyst:	Whole cell biotransformation
Biocatalyst:- Substrate kinetics:	First order (< 0.1g/ L) Zero order (0.1 < 0.8g/ L) Inhibitory (> 0.8g/ L)
Biocatalyst:- Product kinetics:	Toxic (>0.2g/ L)
Biotransformation:	Two step reaction Molecular oxygen required

Table 3.1 Biotransformation characterization summary

The most important issues addressed in process option development included that one of the substrates, fluorobenzene, was poorly aqueous soluble (solubility of 1.52g/L at 25 C), it was a liquid at 30 C (normal biotransformation operating temperature) but it was quite volatile with a vapour pressure of 72mmHg. First order kinetics were observed at low fluorobenzene concentrations (<0.1g/ L) above which zero order kinetics prevailed (0.1ú0.8g/L). Fluorobenzene was inhibitory at 0.8g/ L and above.

The product, fluorocatechol, was completely aqueous soluble and toxic to the biocatalyst at very low levels (0.2g/ L).

This core information defined a BPD flow sheet (Figure 1.3) which was used to develop a number of process options.

Substrate supply (for fluorobenzene) can be approached in many different ways (Section 1.2.5.2). Examples include vapour phase addition, use of a solvent (organic or aqueous), a solid adsorbent, membrane addition or, as in this case, continuous aqueous addition to the bioreactor ensuring that aqueous fluorobenzene concentrations do not exceed biocatalyst inhibitory levels (0.8g/ L).

The oxygen supply mode was required to supply oxygen to the biocatalyst at a rate sufficient to meet the biotransformation requirements of the biocatalyst (Section 1.2.5.3). It was also important, when deciding on a mode of oxygen supply, to take into consideration the volatility of fluorobenzene and employ a mode of oxygen supply which would minimise the loss through volatisation of fluorobenzene.

Since fluorocatechol was toxic at very low concentrations (0.2g/ L) it was necessary to develop a bioreactor design which would facilitate the continuous recovery of product. This meant that the two separate topics of bioreactor design and product recovery (Sections 1.2.5.1 and 1.2.5.4) would have to be approached in parallel rather than in isolation

The purpose of Figure 1.1 was to provide a structured approach to process option evaluation. The process option selection procedure is reported in 3.2.5 where emphasis was placed on an elimination process rather than the evaluation of all possible considerations.

3.2.5 Process options

3.2.5.1 Product recovery

0.2g/ L fluorocatechol was the maximum product concentration which would allow sustained cellular activity for prolonged periods of time (3.2.3.3). The biotransformation and bioreactor design needed to enable product removal to prevent the deleterious effects associated with the accumulation of fluorocatechol in solution.

Figure 3.10 illustrates a number of potential reactor configurations which satisfied the objectives of this search. All configurations are a variation of the stirred tank reactor (STR). For reasons already outlined in Section 1.2.5.1, the STR (or variations thereof) is still one of the most common bioreacter designs.

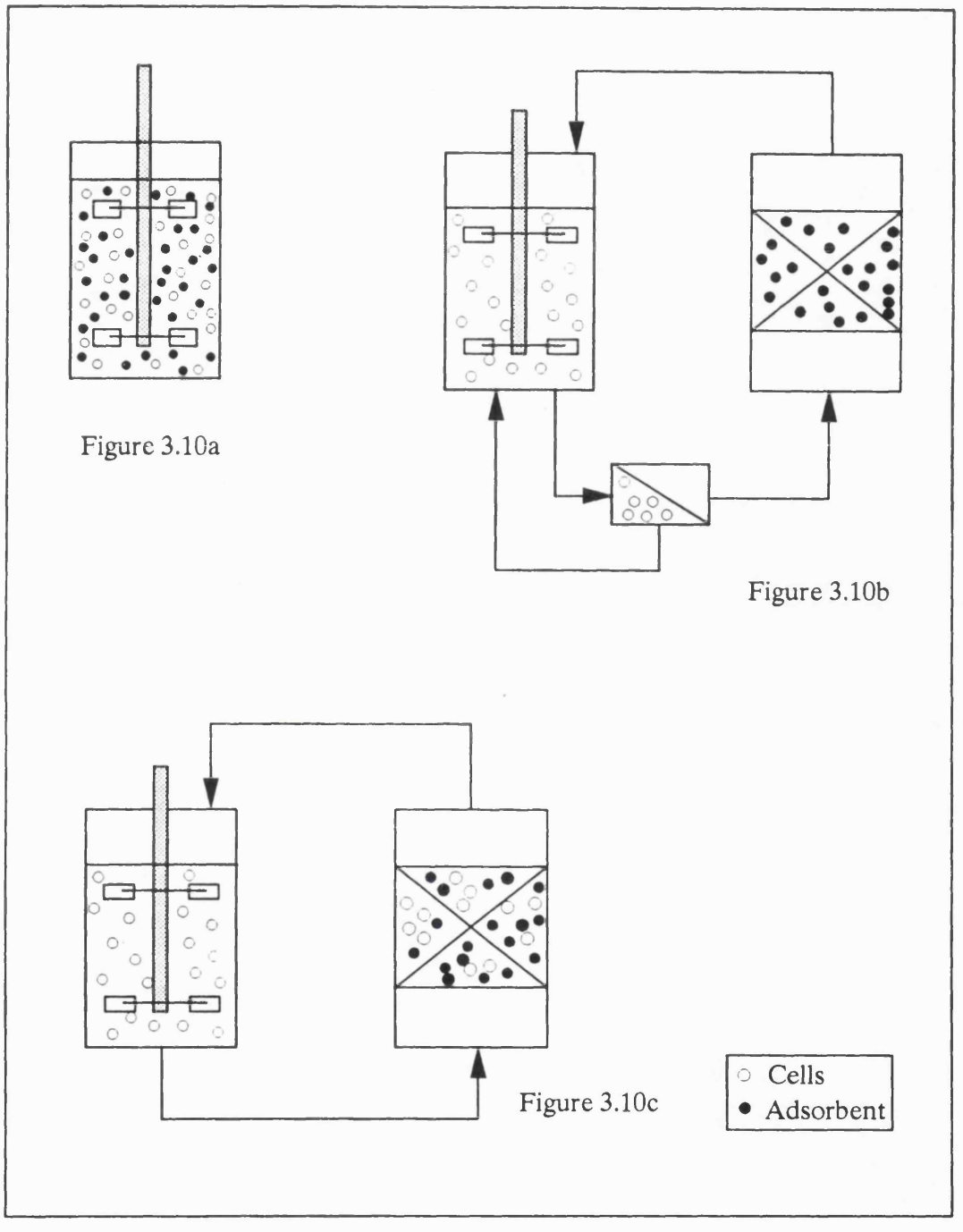

Figure 3.10 Reactor configurations for product recovery

Figure 3.10a illustrates *in situ* product removal where biocatalyst, adsorbent and substrates (fluorobenzene and oxygen) are contacted within the bioreactor. After the biotransformation the biocatalyst and adsorbent are separated from one another. Product is then recovered by extraction into a solvent (for example).

Potential disadvantages with this design include attrition and shear damage to the adsorbent causing it to fragment during biotransformation operation. This would increase its adsorption capacity (greater surface area) but cause difficulties in post biotransformation separation. If the adsorbent is not entirely product specific, then it may also non selectively bind with reactants and cell debris.

In Figure 3.10b, the biocatalyst and reactants are contacted in one vessel while the adsorbent is contained in a column. During the biotransformation, the product loaded cell suspension is pumped to a filtration or a centrifugation step.

The biocatalyst and product are separated and the cells are recycled back to the bioreactor to continue with the biotransformation. The product loaded liquid is pumped to the column where the adsorption process takes place. The product free liquid is then pumped back to the bioreactor from the column.

The two additional processing steps in this design can result in extra operating costs, shear effects and oxygen limitation effects, during the separation stage and pressure drops across the column necessitating high pumping forces.

Figure 3.10c details a compromise between the two designs already outlined. Substrate and biocatalyst are contacted in the bioreactor while the adsorbent is contained within the column. If the adsorbent is of a size large enough to allow the cells suspension to pass through the inter-particle void areas (with a relatively low pressure drop) then the reactor can be operated without a pre-column separation stage. The length of the column feed stream would have to be designed to prevent oxygen depletion during recirculation and also to promote maximum substrate conversion before entering the column (to minimise undesirable substrate adsorption).

It is evident that the choice of an adsorbent formed an integral part of this process option selection and design. The adsorbent would exploit the physical or chemical adsorption capacity of fluorocatechol (interacting with the catechol or benzene moiety). The actual adsorbent selection process was therefore important.

3.2.5.2 Adsorbent selection

A number of adsorbents were short-listed on the basis of chemical properties and their potential to remove (selectively) fluorocatechol from solution. These adsorbents included XAD resins (2, 4, 8, 16, 1180), Spherosil C and QMA, Duolite S861, Rohm and Haas A568, Amberlite IA35 and Norit pK13. These adsorbents derived their adsorptive properties from non-ionic hydrophobic interactions, macroreticular structure and pore size, surface area, cation and anion exchange and physical adsorption respectively.

These adsorbents were placed in contact with fluorocatechol and separately, a mixture of fluorocatechol and fluorobenzene to determine the adsorbent with the greatest affinity for fluorocatechol. The results are illustrated in Figure 3.11, which is a plot of C_X (the equilibrium solid phase concentration) for fluorocatechol in the absence and presence of fluorobenzene, against adsorbent type.

C_X = mass adsorbed onto adsorbent / mass of adsorbent

The larger the value of C_X, the greater its capacity to adsorb a material at a given concentration, mass of adsorbent and volume of liquid

$$C_X = V.DC/ x$$

where:

V: volume of sample

DC: difference from initial to final liquid concentration

x: mass of adsorbent

It was found that a range of adsorbents were suitable for adsorbing pure fluorocatechol and performed effeciently in removing it from solution. However, for a solution of fluorobenzene and fluorocatechol, fluorobenzene preferentially and competitively bound to most of the adsorbents, thus eliminating many of them from the selection procedure, only three candidates remained to choose from, namely; Rohm & Hass A568, Amberlite IA35 and activated carbon, Norit pK13.

The final adsorbent selection for column use was based on ease of regeneration, product removal, potential pressure drop across the column and biocompatability. Both the former resins were available in particle sizes in the range of 0.1 to 0.3mm which would have given rise to a large pressure drop in a column.

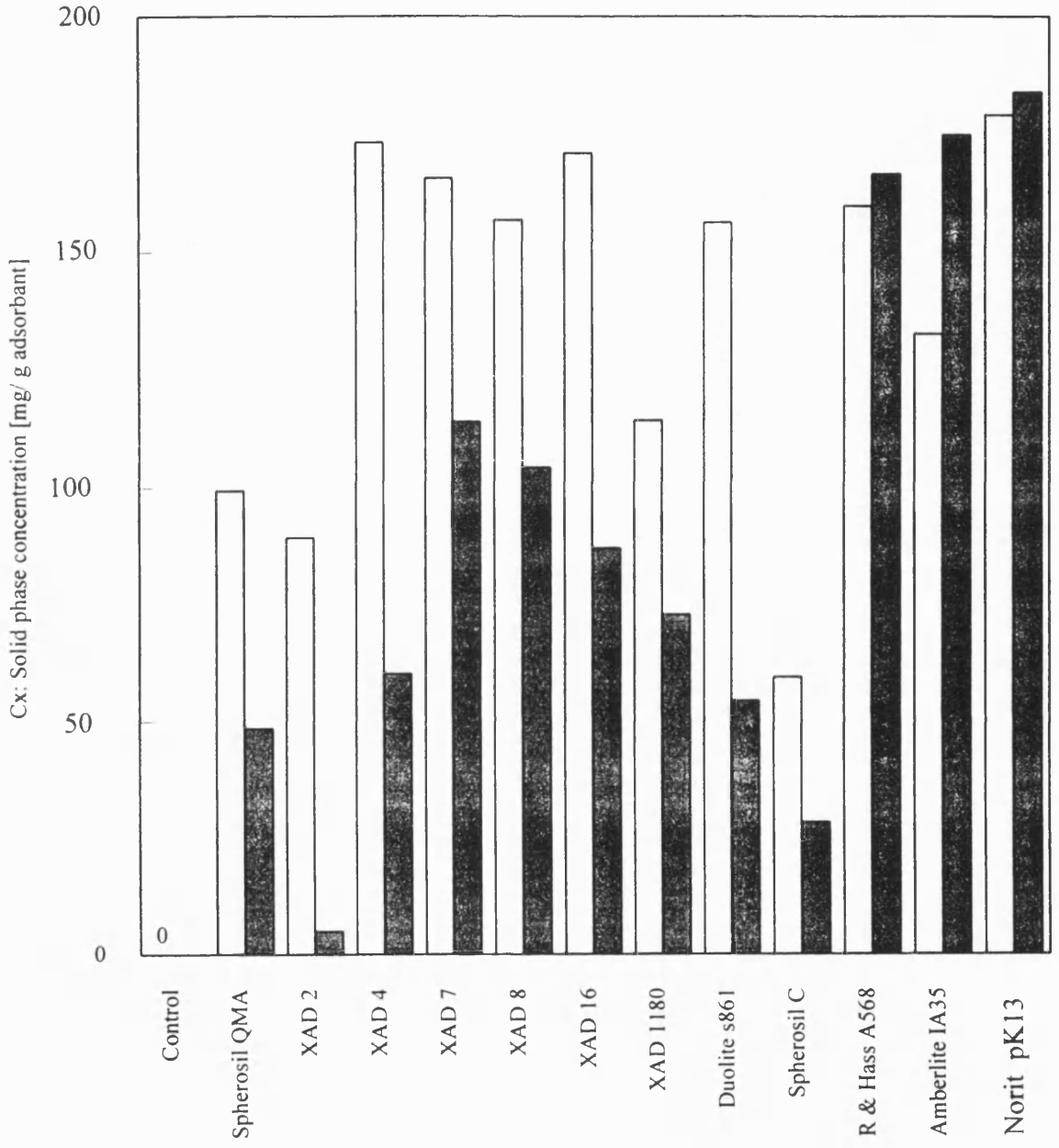

Figure 3.11 Adsorption characterization for different adsorbents

□ Fluorocatechol adsorption in the **absence** of fluorobenzene

■ Fluorocatechol adsorption in the **presence** of fluorobenzene

The size of Norit pK13 (1 to 3mm) was such that a whole cell suspension could be passed through the column without causing a significant pressure drop. It was also biocompatible and its high regeneration capacity meant that a product removal protocol could be developed. For these reasons, activated carbon (Norit pK13) was selected as the adsorbent material to be used in an external loop contactor similar to the one outlined in Figure 3.10c.

3.2.5.3 Equilibrium determination of fluorocatechol adsorption

It was necessary to model and simulate the adsorption of fluorocatechol onto Norit pK13 to predict process behaviour. The behaviour of a fixed bed adsorption column, such as that outlined in Figure 3.10c, depended on kinetic factors (film mass transfer coefficients, intraparticle effective diffusivity) and hydrodynamic factors (axial dispersion) with the most prominent being equilibrium factors. Experimental data were more accurately described by the general Fritz & Schundler isotherm equation **(Fritz & Schundler, 1974)**:

$$C_X = K_L C_L / (A+BC_L)^D \qquad (1)$$

where:

C_X: equilibrium solid phase concentration
K_L: adsorption equilibrium constant
C_L: equilibrium fluid phase concentration
A, B, D: constants

Many previous studies have been carried out to simplify equation (1) and facilitate the evaluation of the isotherm constants. These have resulted in many formulae, one of which is the Langmuir isotherm.

The Langmuir isotherm assumes an energetically homogeneous sorbent surface with equivalent sites, i.e. the energy of adsorption is constant for all sites. This results in monolayer adsorption on the sorbent surface and the formation of a plateau or a constant (saturation) sorbent capacity. Mathematically, the Langmuir isotherm is obtained by setting A=D=1 in equation (1) or:

$$C_X = K_L C_L / (1+a_L C_L) \qquad (2)$$

where:

a_L: saturation concentration
K_L: Langmuir equilibrium constant
C_X: equilibrium solid phase concentration
C_L: equilibrium fluid phase concentration

Equation (2) can be linearised to form:

$$1/C_X = a_L/K_L + 1/a_L C_L \qquad (3)$$

Hence, a plot of $1/C_X$ versus $1/C_L$ gives a straight line with a slope of $1/K_L$ and intercept of $1/a_L$. Figure 3.12 is a plot of C_X versus C_L for fluorocatechol using Norit pK13.. This plot was an accumulation of data from six different adsorption isotherms (i.e. exposing different fluorocatechol concentrations to a given mass of adsorbent and monitoring the reduction in aqueous fluorocatechol concentration i.e. its adsorption onto Norit pK13). The reciprocal plot for data in Figure 3.12 is illustrated in Figure 3.13.

The r^2 value (best fit line) for this plot was 0.997. Consequently, it was concluded that the adsorption of fluorocatechol onto activated carbon (Norit pK13) was best kinetically described by the Langmuir isotherm.

From this plot the saturation concentration (a_L) of fluorocatechol onto activated carbon was 0.183g/ g dry solid (i.e. Norit pK13 has a solid phase equilibrium saturation concentration of 0.183g/ g dry solid or approximately 0.2g fluorocatechol per gram of adsobent).

To verify that adsorption saturation concentration could be translated to continuous operation, a column was packed with 25g dry weight of Norit pK13 and 5.2g/ L fluorocatechol was pumped through the column. Its rate of adsorption was monitored (Figure 3.14). This figure plots the rate of decrease of fluorocatechol within the reactor solution with respect to time. The concomitant equilibrium solid phase concentration (C_X) increase is demonstrated in Figure 3.15.

The fluorocatechol concentration decreased rapidly in the first 40 minutes of operation resulting in a decrease in fluorocatechol concentration from 5.2g/ L to 0.2g/ L. The product loaded liquid continued to circulate through the column for 15 hours resulting in a final fluorocatechol concentration of 0.065g/ L.

From this information the adsorption saturation concentration of fluorocatechol onto activated carbon (Norit pK13) was calculated as 0.208g/ g dry solid. This value compared favourably with that predicted from batch adsorption experiments (0.182g/ g dry solid).

The column breakthrough concentration was calculated as 18.2% (w/w).

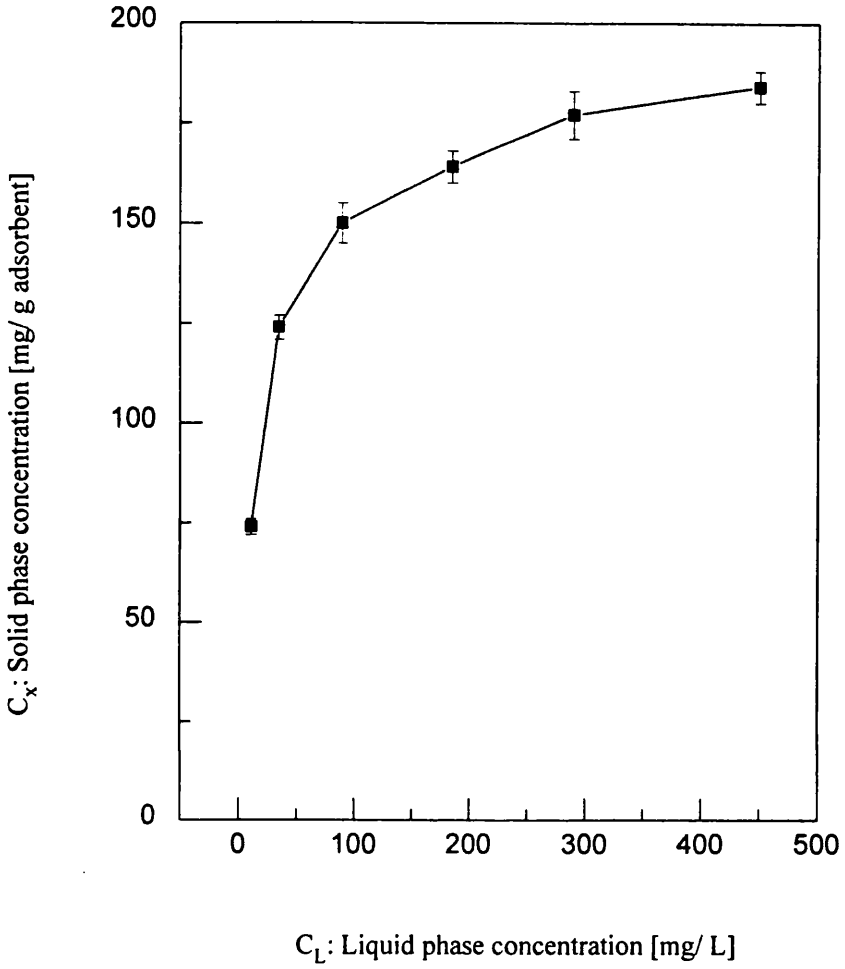

Figure 3.12 Adsorption of fluorocatechol onto Norit pK13

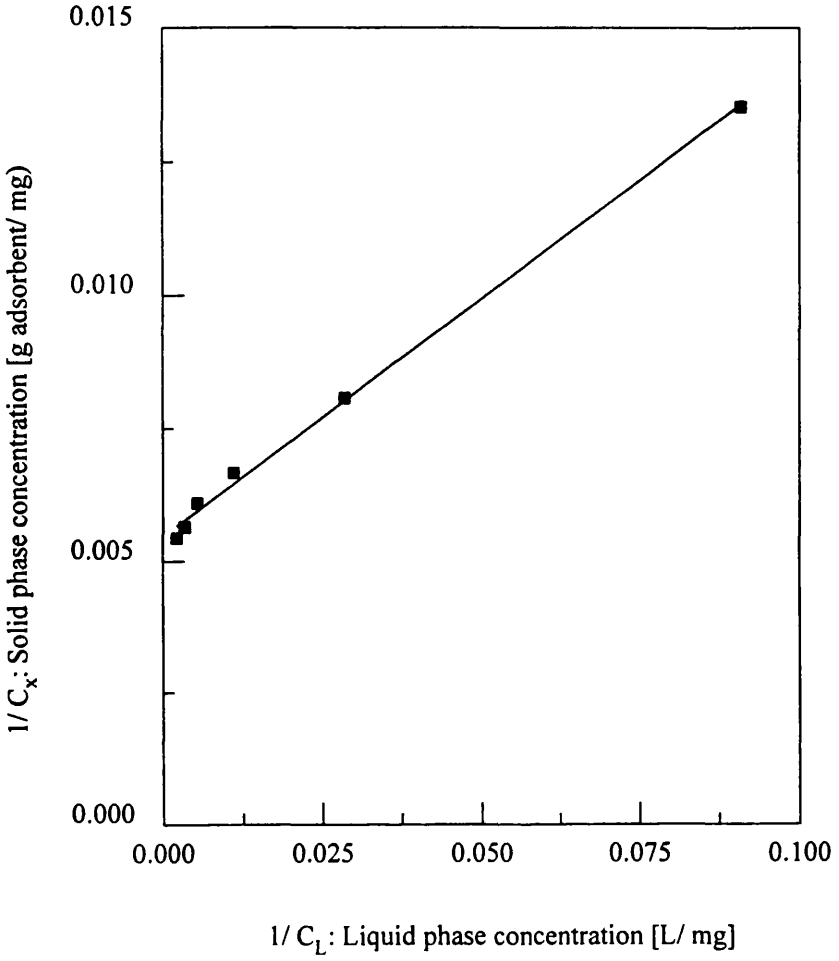

Figure 3.13 Langmuir adsorption plot

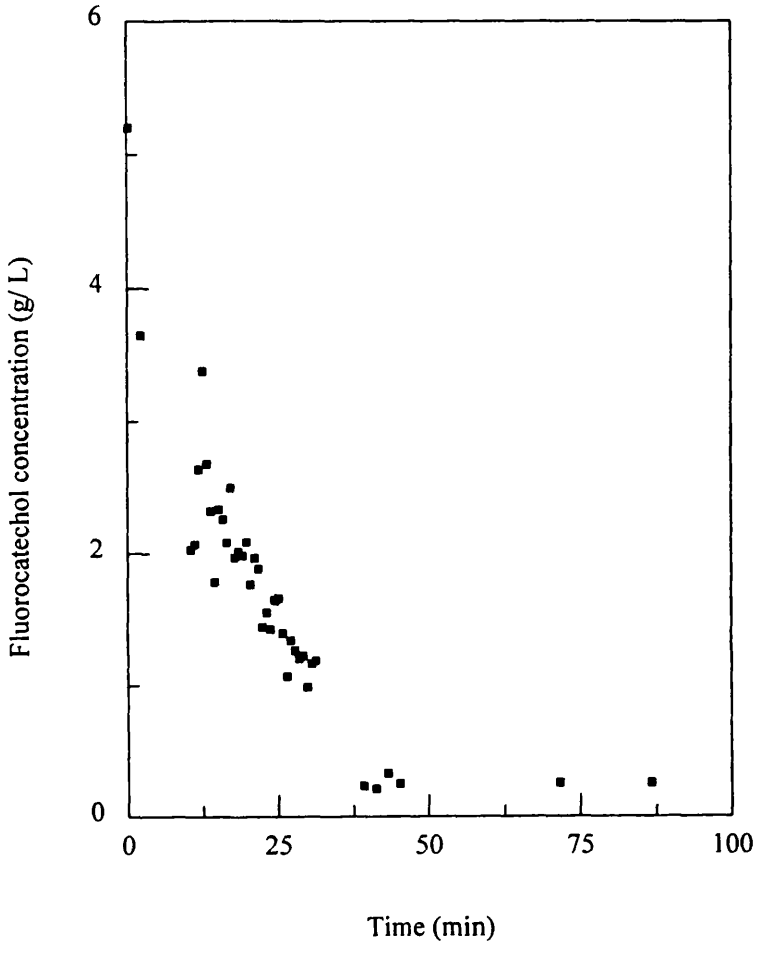

Figure 3.14 Flurorocatechol (liquid phase) adsorption onto a column containing Norit pK13

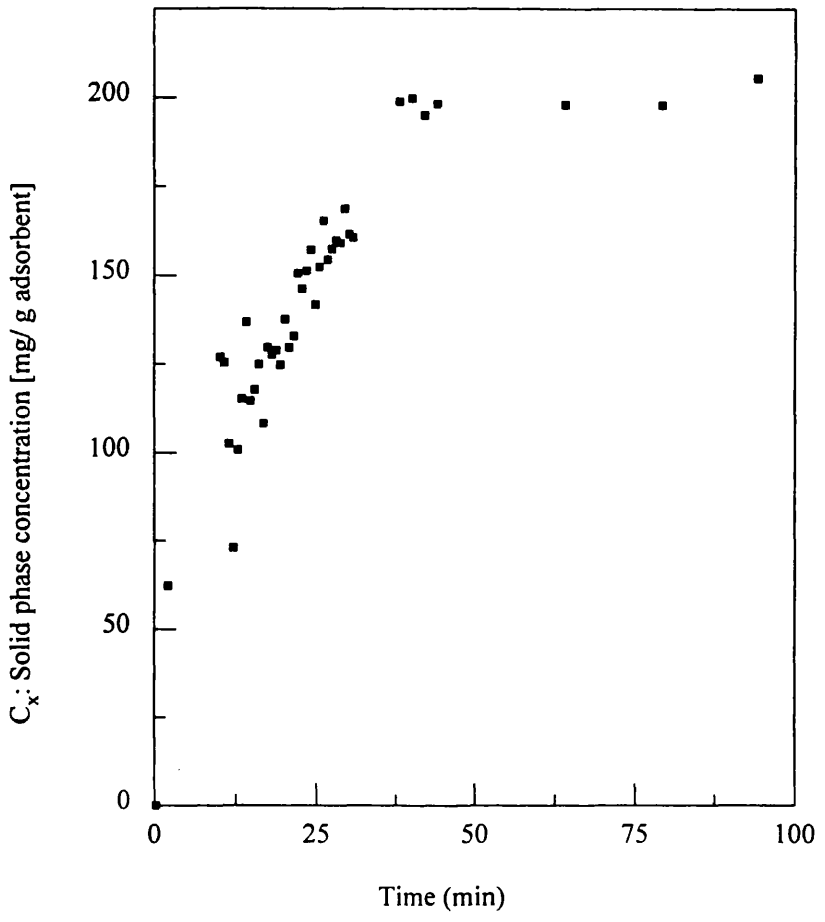

Figure 3.15 Flurorocatechol (solid phase) adsorption onto a column containing Norit pK13

3.2.6 Process option evaluation

3.2.6.1 Bioreactor design

The data contained in Figure 3.9 implied that two bioreactor configurations should be considered for biotransformation operation. The biotransformation could be operated, allowing fluorocatechol to accumulate above 0.8g/ L (without any product recovery) until the biotransformation terminated with complete activity loss.

Alternatively, if the fluorocatechol concentration could be maintained below 0.2g/ L, using continuous product recovery, the biotransformation could be operated over a prolonged period of time without activity loss (at least 16 hours). This would compare favourably with total activity loss in the former mode of operation.

The issue of fluorobenzene inhibition could be addressed by controlling the feed rate of fluorobenzene to the biotransformation, i.e. continuously monitoring by GC to maintain sub-inhibitory levels. This was not a difficult task to accomplish.

A particular challenge arose in addressing fluorocatechol toxicity. Work focused on developing a means of continuous recovery of fluorocatechol from the bioreactor medium using activated carbon (Norit pK13). Activated carbon was chosen for the reasons outlined in Section 3.2.5.2.

A process configuration was developed (Figure 3.16) where biocatalyst and substrate were contacted in the bioreactor while the adsorbent (loosely packed in a column) was connected in an external loop.

During the biotransformation, the product loaded cell suspension was pumped from the bioreactor to the column containing the activated carbon. The loosely packed activated carbon particles (1mm to 3mm) resulted in a porous packed bed which allowed passage of the cell suspension through the column without any significant pressure drop. The fluorocatechol adsorbed onto the activated carbon and the product free stream containing the cells was pumped back to the bioreactor.

The technical feasibility of this process design procedure has been demonstrated for fluorocatechol recovery at 2 litre laboratory scale biotransformations.

The continuous removal of product minimised the risk of further biological or chemical oxidation leading to a loss of yield, unlike the situation in a batch process.

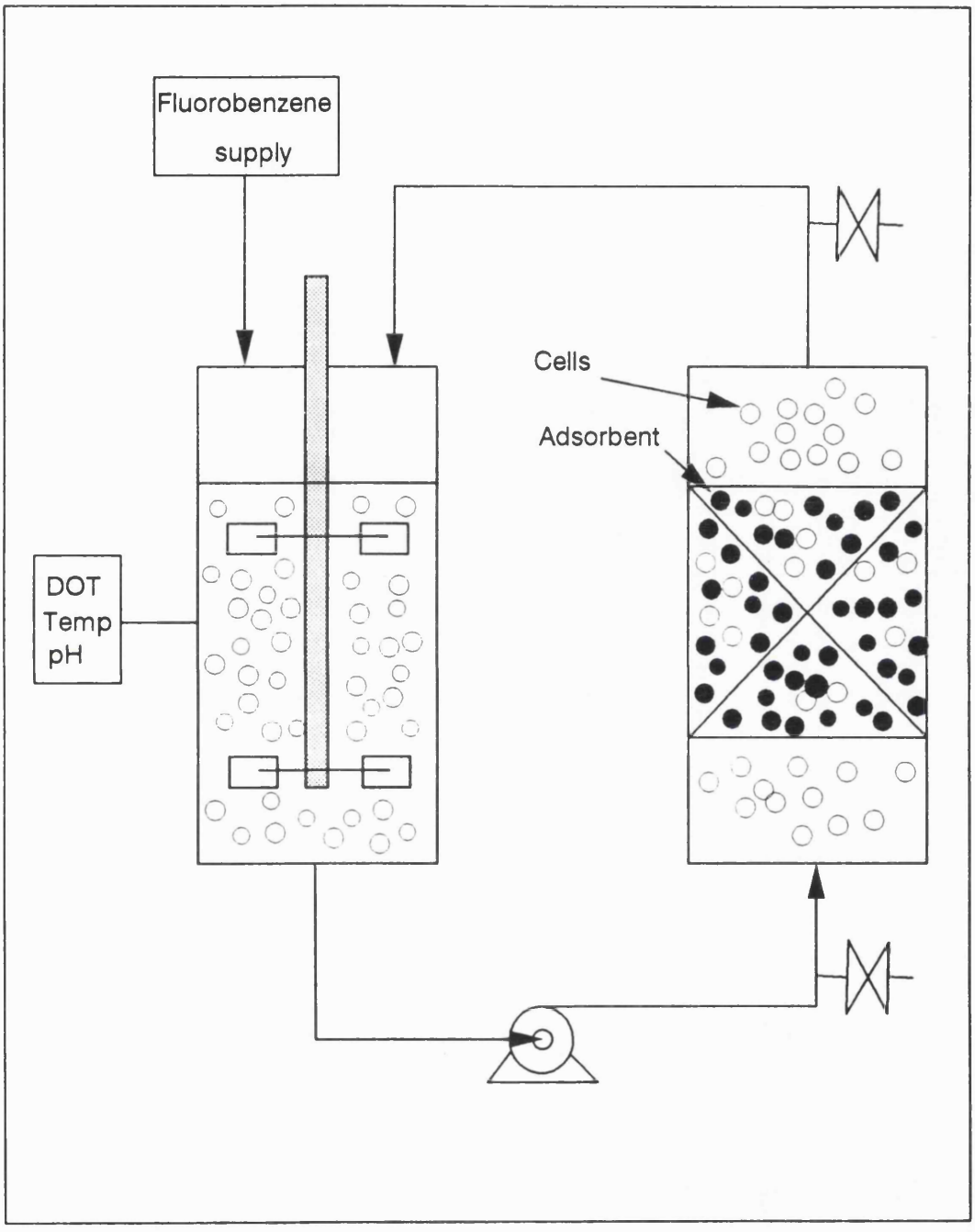

Figure 3.16 Bioreactor configuration for prolonged biotransformations

Figure 3.17 plots DOT, cellular productivity and aqueous dissolved fluorobenzene concentration against time. When the fluorobenzene pump was switched on it pumped a 50% (by volume) solution of fluorobenzene and ethanol to the bioreactor at a rate of 0.0132 L/ hr. The DOT began to decrease reaching a level of 63% after 1.2 hours. The DOT remained constant at this value for 11.9 hours.

After 11.4 hours, the fluorobenzene pump was switched off. 0.5 hours later, the DOT began to increase again to 71%. This increase was associated with a concomitant reduction and subsequent decrease in the fluorobenzene level to zero. The DOT slowly continued to rise to a final value of 95%.

Once the fluorobenzene pump was switched on, samples were taken from the inlet to (C_i), and outlet from (C_o) the activated carbon column every 0.25 hours. These samples were analysed by GC for fluorobenzene and fluorocatechol.

One of the recorded fluorocatechol concentrations in the stream leading to the column inlet was 1.32g/ L. Because it was a well mixed system, 1.32g/ L was also the bulk bioreactor fluorocatechol concentration.

The outlet fluorocatechol concentration from the column was 0.046g/ L.

The amount adsorbed by the activated carbon in the column was therefore 0.086g/ L

The recycle flowrate to the column was 25.8L/ hr.

Therefore, ((0.086g/ L)(25.8L/ hr)) = 2.22g/ hr was adsorbed on the column.

At 6g dry wt/ L, operating volume of 2L, the average productivity was (2.22/12) 0.185g fluorocatechol/ g dry wt/ hr.

At the end of the biotransformation samples taken indicated that column breakthrough did not occur ($C_i > C_o$) illustrating that the column was still functioning efficiently in removing fluorocatechol from solution.

When the activated carbon was removed from the column, butyl acetate (10% v/v) was used to extract the fluorocatechol as detailed in Section 2.8.4. GC analysis determined the quantity of fluorocatechol adsorbed.

At an average productivity of 0.185 g FC/ g dry wt/ hr, for 12 g biocatalyst and an operating time of 11.9 hours, the total fluorocatechol mass adsorbed was 26.42 g (as evaluated by GC). The total mass adsorbed, as measured by solvent extraction into butyl acetate, was 22.4g.

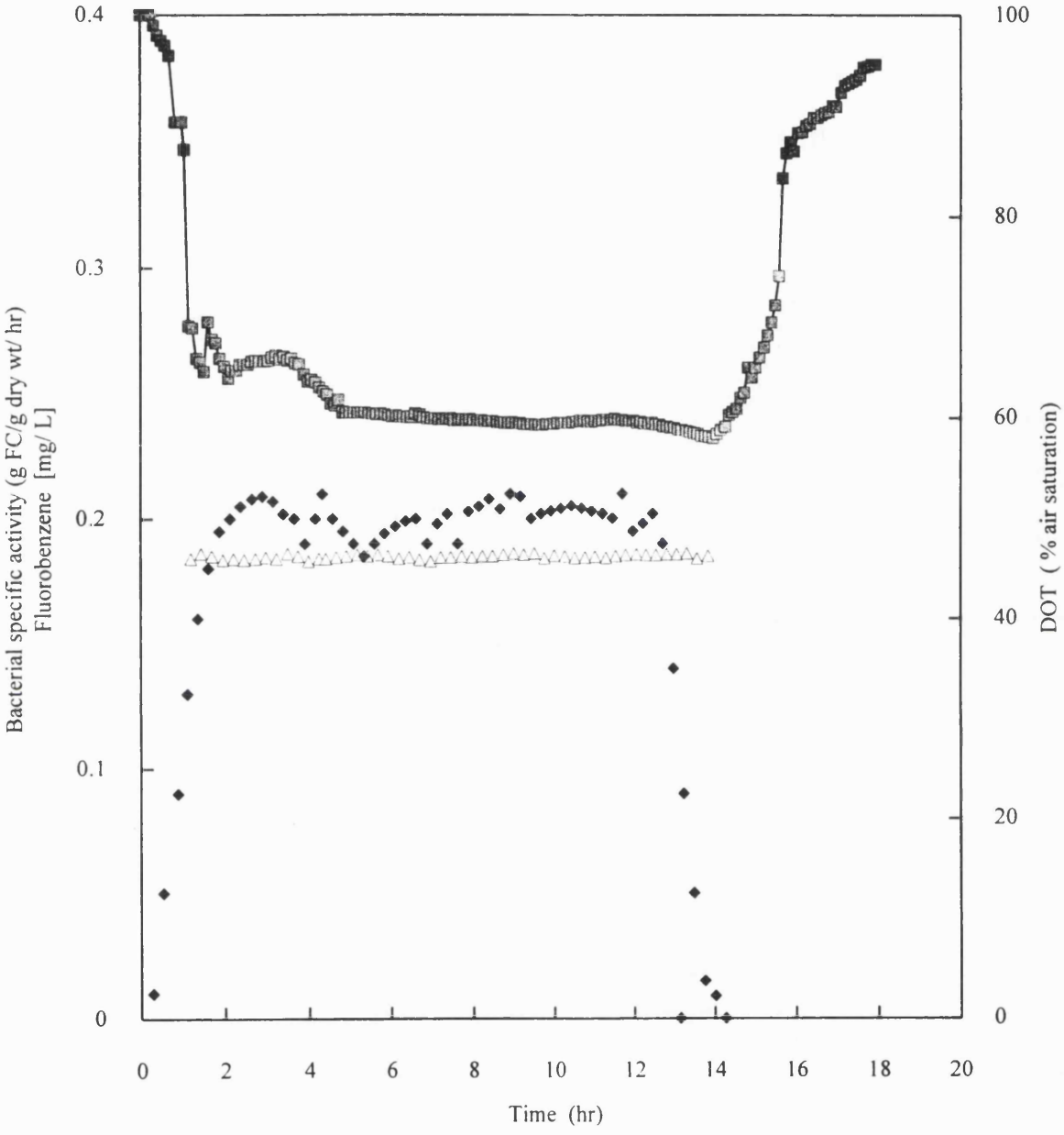

Figure 3.17 Biotransformation profile for an air aerated STR (6g dry wt/ L)

■ DOT

◆ Fluorobenzene concentration

Δ Specific activity

This level of productivity endorses the configuration detailed in Figure 3.16. Using this bioreactor design it was possible to achieve a productivity which was almost 70 times the natural biocatalyst tolerance to fluorocatechol.

The medium was checked for contamination by carrying out plate counts on samples taken from the biotransformation. The optical density of the biotransformation media had been routinely checked during operation to establish if contamination had taken place. The absence of foreign colonies and a negligible optical density increase inferred that the biotransformation had not been contaminated.

Cells taken at the end of the biotransformation had an activity of 0.19g FC/ g dry wt/ hr. (The average productivity recorded throughout the biotransformation was 0.185g FC/ g dry wt/ hr). These assay figures confirmed that the cells performed optimally with no loss of activity throughout the biotransformation.

It can therefore be concluded that the process configuration illustrated in Figure 3.16 represents a process configuration under which the uninhibited biotransformation of fluorobenzene to fluorocatechol using *Pseudomonas putida* ML2 could be carried out. This exemplifies the approach to biotransformation process design detailed in Section 1.2.

3.2.6.2 Increased biomass concentration (7g dry wt/ L)

Another biotransformation was carried out using 7g dry wt/ L to determine the effect of increased bacterial concentration. Figure 3.18 plots DOT, cellular productivity and aqueous dissolved fluorobenzene concentration against time. When the fluorobenzene pump was switched on, pumping a 50% (by volume) solution of fluorobenzene and ethanol into the bioreactor at a rate of 0.0132 L/ hr, the DOT began to decrease, reaching a level of 42% after 0.66 hours. The DOT remained constant at 42% for 12 hours, after which the fluorobenzene/ethanol solution pump was switched off. The DOT then began to increase to a final level of 93%.

The average productivity recorded during this biotransformation was 0.182g/ g dry wt/ hr. Column breakthrough did not occur. Extraction of the activated carbon into butyl acetate yielded a mass of 27.1g fluorocatechol. This compared with a mass of 30.58g as evaluated by GC.

No increase in biomass was observed. There was no evidence to suggest the presence of contaminating organisms.A sample of the cells was taken from the reactor at the end of the biotransformation and their biotransformation rate activity was assayed. The cells had an activity of 0.188 gFC/ g dry wt/ hr.

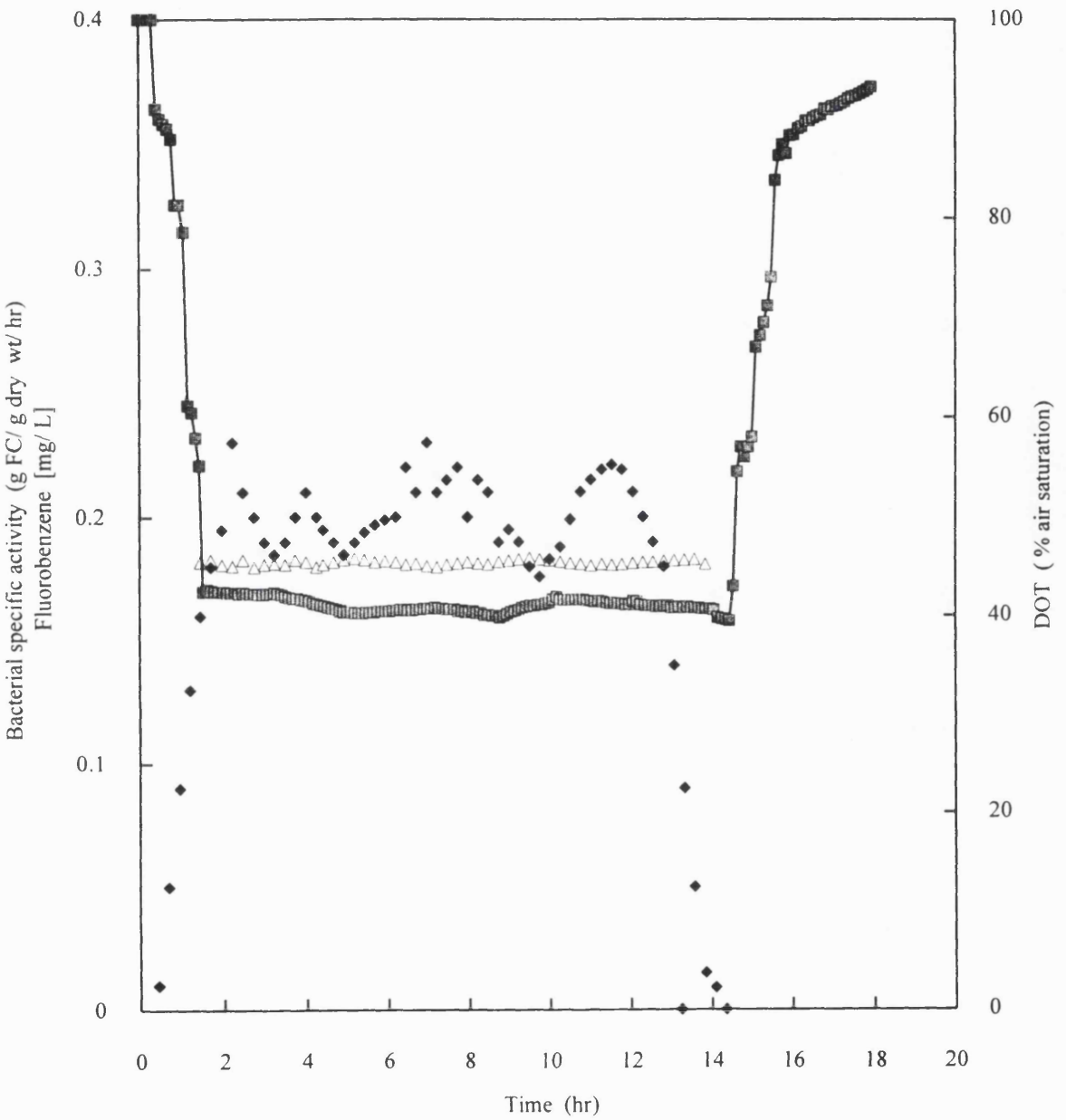

Figure 3.18 Biotransformation profile for an air aerated STR (7g dry wt/ L)

■ DOT

◆ Fluorobenzene concentration

Δ Specific activity

3.2.6.3 Increased biomass concentration (10g dry wt/ L)

Figures 3.19 and 3.20 plot DOT, cellular productivity and aqueous dissolved fluorobenzene concentration against time for two biotransformtions using 10g dry wt/ L. After the fluorobenzene pump was switched on (Figure 3.19) pumping a 50% (by volume) solution of fluorobenzene and ethanol into the bioreactor at a rate of 0.0132 L/ hr, the DOT began to decrease reaching a level of 94% after 0.26 hours when its rate of decrease slowed down.

After 2.33 hours the DOT resumed its more rapid decrease to a value of 36% after another 2.5 hours. It remained at this level for 2.4 hours before increasing to 33% DOT for 3 hours. It then rapidly increased again to 87% before finally finishing at 95%.

In Figure 3.20 the observations made were similar. One of the observed differences was that the period at 3.6% DOT lasted one hour longer. The plateau DOT observed was lower than that in Figure 21 representing a DOT of 11%.

In Figures 3.19 and 3.20 the fluorobenzene supply rate had to be reduced on several occasions to maintain a level of approximately 0.2g/ L within the bioreactor because of low productivity levels.

An average productivity of 0.0175 g FC/ g dry wt/ hr was recorded during the biotransformation phase. This rate is approximately 10% of the maximum biocatalyst activity levels previously observed.

Biotransformation rate assays were carried out on samples taken from both biotransformations. The cells had an average activity of 0.04gFC/g dry wt/hr (the maximum activity level is 0.2 gFC/g dry wt/hr). This activity rate suggested that the bocatalyst had lost most of its biocatalytic activity during these biotransformations.

The observation that the biocatalyst had lost its biocatalytic activity during both these biotransformations was consistent with results already reported in Section 3.2.2.2 where it was found that if the DOT decreased below 26% (during fermentation) an irreversible loss in biocatalytic activity was recorded. In both biotransformations the DOT decreased below 26% to a level of 3.6% for a prolonged period of time (2.5 to 3 hours). This reduction in DOT resulted in an irreversible loss in activity which accounted for the poor activity levels recorded.

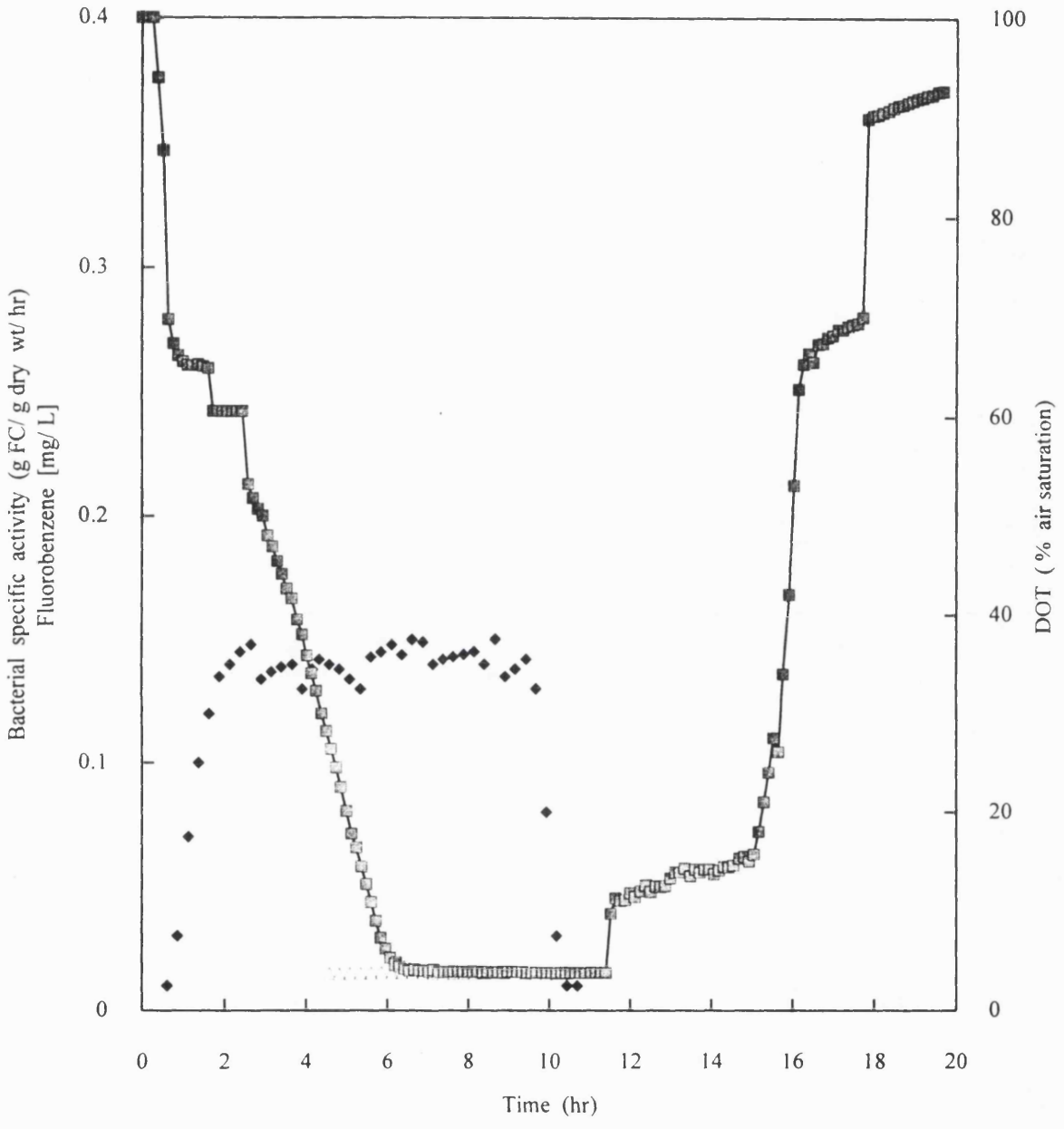

Figure 3.19 Biotransformation profile for an air aerated STR (10g dry wt/ L)

■ DOT

◆ Fluorobenzene concentration

Δ Specific activity

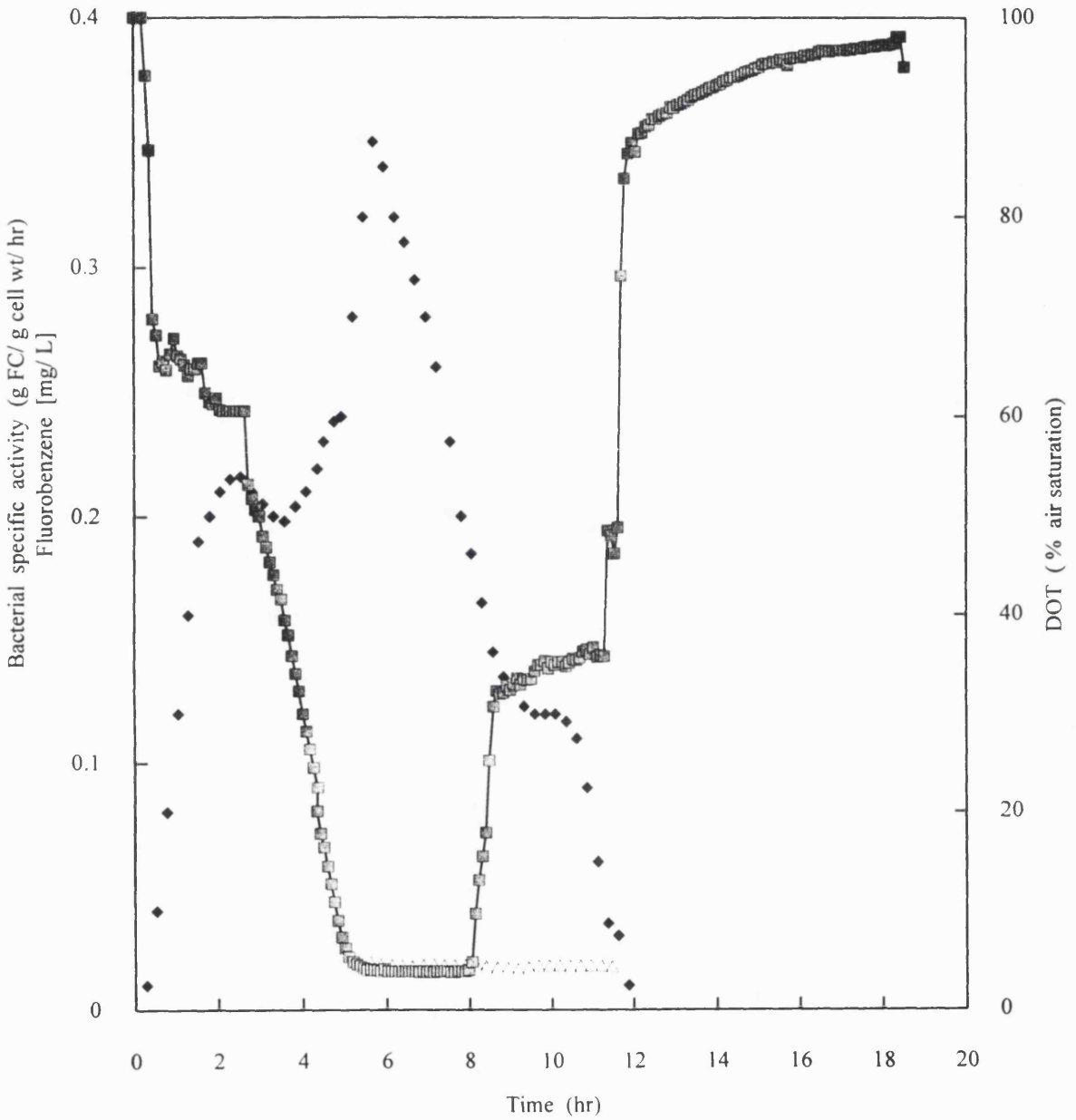

Figure 3.20 Biotransformation profile for an air aerated STR (10g dry wt/ L)

■ DOT

◆ Fluorobenzene concentration

Δ Specific activity

3.2.6.4 Increased biomass concentration (6, 7 and 10g dry wt/ L)

In most cases the difference between operating with 6, 7 and 10g dry cell wt/L would be negligible. However, as Figures 3.17, 3.18, 3.19 and 3.20 illustrate, there was a considerable difference between the DOT profiles under identical aeration conditions (2.5 vvm, 1100rpm). This suggested that operating at 7g dry wt/ L was close to the oxygen supply limit with the bioreactor configuration illustrated in Figure 3.16, whereas not enough oxygen (in the form of air) can be supplied to a biotransformation containing 10g dry wt/L.

3.3 DISCUSSION

In this thesis, the microbial oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida* ML2 was chosen to illustrate the benefits of approaching biotransformation process design in the structured manner detailed in Section 1.2. This approach was proposed by Woodley and Lilly (**Woodley and Lilly, 1994**).

By adopting a BPD framework (Figure 1.1) the biotransformation has been characterized, a number of process constraints have been established and process options defined, leading to the development of a bioreactor configuration capable of promoting optimal biotransformation operation.

A standardised fermentation protocol was developed for *Pseudomonas putida* ML2, resulting in a fermentation profile with a biocatalyst yield of 10g dry wt/ L.

Fermentation DOT was maintained in excess of 20% air saturation to prevent irreversible loss in biocatalytic activity. This irreversible loss in activity has previously been described by Geary and co-workers (**Gibson *et al*, 1970; Axcell and Geary, 1973; Crutcher and Geary, 1979; Geary *et al*, 1984; Whited *et al*, 1986; Zamanian and Mason, 1987**) and has been associated with the degradation of the iron sulphur subunits which constitute part of the complex dioxygenase enzyme system. This catabolic process was irreversible below certain DO levels. It has been established that this DOT level was 20%. The fermentation protocol was subsequently designed so that growth would terminate after 30% DOT.

Biocatalyst can be stored for up to 32 hours after harvesting without any loss in activity.

A biotransformation rate assay, unique to this biotransformation system has been developed. This assay was capable of accurately and reproducibly reporting activity levels for *Pseudomonas putida* ML2 independently of the biotransformation itself. Fluorobenzene was inhibitory to the biocatalyst, causing reversible activity loss above 0.8g/ L. If fluorobenzene concentrations exceeded this level, biocatalyst activity could be regained by reducing fluorobenzene levels to below 0.8g/ L.

As the substrate concentration was increased to 0.1g/ L, zero order kinetics were observed where there was an observed doubling in biocatalyst activity with substrate concentration. As the fluorobenzene concentration was progressively increased to 0.8g/ L, the biocatalytic activity was found to be independent of substrate concentration, indicating that zero order kinetics were observed. Above 0.8g/ L resulted in reversible inhibition.

Establishing a profile similar to that illustrated in Figure 3.7 was important since it was necessary to establish the maximum biocatalytic reaction rate in the absence of a second liquid phase (**Woodley, 1990**). Such profiles have been developed for a range of aromatic biotransformation systems and have proved necessary for optimal biotransformation operation (**Harrop *et al*, 1992; Hack, 1992; Brazier, 1989**). These authors have observed a similar sharp transition from first to zero order kinetics suggesting that it may be unique to aromatic biotransformaion systems.

The biotransformation product, fluorocatechol, was toxic to the biocatalyst at low levels (0.2g/ L). In an investigation into the effect of catechol on lipoxygenases, which are single subunit enzymes containing an iron-sulphur centre (similar to the multiple subunits in the complex dioxygenase system) irreversible inactivation of the lipoxygenases occurred through coordination of the ferric co-factor (**Galpin et al, 1976**) or by reduction of the catalytically active ferric enzyme to the catalytically inactive ferrous form (**Kemal *et al*, 1987**). It is possible that a similar mode of inactivation was responsible for disrupting the electron transfer from the cofactor (NADH) to the oxidation of the aromatic substrate.

Twelve different adsorbents have been evaluated for their capacity to adsorb fluorocatechol. Activated carbon, Norit pK13, was chosen since it was biocompatible, had a high regeneration capacity, was efficient at adsorbing fluorocatechol and was reasonably simple to extract product from, using a solvent (butyl acetate).

The adsorption equilibria of fluorocatechol onto Norit PK13 followed the Langmuir adsorption isotherm principle, i.e. monolayer adsorption. The maximum adsorption capacity of activated carbon, Norit pK13, was 1.83g fluorocatechol/ g dry adsorbent weight.

This characterization approach has been verified by the development and validation of a bioreactor design specification capable of carrying out the biotransformation under optimal conditions.

More recent authors have focussed on the need to characterize biotransformations resulting in reduced bioreactor sizes, increased biocatalytic activity and enhanced mass transfer conditions (**Zahradnik *et al*, 1985; Folsom and Chapman, 1991; Alvarez-cohen and Mc Carthy, 1991; Meyer, 1993; Holland *et al*, 1993**). General characterization work identified the selectivity of substrate processing in whole cell biotransformations based on the nature, position and size of substrate molecules, substituent side chains and sites of reaction.

Hardman (**Hardman, 1991**) reviewed the characterization of halogenated compound biotransformations with reference to biochemical characterization leading to physiological studies of their genetic and activity levels. Long (**Long and Ward, 1989**) has characterized the fermentation and toxic effects of substrates and products in the biotransformation of benzaldehyde by *Saccharomyces cerevisiae*. Robinson (**Robinson *et al.*, 1992**) characterized different strains of *Pseudomonas putida* with respect to the substrates they were capable of metabolizing and biotransforming.

None of the authors, however, have used the characterization information to design a biotransformation process capable of continuously carrying out their respective biotransformations in the manner exemplified in this thesis.

A wholly biocatalytic route to the formation of fluorocatehol and the techincal feasibility of a bioreactor configuration (Figure 3.16) has been demonstrated at 2L laboratory scale biotransformation (using 6g dry wt/ L).

This process configuration was capable of carrying out the biotransformation over a prolonged period of time and maintaining conditions of uninhibited biocatalytic activity within the bioreactor so that the biocatalyst could operate at an optimal level of productivity. Fluorocatechol produced by this means is up to 20% less expensive to produce than normal chemical methods (**Johnston *et al*, 1987**) inferring that the process may have commercial potential.

Not enough oxygen (in the form of air) could be supplied to the biotransformation, however, at high biocatalyst concentrations (10g dry wt/ L). Furthermore, this mode of oxygenation (air aerated STR) did not address the issue of substrate volatility either, resulting in a large loss (through volatization) of fluorobenzene from the bioreactor over prolonged continuous operation. Chapter IV will address these challenges while attempting to maintain optimum biocatalytic activity during prolonged operation.

CHAPTER IV

OXYGEN SUPPLY MODE EVALUATION FOR CONTINUOUS BIOTRANSFORMATION OPERATION

4 OXYGEN SUPPLY MODE EVALUATION FOR CONTINUOUS BIOTRANSFORMATION OPERATION

4.1 Introduction

4.1.1 Oxygen mass transfer dynamics

Providing an adequate oxygen supply for aerobic biotransformations is a critical requirement for optimum biocatalytic activity. Unfortunately, oxygen mass transfer is often a major limiting factor because of oxygen's low solubility in an aqueous environment (approximately 1.26mmol/ L at ambient temperature and pressure).

The measured oxygen transfer rate (OTR), taken as the difference between the oxygen consumed by the microbial biomass and the amount being transferred to the liquid phase is given by the following equation:

$$OTR = K_L A(C^* - C) - q O_2$$

where:

$K_L A$ = oxygen mass transfer coefficient (hr^{-1})

qO_2 = The measured microbial oxygen uptake rate

C^* = Dissolved oxygen concentration in equilibrium with the gas phase

C = bulk dissolved oxygen concentration.

This dynamic equation is based on the oxygen mass balance around a stirred bioreactor containing a microbial biomass. The term $K_L A$ cannot be clearly separated into "K_L" and "A" individually. Nevertheless, "A" is largely dependent on the bioreactor configuration (degree of mechanical agitation and sparging) whereas "K_L" is closely associated with convection, turbulence and liquid properties.

C^* is the dissolved oxygen concentration in the liquid phase at equilibrium with the inlet gas, a temperature dependent thermodynamic parameter that can be calculated from Henry's Law on the basis of the oxygen partial pressure of the gas phase when deviation from an ideal solution is minor. The value of C^* can be obtained from a wide range of tables.

The value for qO_2 was measured using the method described in Section 2.7.3 as 5mmol/ L hr for 1g dry wt/ L of *Pseudomonas putida* ML2.

4.1.2 Rationale for study

The objective in a biotransformation is to implement actions that lead to a higher value for the term $K_L A$ (C* - C) while maintaining C at a level satisfactory for cell growth and maintenance.

Traditionally, oxygen is supplied to a biotransformation by bubbling air through the medium accompanied by vigorous agitation to effect a fine bubble dispersion. One of the limitations associated with this mode of oxygen supply is its finite capacity to supply oxygen at a rate which will meet the requirements of the biotransformation.

This limitation was demonstrated in the previous chapter when the air aerated STR was unsuccessful in meeting the oxygen requirements for biotransformations carried out using 10g dry wt/ L (Section 3.2.6.3). In addition, this bioreactor configuration was incapable of providing a solution for the large loss of fluorobenzene (through volatization) during prolonged biotransformation operation.

It was decided, therefore, to investigate several modes of oxygen supply and evaluate the oxygen transfer rates achieved under different operating conditions (stirrer speed and air flow rate). This would provide an empirical basis for comparison and enable a decision to be made on the optimum air flow rate (AFR) and agitation rate for a given mode of oxygen supply.

Modes of oxygen supply which merited further investigation (e.g. on the basis of increased OTR or reduced substrate volatisation) were evaluated during continuous biotransformation operation using the configuration described in Section 3.2.6 (3.16).

The oxygen transfer rate was measured for ten different modes of oxygen supply, including a novel membrane oxygenator, a perfluorocarbon, a solvent, head pressurisation and oxygen enrichment.

The aim of this work was to establish which mode of oxygen supply was most suitable for a whole cell catalysed aromatic oxidation with a volatile inhibitory poorly aqueous soluble substrate and a toxic completely aqueous soluble product.

4.2 Oxygen mass transfer

4.2.1 Oxygen mass transfer using a sparged STR

The oxygen transfer rate was evaluated for *Pseudomonas putida* ML2 (6g dry wt/ L) using a 2L (working volume) MBR bioreactor over a range of stirrer speeds (350, 750 and 1100 rpm) combined with a range of gas flow rates (3, 6 and 9L/ min or 1.5, 3 and 4.5vvm) using the technique described in Section 2.7.

The modes of oxygen supply evaluated under these conditions are as follows:

-Air

-Pure oxygen

-50% air / 50% pure oxygen mixture (60% oxygen content)

-Air with a 5psi STR head pressure

-Pure oxygen with a 5 psi STR head pressure

-50% air / 50% pure oxygen mixture with a 5 psi STR head pressure

Figures 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6 illustrate the results of this work.

Each point on these graphs (4.1 to 4.6) represents the mean and standard deviation of six OTR measurements under specific aeration and agitation conditions. The data illustrated in Figures 4.1, 4.2 and 4.3 are the same as that illustrated in Figures 4.4, 4.5 and 4.6. Both sets of graphs (Figures 4.1 to 4.6) clearly illustrate the effect of agitation rate on oxygen mass transfer.

A number of conclusions can be drawn from this work:

1. Small increases in agitation rates have a much greater effect on oxygen mass transfer than large increases in gas flow rates.

2. Figures 4.1, 4.2 and 4.3 illustrate that operating at 1100 rpm (at any given gas flow rate) will result in high oxygen transfer rates.

3. Figures 4.4, 4.5 and 4.6 illustrate that large increases in gas flow rates at a given agitation rate will result in only a very slight increase in oxygen mass transfer rate, i.e. the OTR at 1.5vvm is only marginally less than that at 4.5vvm at the same agitation rate.

4. Increasing the gas flow rate from 1.5 to 4.5vvm, combined with an increasing agitation rate does not result in a decrease in OTR. Thus it can be concluded that impeller flooding does not occur under these operating conditions with these modes of oxygen supply.

5. The optimum conditions of operation for this configuration are a gas flow rate of 1.5vvm and an agitation rate of 1100 rpm.

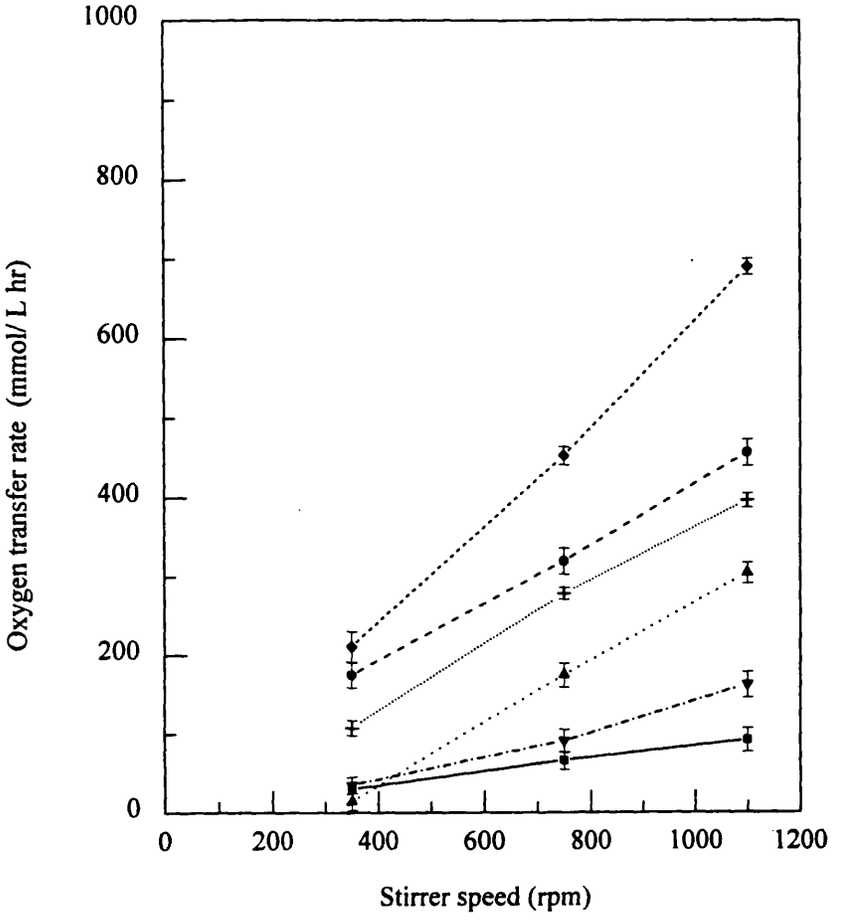

Figure 4.1 OTRs at 1.5vvm, as a function of stirrer speed

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ▼ Air @ 5psi
- ◆ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

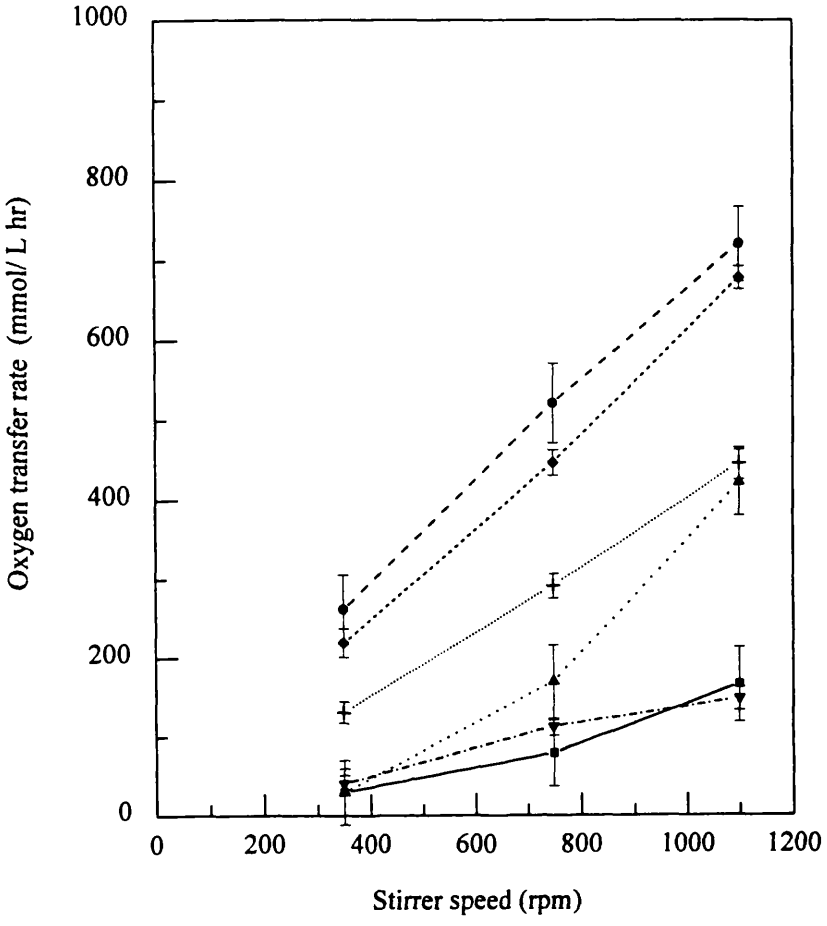

Figure 4.2 OTRs at 3vvm, as a function of stirrer speed

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ▼ Air @ 5psi
- ◆ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

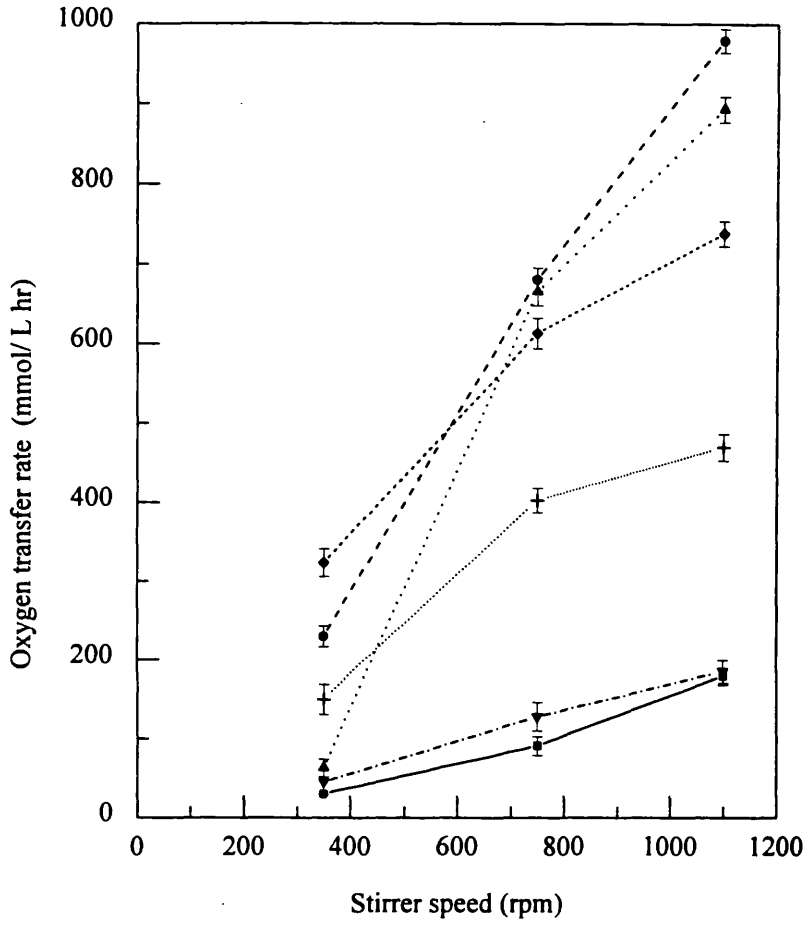

Figure 4.3 OTRs at 4.5vvm, as a function of stirrer speed

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ▼ Air @ 5psi
- ◆ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

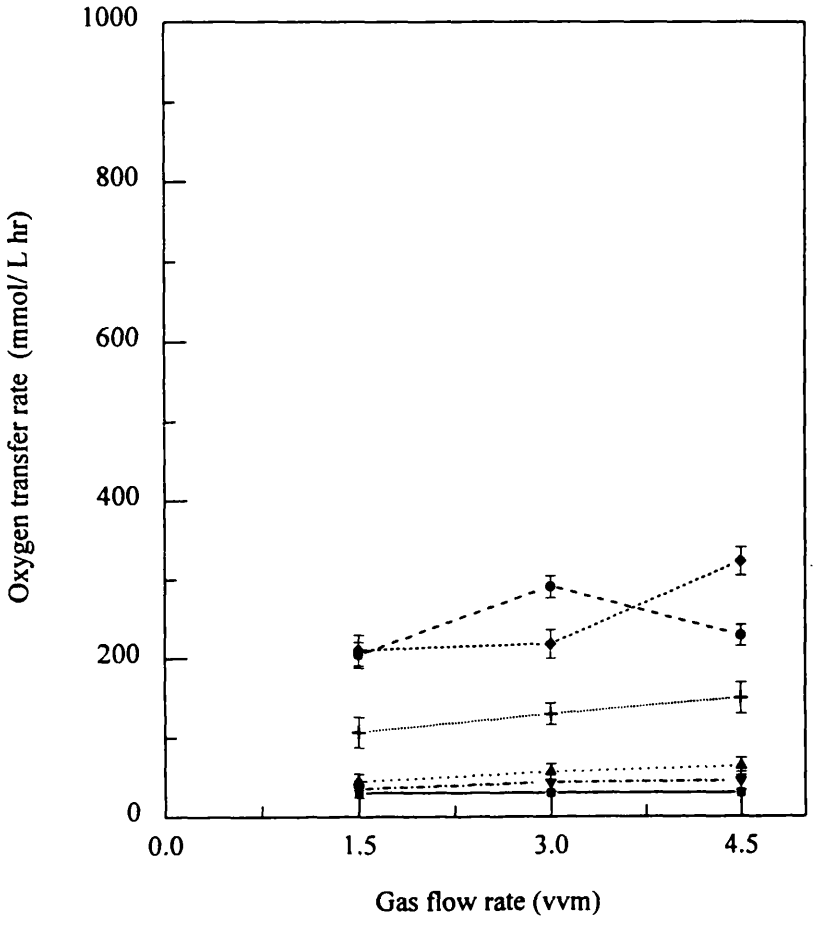

Figure 4.4 OTRs at 350 rpm, as a function of gas flow rate

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ♥ Air @ 5psi
- ♦ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

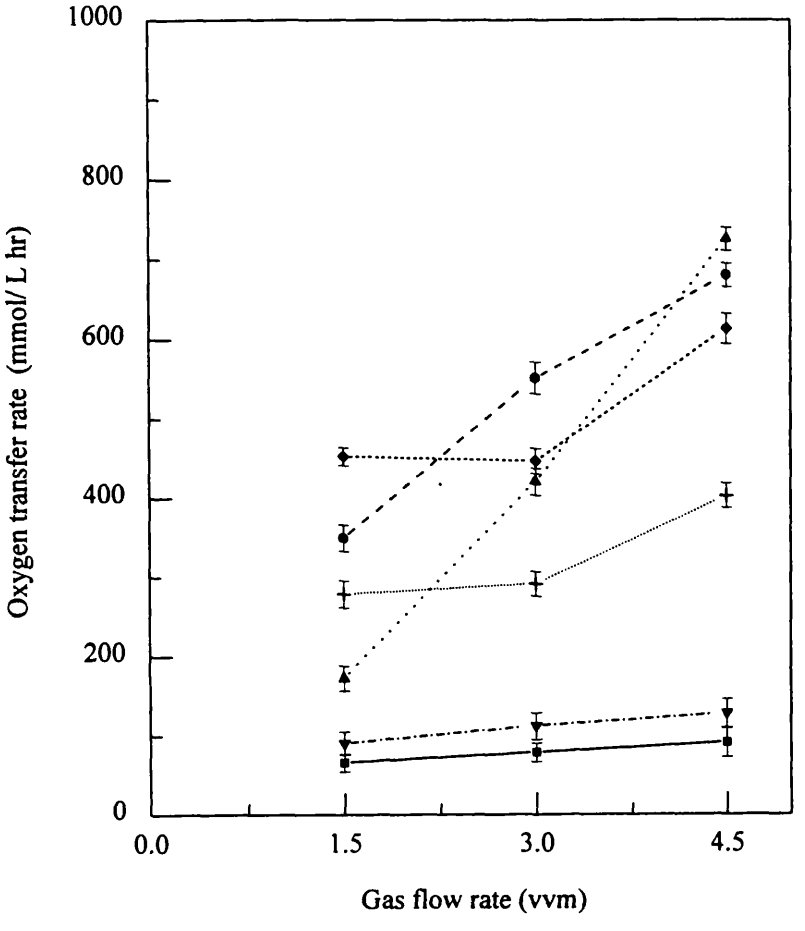

Figure 4.5 OTRs at 750 rpm, as a function of gas flow rate

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ♥ Air @ 5psi
- ◆ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

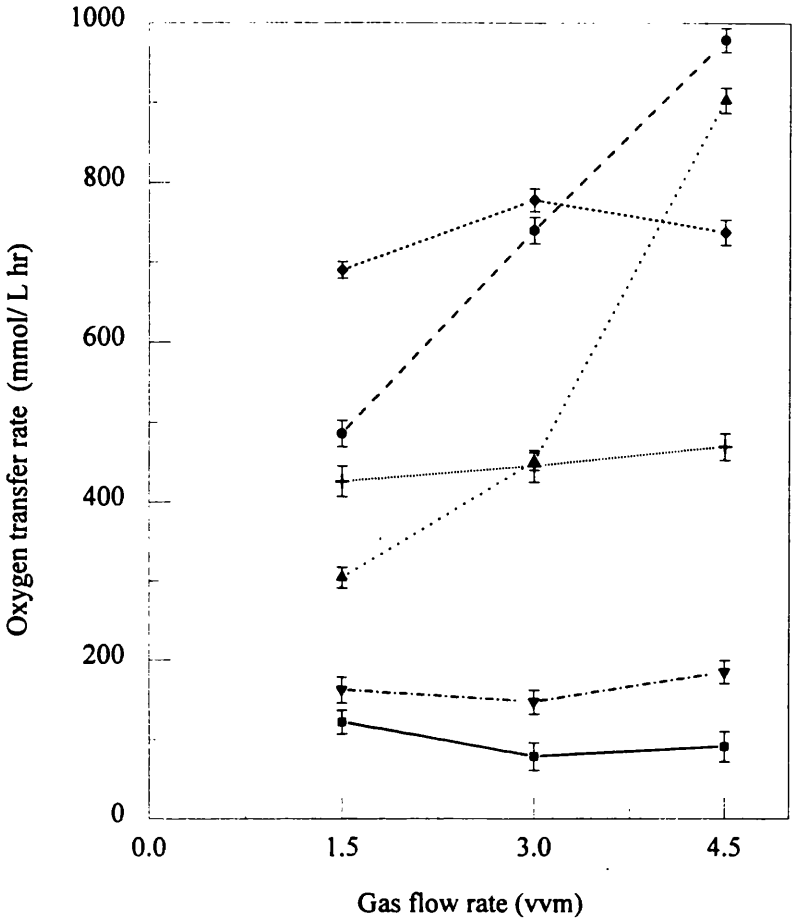

Figure 4.6 OTRs at 1100 rpm, as a function of gas flow rate

- ■ Air
- ● Oxygen
- ▲ Air / Oxygen
- ♥ Air @ 5psi
- ♦ Oxygen @ 5psi
- \+ Air / Oxygen @ 5psi

The five conclusions listed on p91 reinforce what is widely known about the dependency of OTR and K_LA on reactor operating conditions. Many correlations for K_LA have been proposed in the literature, (**Robinson and Wilke, 1973; Zlokarnik, 1978; Van't Riet, 1979; Moo-Young and Blanch, 1981; Judat, 1982; Henzler and Kauling, 1985; Kawase and Moo-Young, 1988, Moresi and Patete, 1988; Schulter and Deckwer, 1992**) most of which can be reduced to two types, namely:

$$K_LA = (P/V_L)^a (qG/A)^b \qquad (1)$$

$$K_LA = (P/V_L)^a (qG/V_L)^b \qquad (2)$$

where:

K_LA = overall volumetric mass transfer coefficient

P = power input (kW)

V_L = liquid volume (m^3)

qG = gassing rate (m^3/sec)

A = area (m^2)

Both correlations predict the same (or similar) dependency on power input (referred to liquid volume). They differ, however, with regard to the effect of aeration.

In equation (1) the linear gas velocity (uG = qG/A) is applied while in equation (2) the gas flow rate referred to liquid volume (qG/V_L) is used. What is evident is that the term $(P/V_L)^a$ has a more profound effect on the value of K_LA (typical values for "a" are between 0.8 and 0.95, although values decrease with increasing scale).

The term $(qG/A)^b$ or $(qG/V_L)^b$ has a much smaller effect on K_LA (typical values for "b" are between 0.4 - 0.6). In other words small adjustments to agitation rate will have large effects on oxygen mass transfer in contrast to large variations in gas flow rate having a relatively small influence.

Another means of representing the information in Figures 4.1 to 4.6 is illustrated in Table 4.1 where OTR values measured at 1.5vvm and an agitation rate of 1100 rpm for different modes of oxygen supply are compared.

The trend observed in Table 4.1, under the particular conditions of operation (1.5vvm AFR and 1100 rpm) is typical of the trend observed at all other agitation and gas flow rates. It illustrates how the different modes of oxygen supply could be expected to perform on the basis of oxygen transfer rate.

Mode of oxygen supply	OTR (mmol/ L hr)
Air (normal)	92
Air (5psi)	132
Air / Oxygen (normal)	274
Air / Oxygen (5psi)	396
Oxygen (normal)	456
Oxygen (5psi)	660

Table 4.1 OTR for different oxygen supply modes

It was significant that a 50% mixture of air / oxygen (resulting in a 60% oxygen concentration) at a head pressure of 5psi could potentially be less expensive, less dangerous and almost as efficient as using pure oxygen.

4.2.2 Fluorobenzene loss from a sparged STR

Results illustrated in Figures 4.1 to 4.6 favoured STR operation at 1100 rpm and a gas flow rate of 1.5vvm. Increasing the gas flow rate did not result in any appreciable increase in OTR. However, fluorobenzene is a volatile biotransformation substrate (vapour pressure of 72mmHg) and so it was decided to investigate the rate of removal of fluorobenzene from solution at different gas flow rates using a stirrer speed of 1100 rpm.

Figure 4.7 illustrates the results of this work. A fluorobenzene solution (0.2g/ L) was monitored for rate of fluorobenzene loss from the MBR (1100 rpm and 1.5, 3, 4.5vvm) by GC at 30 second intervals. At 4.5vvm there was a rapid, almost instantaneous, decrease in fluorobenzene concentration, with a much slower, more gradual loss at the lower gas flow rates of 1.5 and 3vvm.

As a consequence of this rapid fluorobenzene loss at high gas flow rates, it was decided to evaluate the different modes of oxygen supply (air, air/oxygen, oxygen etc.) under continuous biotransformation operation (1.5vvm and 1100 rpm).

Another reason for using the lower gas flow rate was the almost negligible increase in OTR associated with the higher gas flow rate. It had also been planned to use a solvent and a perfluorocarbon to enhance oxygen mass transfer. Operating at a high gas flow rate would aid the loss, through volatization and "blow-off", of these oxygen transfer catalysts.

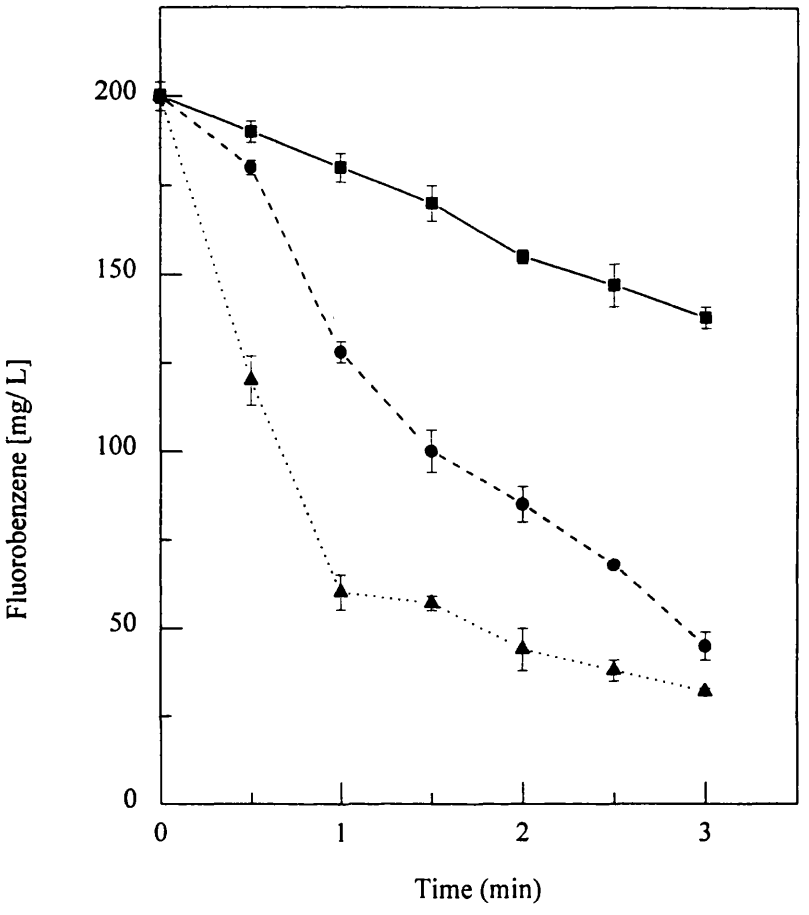

Figure 4.7 Fluorobenzene loss from a sparged STR

■ 1.5vvm

● 3vvm

▲ 4.5vvm

4.2.3 Oxygen mass transfer using the membrane oxygenator

Figure 4.8 is a schematic representation of the membrane oxygenator as descrbed in Section 2.7.2 Oxygen transfer rates, measured as a function of recycle flow rate past the membrane, and membrane oxygen supply pressure are detailed in Figure 4.9.

There are five graphs representing different oxygen supply pressures (10, 20, 30, 40, 50psi) within the membrane oxygenator. There is a direct relationship between the membrane oxygen supply pressure and OTR. OTR increased with increasing membrane pressure.

At the outset, the rates of oxygen mass transfer for the membrane oxygenator did not compare favourably with the STR modes of oxygen supply described above. However, this membrane oxygnation module was designed to supply oxygen at a rate of 100mmol/ L hr. The module was successful in fulfilling this criterion. An increase in the oxygen transfer rate could be achieved by:

1. Increasing the membrane surface area
2. Increasing the membrane oxygen supply pressure (0 to 80psi)
3. Increasing the recycle flow rate
4. Using a different membrane material
5. Reducing the module housing size to accomodate a higher superficial velocity

All of which were possible either during operation or in the design of the module. The unique advantage associated with the membrane oxygenation module is that it provided *bubble free oxygenation* (**Cote *et al.*, 1988; Semmens *et al.*, 1989; Ahmed and Semmens, 1992a,b; Henzler and Kauling, 1993**).

This mode of oxygenation could address the challenge of substrate volatility by removing one of the primary driving forces for volatization, namely the oxygenation source gas bubbles (although CO_2. evolution could still have an influence).

A biotransformation rate assay was carried out on the cells prior to the oxygen transfer rate evaluation work. Another biotransforamtion rate assay carried out at the end of the work (4 hours later) indicated that no loss in biocatalytic activity occurred.

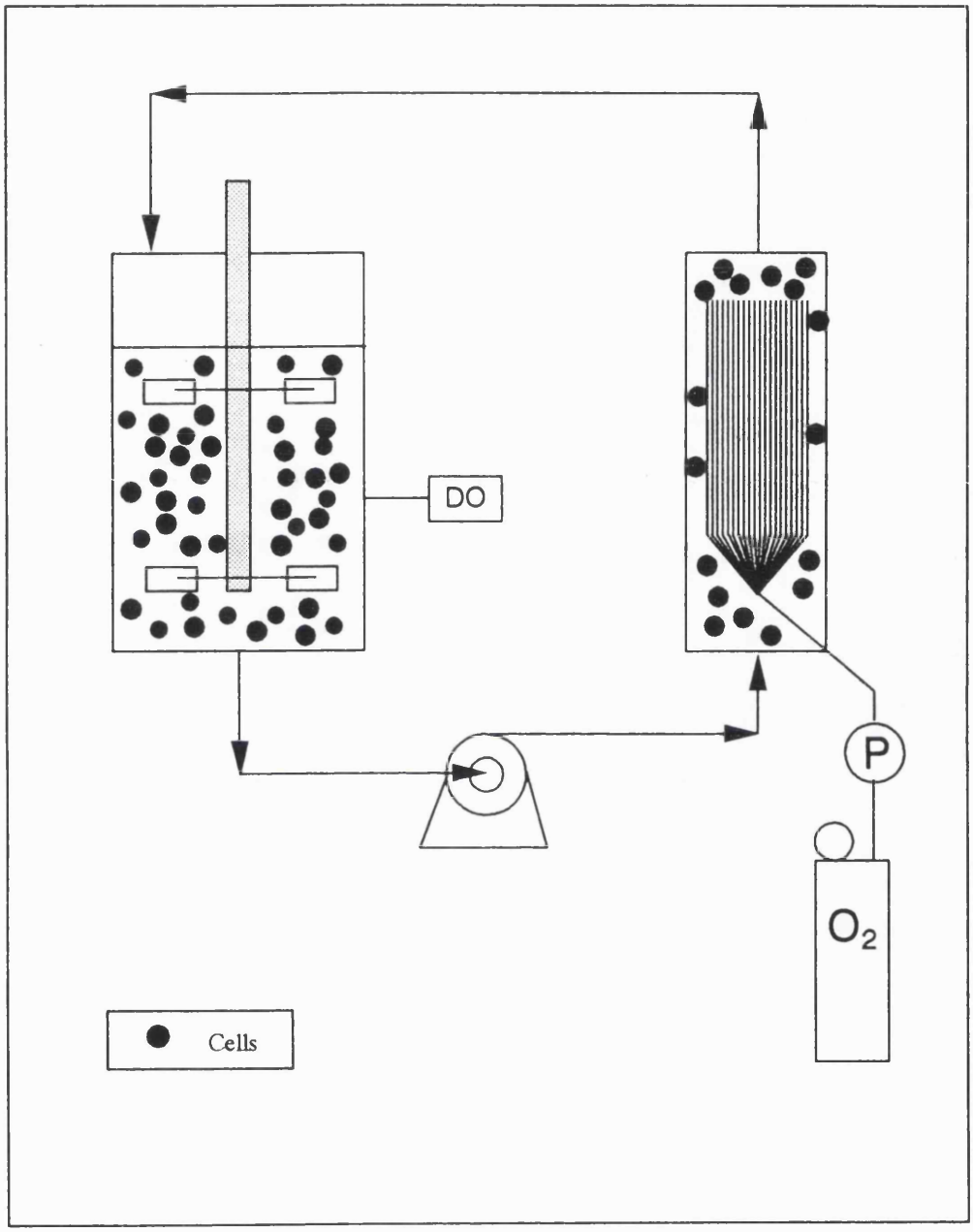

Figure 4.8 Membrane oxygenated STR, used for OTR evaluation

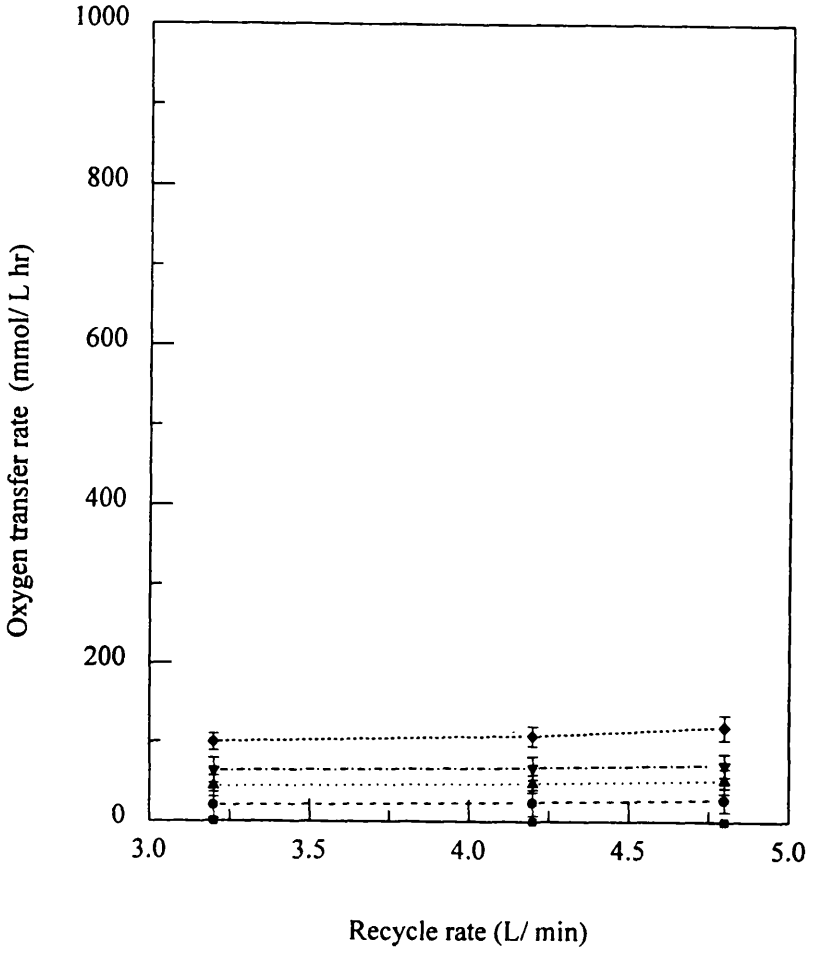

Figure 4.9 OTRs for the membrane oxygenator

- ■ 10psi
- ● 20psi
- ▲ 30psi
- ▼ 40psi
- ◆ 50psi

4.2.4 Oxygen mass transfer using perfluorocarbon and air aerated STR

An aqueous immiscible perfluorocarbon liquid, perfluoromethyldecalin, PP9, ($C_{11}F_{20}$) as described in Section 2.7.4 was chosen for this work. It is a colourless, odourless, stable, inert, biocompatable liquid and has an oxygen solubility of 144mg/ L. This compares with the solubility of oxygen in water of 8.24mg/ L (1 bar air pressure @ 25 C).

The oxygen transfer rates were measured using different phase ratios of PP9 in an air aerated STR (1.5vvm, 1100 rpm) with 6g dry wt/ L. The results of this work are illustrated in Figure 4.10. A phase ratio of 0.1 refers to a 10% (by volume) content of PP9 in the total aerated liquid volume.

The OTR did not increase until a phase ratio of 0.6 was employed, i.e. 60% PP9 by volume. At 0.6 the OTR was three times that of a normal air aerated STR (phase ratio of 0) and remained at this level for further increases in phase ratio.

It was necessary to operate at a phase ratio of 0.6 or higher to obtain any appreciable increase in oxygen transfer rates using PP9. This was quite a high phase ratio and was expected to prove quite expensive since PP9 costs £150 per litre. The high phase ratio required eliminated PP9 from further evaluation.

It took 5 minutes to separate both phases at a phase ratio of 0.1. The time for separation decreased with increasing phase ratio until, at a phase ratio of 0.5, separation was almost instantaneous once agitation ceased.

A biotransformation rate assay was carried out on the cells prior to the oxygen transfer rate evaluation work. Another biotransforamtion rate assay carried out at the end of the work indicated that no loss in biocatalytic activity occurred.

4.2.5 Oxygen mass transfer using tetradecane and an air aerated STR

It was decided to investigate the use of a hydrocarbon solvent as a potential oxygen transfer catalyst and oxygen reservoir. Tetradecane was chosen for this work because it is non toxic to P*seudomonas putida* (**Hack, 1992**); fluorobenzene is completely soluble in tetradecane whereas fluorocatechol is not. The solubility of oxygen in tetradecane is 35 times that in water. The properties of tetradecane are listed in Section 2.7.5.

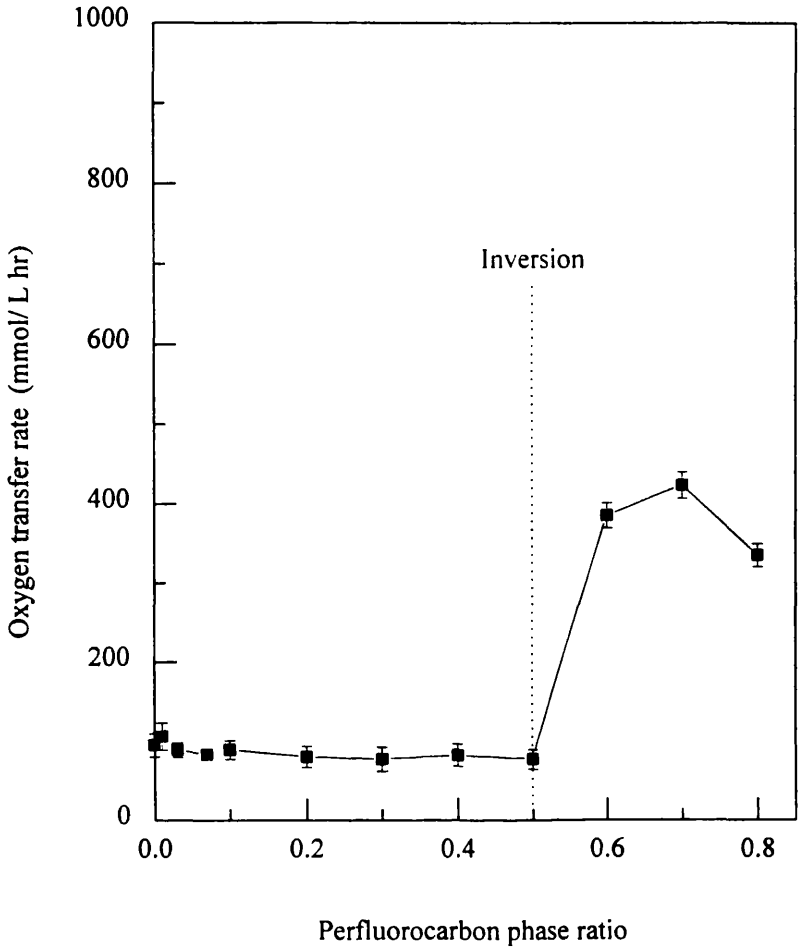

Figure 4.10 OTR for PP9, as a function of phase ratio

The oxygen transfer rate was measured using different phase ratios of tetradecane. Results are illustrated in Figure 4.11. A phase ratio of 0.1 referred to a 10% (by volume) content of tetradecane in the total aerated liquid volume. The OTR increased when a phase ratio of 0.1 was employed, i.e. 10% tetradecane by volume. At 0.1 the OTR was twice that of a normal air aerated STR (phase ratio of 0) and remained at this level for further increases in phase ratio.

A phase ratio of 0.1 was all that was necessary to double the rate of oxygen mass transfer using tetradecane. This mode of oxygen supply seemed promising, especially in view of the advantages associated with it, listed above. However, because of time constraints it was not possible to go into any more detail than is described here.

Separation of both phases occurred after 5 minutes (at all phase ratios), once agitation ceased. Phase inversion (visual inspection) took place at a phase ratio of 0.5.

A biotransformation rate assay was carried out on the cells prior to the oxygen transfer rate evaluation work. Another biotransformation rate assay carried out at the end of the work (4 hours later) indicated that no loss in biocatalytic activity occurred.

4.2.6 Oxygen transfer with perfluorocarbon and membrane oxygenation

It was decided to investigate if the use of PP9 at different phase ratios in parallel with the membrane oxygenator would enhance the rate of oxygen mass transfer (in comparison to the individual systems described above).

In Figure 4.9, it is obvious that the highest membrane oxygen transfer rates are associated with a recycle flow rate of 4.8L/ min and a membrane oxygen pressure of 50psi. Oxygen transfer rates were evaluated using these membrane operating conditions and different PP9 phase ratios (Figure 4.12).

The rate of oxygen mass transfer remains constant with increasing phase ratio up to 0.3. After 0.3 the rate of oxygen mass transfer decreases, presumably because the rate of oxygen transfer to solution from the perfluorocarbon, is limited by the rate of transfer from the membrane oxygenator to the perfluorocarbon.

Since the rate of oxygen transfer associated with this system was so low, it was not considered for any further evaluation.

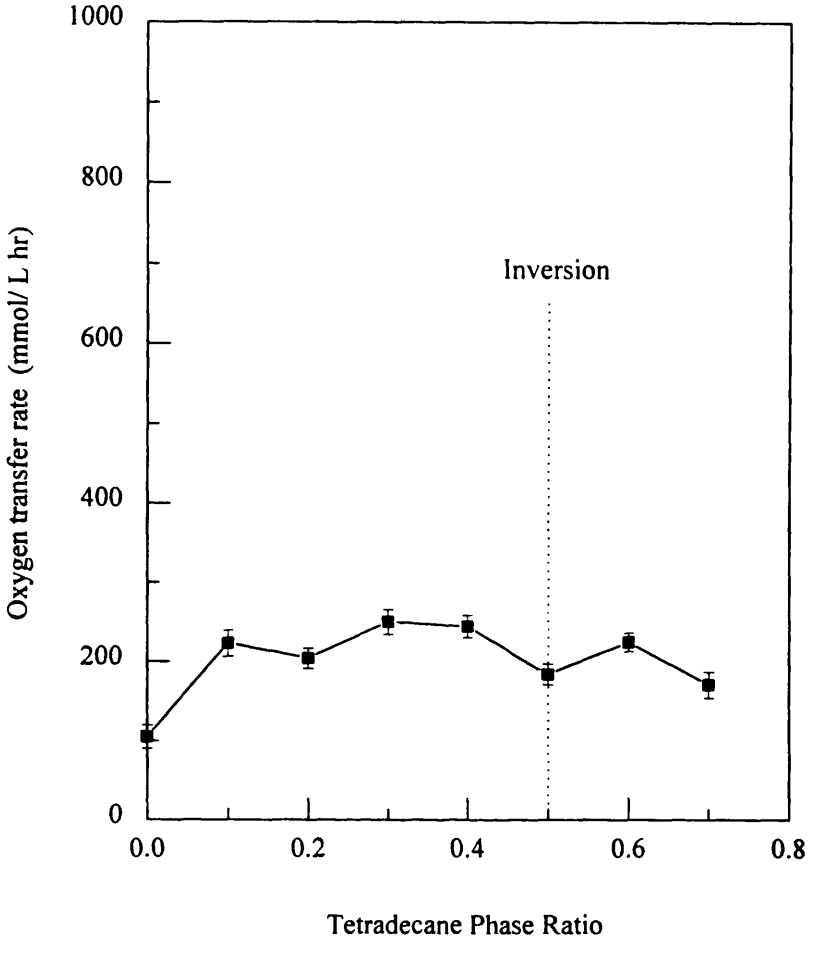

Figure 4.11 OTR for tetradecane, as a function of phase ratio

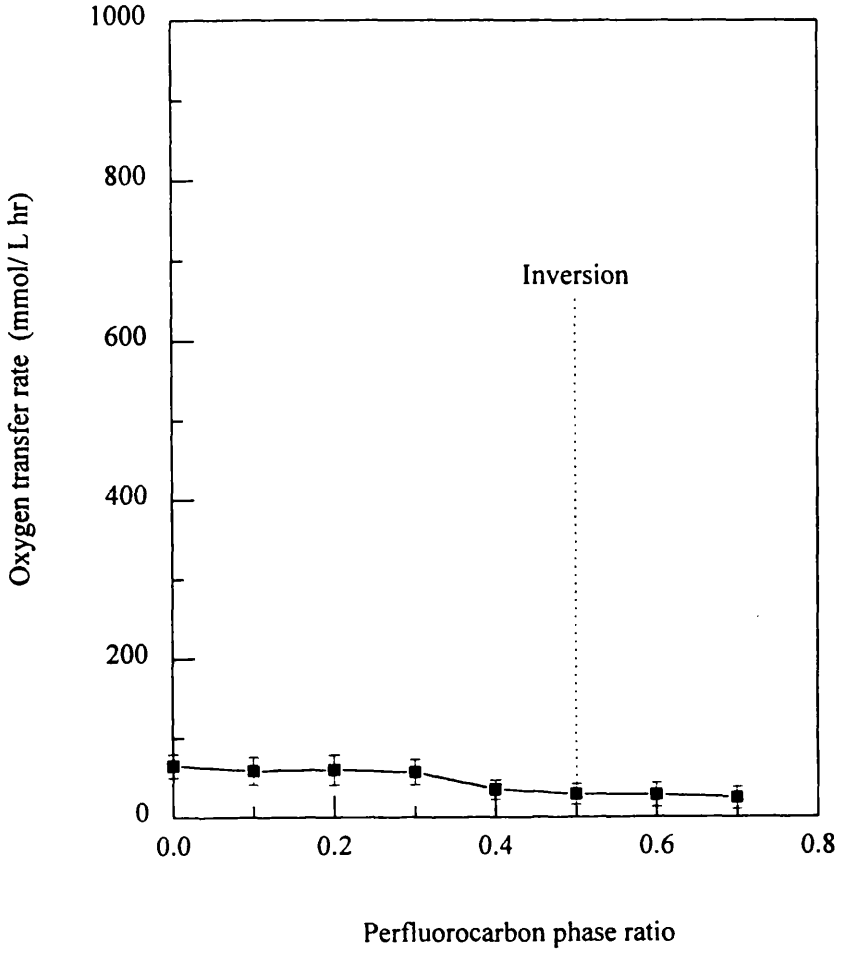

Figure 4.12 OTR for PP9, as a function of phase ratio, with membrane oxygenation

4.2.7 Oxygen mass transfer rates for different modes of oxygen supply

The oxygen transfer rates evaluated for the modes of oxygen supply (described above) are illustrated in Table 4.2 where the rates are graded in ascending order of oxygen transfer capacity as:

Mode of oxygen supply	OTR (mmol/ L hr)
Perfluorocarbon in parallel with membrane oxygenation	60
Air aerated STR	92
Membrane oxygenation	120
Air with a 5psi head pressure	132
Tetradecane with air aerated STR (phase ratio of 0.1)	250
50% air / 50% pure oxygen (60% oxygen content) aerated STR	274
50% air / 50% pure oxygen aerated STR with a 5psi head pressure	396
Perfluorocarbon and air aerated STR (phase ratio of 0.6)	423
Pure oxygen aerated STR	456
Pure oxygen aerated STR with a 5 psi head pressure.	660

Table 4.2 Comparison of OTRs for oxygen supply modes (1.5vvm, 1100 rpm)

It was decided to carry out continuous biotransformation evaluation work, based on this table, using pure oxygen and then separately, the membrane oxygenation configuration.

4.3 Validation of oxygen mass transfer modes

4.3.1 Continuous biotransformation with pure oxygen

Using pure oxygen as the supply source, 6g dry wt/ L of *Pseudomonas putida* ML2 were added to the bioreactor configuration illustrated in Figure 3.16, to make up a 2L working volume. Stirrer speed was set at 1100 rpm and the oxygen probe calibrated as described in Section 2.7.3. The DOT was controlled at 50% air saturation by controlling the inlet oxygen supply flowrate.

Figure 4.13 is a plot of DOT, cellular activity and the aqueous dissolved fluorobenzene concentration against time for a pure oxygen sparged STR biotransformation (using the configuration illustrated in Figure 3.16).

The DOT was initially controlled at 50%. After 0.5 hours the fluorobenzene pump was switched on and the fluorobenzene / ethanol solution (50% by volume) was pumped to the bioreactor at a rate of 0.0132 L/ hr. The DOT was controlled at 50%.

Samples taken at the inlet to and outlet from the activated carbon column were monitored by GC. An initial activity of 0.188 g FC/ g dry wt/ hr was recorded.

After 3 hours, the DOT was increased to 100% (by increasing the mass flow rate of oxygen). The cells continued to produce fluorocatechol at an average rate of 0.188 g FC/ g dry wt/ hr.

After 5 hours, the DOT was increased to 150%. No difference in cellular activity was observed.

After 7 hours, the DOT was increased to 200%. No difference in cellular activity was observed.

After 9 hours, the DOT was increased to 250%. After 11.3 hours cellular activity began to decrease. After 12.8 hours, the DOT was decreased to 200%. Cellular activity stabilised at 0.1 g FC/ g dry wt/ hr. Subsequent progressive decreases in DOT did not result in a restoration of cellular activity, implying that an irreversible loss in activity had taken place.

A sample was removed from the biotransformation and assayed for biocatalytic activity using the biotransformation rate assay detailed in Section 2.6. The cells had an activity of 0.11 g FC/ g dry wt/ hr, confirming that a DOT of 250% resulted in irreversible activity loss to the cells during the biotransformation.

The same configuration was previously successfully employed to carry out a biotransformation over 12 hours (Section 3.2.6.1) without any loss in activity, inferring that activity loss was dependent upon oxygen concentration.

4.3.2 Continuous biotransformation with membrane oxygenation

Using the membrane oxygenator as the oxygen supply source, 6 g dry wt/ L of *Pseudomonas putida* ML2 were added to the bioreactor to make up a 2L working volume. Figure 4.14 illustrates the bioreactor configuration used in this work.

The bioreactor stirrer speed was set at 350 rpm, the membrane oxygen supply pressure at 40psi and the membrane recirculation pump was switched on.

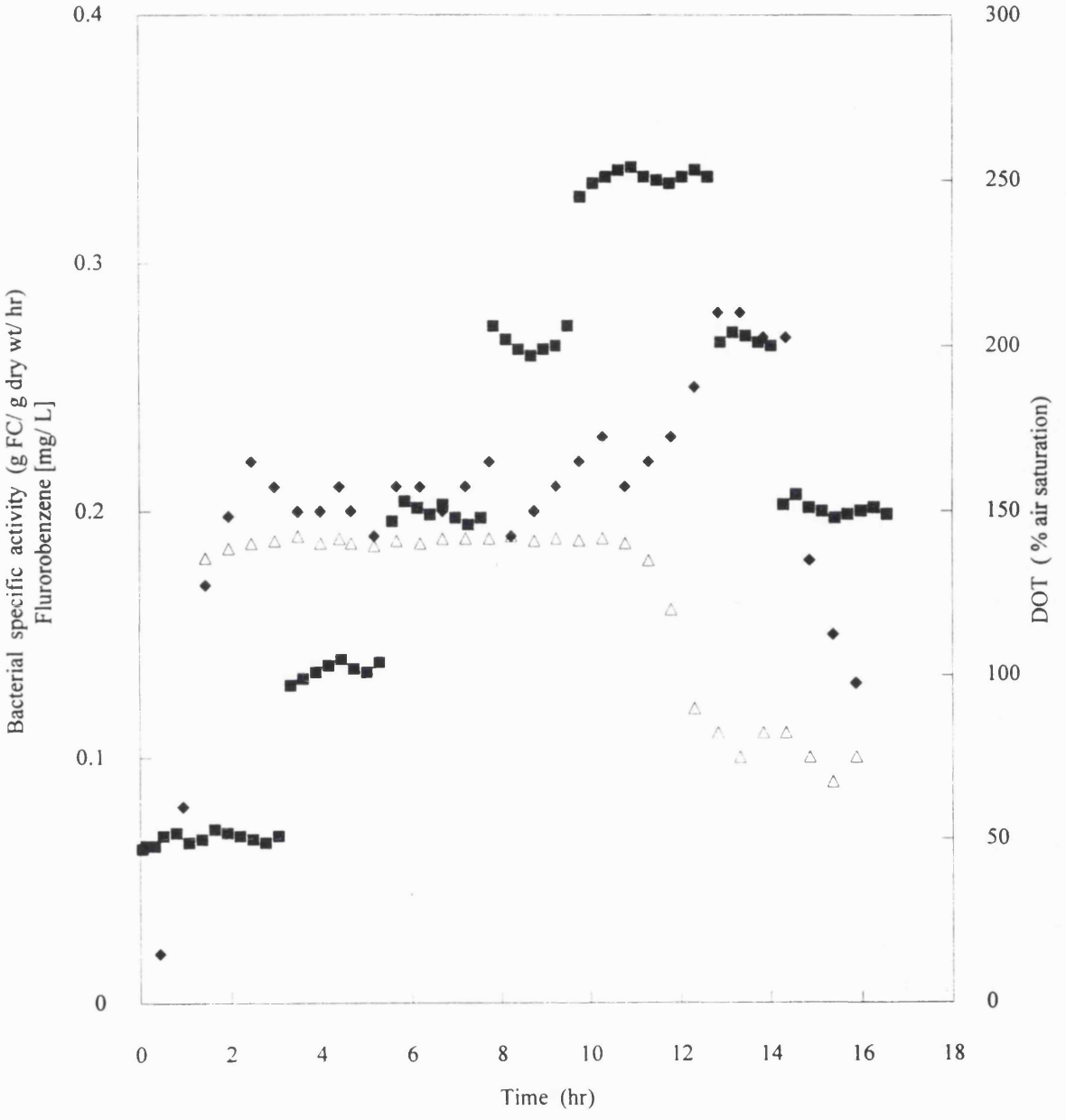

Figure 4.13 Biotransformation profile for an STR using pure oxygen (6g dry wt/ L)

■ DOT (% air saturation)

◆ Fluorobenzene

Δ Specific activity

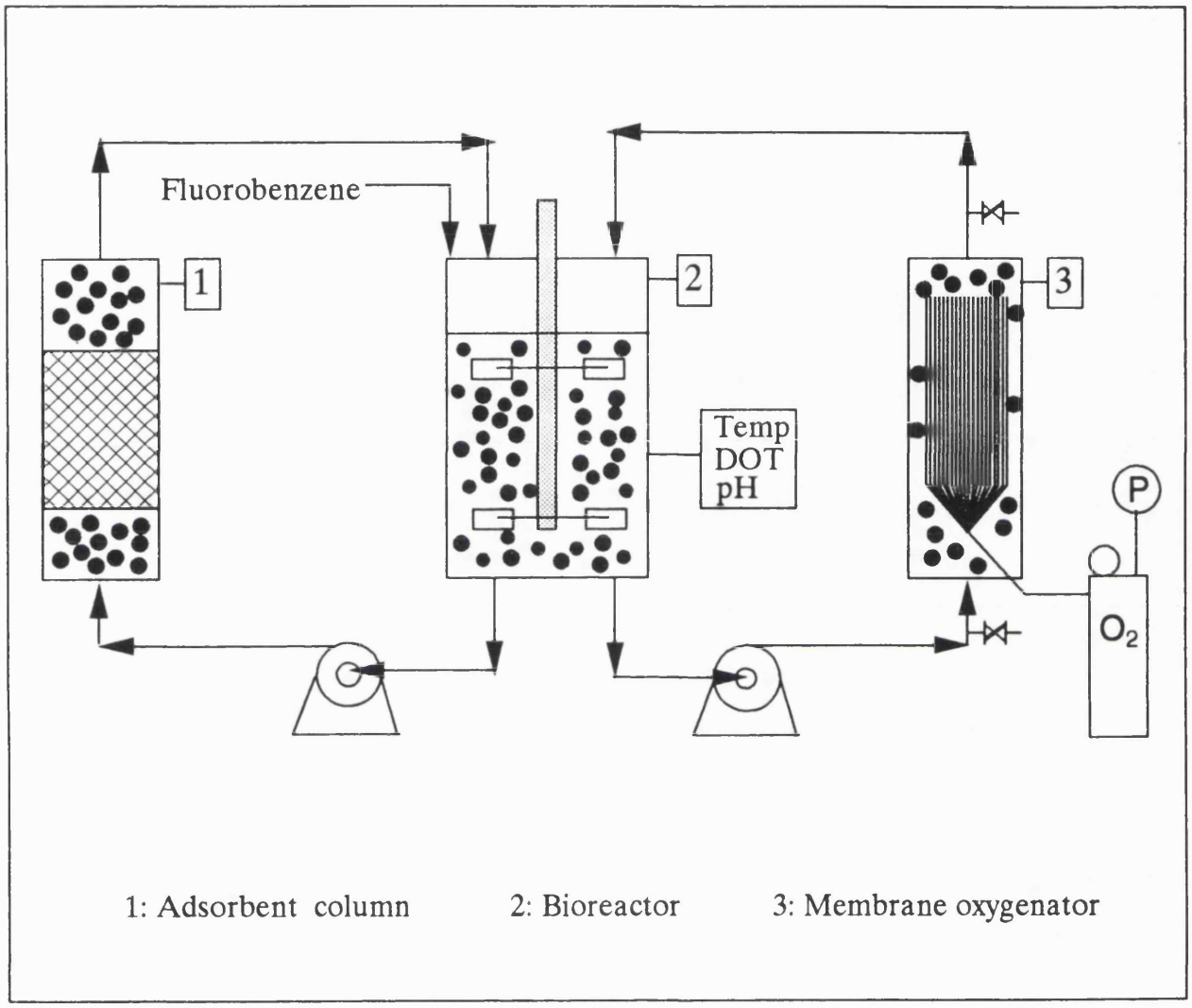

Figure 4.14: Membrane oxygenated biotransformation configuration

Figure 4.15 is a plot of DOT, cellular activity and the aqueous dissolved fluorobenzene concentration against time for a membrane oxygenated biotransformation (using the configuration in Figure 4.14).

After 0.4 hours of conditioning, the fluorobenzene/ethanol solution (50% by volume) was pumped to the bioreactor at a rate of 0.0044 L/ hr.

DOT was maintained at 70% by adjusting the membrane oxygen supply pressure. This method of DOT control was successful in maintaining a constant DOT for the duration of the biotransformation. The suspension DOT on return from the adsorbent column never decreased below 30%. After 14.1 hours the fluorobenzene pump was switched off.

Once the fluorobenzene pump was switched on, samples were taken at the inlet to and outlet from the activated carbon column every 30 minutes. A productivity of 0.183 g FC/g dry wt/ hr was recorded throughout the biotransformation.

Fluorocatechol samples taken at the end of the biotransformation indicated that column breakthrough did not occur. Cellular activity was measured as 0.19 g FC/ g dry wt/ hr. These figures confirm that the cells carried out the biotransformation under optimal conditions with no loss of activity throughout the biotransformation. No fouling of the membrane was recorded.

4.4 Discussion

The total oxygen requirement for *Pseudomonas putida* ML2 can be divided into four parts. These are:

1. Endogeneous (self digestion in the absence of a carbon source)
2. Maintenance (cell organelle turnover and repair)
3. Growth
4. Biotransformation

For biotransformation purposes, only two of these are required (maintenance and biotransformation). Jones (**Jones, 1994**) has measured the maintenance requirement of *Pseudomonas putida* ML2 as 5mmol/ L hr for a 1g dry wt/ L suspension.

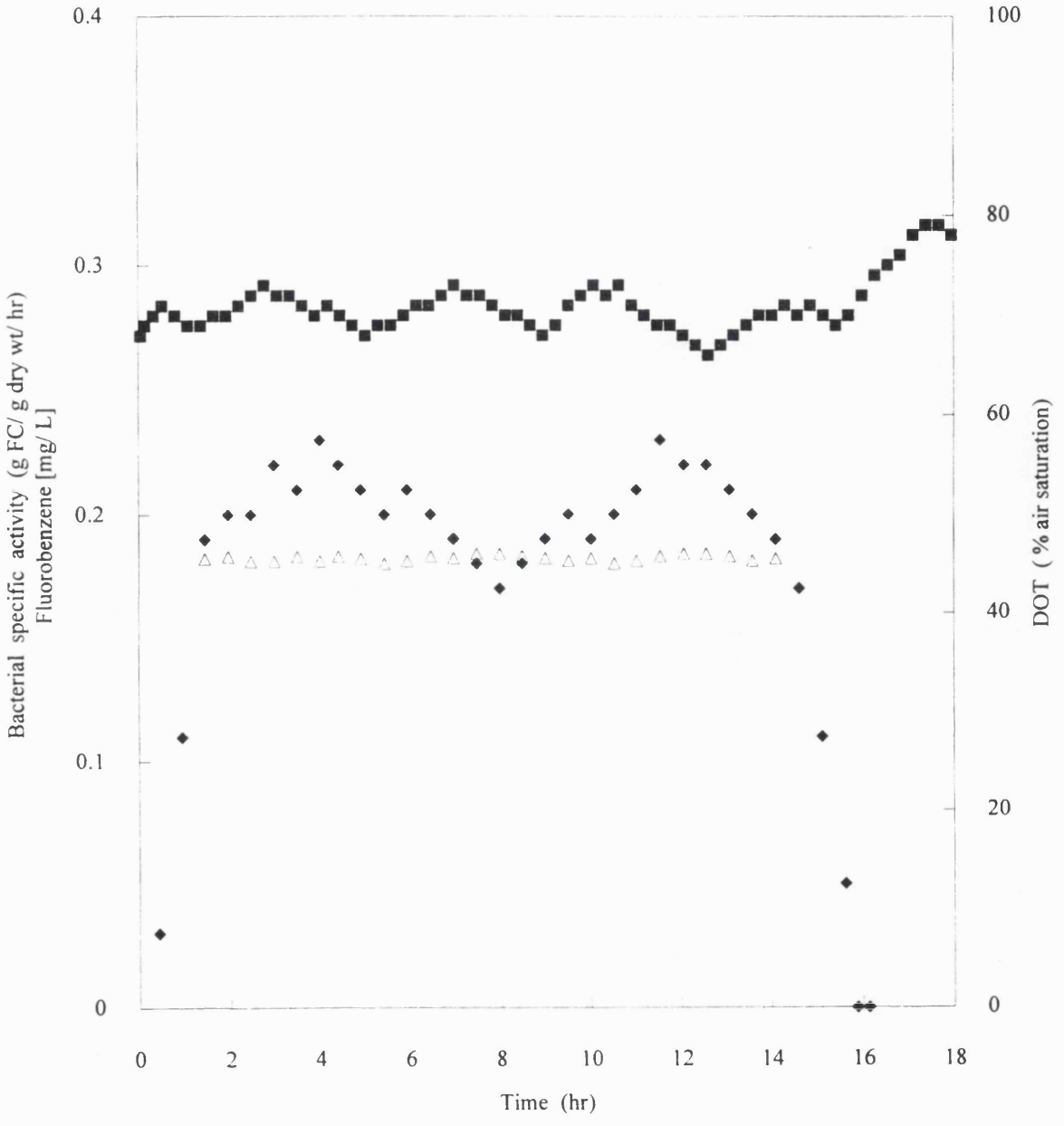

Figure 4.15 Biotransformation profile for a membrane oxygenated STR (6g dry wt/ L)

■ DOT (% air saturation)

◆ Fluorobenzene

Δ Specific activity

The oxygen requirement just to convert fluorobenzene to fluorocatechol can be calculated.

If 0.2g flurocatechol are produced per gram dry cell weight per hour, then ((0.2/ 128) 1000) = 1.56 mmol fluorocatechol produced per hour. This is equivalent to 1.56 mmol fluorobenzene used per gram dry cell weight per hour.

This is equivalent to 1.56 mmol oxygen used per hour (there is an equimolar equivalence of oxygen to fluorobenzene consumption) just to convert fluorobenzene to fluorocatechol.

Oxygen is also required to consume ethanol and fuel the complex dioxygenase enzyme system responsible for the biotransformation. If a factor of 4 (**Jones, 1994**) is considered as sufficient to take this oxygen requirement into consideration, then the total biotransformation oxygen requirement is:

$$((4 \times 1.56) + 5) = 11.24\text{mmol oxygen/ g dry cell weight/ hr.}$$

This value corresponds to an oxygen consumption rate of 10.1 mmol/ g dry wt/ hr, measured in a Clark oxygen electrode.

The air aerated STR configuration illustrated in Figure 3.16 was capable of supplying 80mmol/ L hr of oxygen to the biotransformation. This was sufficient to satisfy the biotransformation oxygen requirement at 6 and 7g dry wt/ L (67.4 and 78.7mmol/ L hr) as described in Section 3.2.6.1 and 3.2.6.2.

It also explains why this reactor configuration (Figure 3.16) was incapable of supplying an adequate quantity of oxygen (in the form of air) to maintain the biotransformation oxygen requirement of 10g dry wt/ L (Section 3.2.6.3). The required amount during continuous biotransformation operation was 112.4mmol/ L hr for 10g dry wt/ L. The air aerated STR was only capable of supplying 92mmol/ L hr explaining why the DOT decreased to zero during the biotransformation.

In Section 4.2.2, fluorobenzene loss from an air aerated STR was evaluated. It was concluded that an air aerated STR resulted in a significant loss of fluorobenzene over a very short period of time. It was not a desirable mode of oxygenation because of the large loss of substrate from the bioreactor, although if the financial and environmental costs were tolerable then it could be used.

There were two ways of addressing this challenge.

One was to evaluate several oxygen supply modes for their capacity to supply oxygen at a low gas flow rate (1.5vvm) and high agitation rate (1100rpm). Fluorobenzene loss was 60% less at 1.5vvm than at 3 or 4.5vvm (Figure 4.7). Consequently, if it was necessary to operate a gas sparged STR, then substrate loss would be greatly reduced although far from eliminated.

The results of this work are contained in Table 4.2. It was concluded from this work that pure oxygen would be used as an oxygen supply mode in a gas sparged STR (such as the configuration in Figure 3.16) since it provided the highest oxygen transfer rates. This was evaluated using continuous biotransformation operation.

25% less fluorobenzene was used when pure oxygen was sparged to the STR in comparison to the air aerated system described in Section 3.2.6. However, it was found that at a DOT of 250% and above, (Figure 4.13) pure oxygen was toxic to the biocatalyst. By maintaining the DOT below 200% (as a percentage of air saturation) prolonged uninhibited biocatalytic activity was observed.

Although this mode of operation did not eliminate fluorobenzene loss during the biotransformation it did reduce the amount lost by an appreciable amount.

The other alternative was to operate with bubble free oxygenation using the membrane oxygenation configuration in Figure 4.14. 63% less fluorobenzene was used (and **none** was lost through volatization) when compared to the aerated system described in Section 3.2.6. This membrane configuration resulted in prolonged uninhibited cellular activity (Figure 4.15).

This membrane configuration has solved the challenge of supplying oxygen to a whole cell catalysed aromatic oxidation with a volatile inhibitory poorly aqueous soluble substrate and a toxic, completely aqueous soluble product.

CHAPTER V

GENERAL DISCUSSION

5 GENERAL DISCUSSION

In the introduction to this thesis, a framework for biotransformation process design was outlined. The microbial oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida* ML2 was chosen to illustrate the benefits of adopting such a structured approach.

Such an approach proved successful in addressing the challenge of supplying oxygen to a whole cell catalysed aromatic oxidation with a volatile inhibitory, poorly aqueous soluble substrate and a toxic, completely aqueous soluble product.

But. how applicable is this process design approach to other biotransformations and can it be used to circumvent the many problems facing researchers in process design?

Any process design must inevitably be a compromise. It is the author's opinion that, by adopting the approach detailed in Section 1.2 of this thesis, process **design** can become a much more exact science.

There is a general lack of realisation that there are two major aspects of process quality. Firstly the quality of design which concerns the degree to which the design suits the purpose. Secondly, the quality of the conformance of the process to the design. **Poor process work can easily debase a good design, but poor design can NEVER result in good process quality.**

It may not be (and usually is not) one of the most direct, time efficient routes to take. However, effort placed in producing biocatalyst of consistent activity, the development of an activity assay and the focused characterization of a reaction system will result in a short-list of defined process configurations which can be evaluated during continuous biotransformation operation.

In the authors experience, the most important part of this work centred on characterizing the reaction system and developing a biotransformation rate assay.

The assay was used as a tool for characterization and was fundamentally important in establishing key parameters for process design

In this case, the established parameters included substrate inhibition and product toxicity. It was necessary not only to identify these parameters, but to quantify the inhibitory and toxic levels as well as establishing the kinetics of the reaction.

Once these parameters were evaluated a number of process options were defined which were assessed during continuous biotransformation operation. The activity assay was instrumental in assessing different process options and their influence on biocatalyst.

When a process configuration was developed for air aerated biotransformations, it was immediately evident that a large loss (through volatization of the substrate) was taking place even though the process was capable of sustaining optimal biocatalytic activity.

Different oxygen transfer modes were evaluated and the high rate transfer modes (using pure oxygen) resulted in lower rates of substrate loss. However, it was also established (through the activity assay) that oxygen was toxic above certain levels (250%).

A novel membrane oxygenator, adapted from environmental engineering applications, eliminated the problem of substrate loss. Bubble free oxygenation removed the primary driving force for volatization (a gas liquid interface) while promoting optimal biotransformation operation.

The approach to biotransformation design outlined in this thesis (and that proposed by Woodley and Lilly (**Woodley and Lilly; 1994**) can be used for a range of biotransformations. Results from this work illustrate the benefits of adopting such a formal structured approach to process design.

CHAPTER VI

CONCLUSIONS

6 CONCLUSIONS

1 A standardised fermentation has been developed for *Pseudomonas putida* ML2 with a yield of 10g dry wt/ L.

2 To prevent irreversible loss in biocatalyst activity, it is necessary to ensure DOT does not fall below 20% during fermentation.

3 Biocatalyst can be stored for a period up to 32 hours after harvesting without any loss in activity.

4 A biotransformation rate assay (unique to this biotransformation) has been developed which can evaluate biocatalytic activity independently of the biotransformation itself. This assay has been used as a tool to characterize the reaction system with the following results:

- Fluorobenzene (the poorly aqueous soluble, volatile substrate) causes reversible cellular activity inhibition at 0.8g/ L.
- First order kinetics are observed for fluorobenzene conversion up to 0.1g/ L, after which, zero order kinetics apply.
- Fluorocatechol (the completely aqueous soluble product) is toxic at 0.2g/ L causing irreversible activity loss.

5 Activated carbon (Norit pK13) is the most efficient adsorbent for fluorocatechol.

6 Product can be removed from the adsorbent in a post biotransformation liquid - liquid extraction protocol using butyl acetate.

7 The maximum adsorption capacity was 1.83g fluorocatechol/ g dry adsorbent weight. Adsorption followed the Langmuir adsorption isotherm principle, i.e. monolayer adsorption.

8 A bioreactor configuration has been developed, with an external loop to an activated carbon column, capable of production levels up to 70 times the cellular toxicity of the biocatalyst during prolonged biotransformation operation.

9 This configuration was not successful in eliminating the loss through volatization of fluorobenzene during biotransformations.

10 Pure oxygen can reduce this loss, but is toxic to the biocatalyst above certain levels (250 % air saturation).

11 Oxygen transfer rates recorded using a perfluorocarbon indicated that no appreciable benefit was to be gained by employing such an oxygen transfer vector.

12 A novel membrane oxygenator completely eliminated the fluorobenzene volatization loss through bubble free oxygenation, while promoting optimum biotransformation operation during prolonged operation.

13 This membrane configuration has solved the challenge of supplying oxygen to a whole cell catalysed aromatic oxidation with a volatile inhibitory poorly aqueous soluble substrate and a toxic, completely aqueous soluble product.

APPENDIX I

APPENDIX 1

A1 Suppliers list

The addresses of all suppliers are given in the following table:

Aldrich Chemical Co. Ltd., Gillingham, Dorset, UK	Fisons Scientific Ltd., Loughborough, Leicestershire, UK	Norit Ltd., Glasgow, Scotland UK
Amicon Ltd., Sotnehouse, Gloucestershire, U.K.	Gelman Sciences, Ann Arbor, Michigan, USA	BNFL fluorochemical Ltd., Preston, Lancashire, UK.
BDH Chemicals Ltd., Poole, Dorset, U.K.	MBR Bioreactors B Braun Biotech, Aylesbury, Buckinghamshire, UK	New Brunswick Scientific, Eddison, New Jersey, USA
BCS Ltd., Maidenhead, Berkshire UK	MSE Scientific Instruments Crawley, Sussex, UK	Sigma Chemical Co. Ltd., Poole, Dorset, UK.
Boehringer Mannheim, Mannheim, Germany	Membran Corp., Minneapolis, USA	Difco Labs, Detroit, Michigan, USA.
BOC Ltd, Guilford, Surrey, UK	Pye Unicam, Philips Scientific, Cambridge, UK	Rohm and Haas, Croydon, Surrey, UK.

Table A.1 List of suppliers and addresses

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