HYDROXYLATION OF NAPHTHALENE BY

PSEUDOMONAS PUTIDA

IN THE PRESENCE OF ORGANIC SOLVENTS

by

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Department of Chemical and Biochemical Engineering
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For my Parents
"All this is a dream. Still, examine it by a few experiments. Nothing is too wonderful to be true, if it be consistent with the laws of nature and in such things as these, experiment is the best test of consistency."

M. Faraday.
I wish to thank Professor Malcolm Lilly and Dr. John Woodley for their continual advice and encouragement during the course of this project. I also wish to thank Mr. J. Betts, Mr. D. F. Montgomery and all his staff for their excellent technical support. I am also grateful to the Science and Engineering Research Council for the funding of this project.
ABSTRACT

The operation of two-liquid phase bioreactors is a relatively new technology by which compounds only sparingly soluble in water can be dissolved to high concentrations in water-immiscible organic solvents so as to bring about bioconversion in an efficient manner. There are many potential advantages of operating in the presence of a second phase, however, there are also drawbacks, including the possible inactivation of the biocatalyst by the organic solvent. With the development of this new technology there comes an increasing need to understand how the catalyst interacts with its environment during the biotransformation and in particular the effect of the potentially toxic organic phase on catalytic activity and stability.

This thesis describes the design of a model system involving the microbial hydroxylation of naphthalene by the Gram-negative bacterium \textit{Pseudomonas putida} in the presence of a selection of organic solvents to determine biocatalyst-solvent interactions and their effects upon biocatalyst catalytic activity.

Optimisation studies for the production of toluene dioxygenase, the enzyme responsible for the hydroxylation of many aromatic compounds including naphthalene, were performed for the constitutive mutant \textit{P. putida} UV4 in order to define the enzyme production process.

A correlation between solvent polarity and biocatalyst activity has been demonstrated, polar solvents appearing more toxic. These findings when compared to the steroid \textit{Δ1-dehydrogenase} system of \textit{Arthrobacter simplex} a Gram-positive bacterium, for the same solvents, show a striking quantitative difference in product formation rates; \textit{P. putida} being able to bring about biotransformation in a wider range of polar organic solvents. This thesis details the biocatalyst-solvent interactions of \textit{P. putida} UV4 including the effects of solvent exposure upon membrane permeability, cell viability and motility. The role of the organic-aqueous liquid-liquid interface in biocatalyst inactivation is also considered.

This Gram-positive - Gram-negative difference is discussed in relation to the differences in cell wall make up. It is proposed that the presence of the outer membrane in Gram-negative bacteria shields the organism from some of the potentially toxic effects of the organic solvents employed. This Gram-positive - Gram-negative difference in reactor productivity is likely to be a key consideration in the selection of biocatalysts for biotransformations in the presence of organic solvents. It is likely that the method of biocatalyst growth will alter operational stability, this along with strain development and design are also discussed including outlines for future work.
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ABREVIATIONS

ATP  adenosine triphosphate
BDH  British Drug Houses
C    celsius
cc   cubic centimetre
DCE  dichloroethane
dcw  dry cell weight
DNA  deoxyribonucleic acid
DOT  dissolved oxygen tension
EDTA ethylene diaminetetraacetate
h, hr hour
g    gram
HPLC high pressure liquid chromatography
IETP intermediate electron transport protein
L    litre
LPS  lipopolysaccharide
μg   microgram
mg   milligram
m, min minute
μl   microlitre
ml   millilitre
mM   millimolar
NAD⁺, NADH nicotinamide adenine dinucleotide
NMR  nuclear magnetic resonance
pmf  proton motive force
psi  pounds per square inch
rpm  revolutions per minute
s    second
1.1 INTRODUCTION

There are many biological reactions of potential industrial interest where the reactants and/or products are only sparingly soluble in an aqueous environment. This necessitates the use of large volume reactors and large amounts of biocatalyst in order to increase the productivity of the reaction.

It is however possible to overcome some of the problems associated with the biotransformation of a water-insoluble organic compound by the addition of an organic phase (Reviews by Lilly, 1982; Lilly, 1983). This organic phase, which can occupy a large proportion of the reactor volume, may consist of the reactant alone or the reactant dissolved in a water-immiscible organic solvent. The use of a two-liquid phase system as described above makes it possible to increase the concentration of water-insoluble organic compound in the reactor, this being due to the greater solubility of the water-insoluble organic compound in the organic phase.

The presence of a second liquid phase has other potential advantages: (1) Higher concentrations of water-insoluble product can be achieved in the reactor, (2) Reactant/product inhibition may be reduced, (3) Biocatalyst and product separation may be enhanced, (4) Oxygen may be able to dissolve to higher levels in the reactor due to increased solubilities in organic solvents, (5) It may also be possible to carry out thermodynamically unfavourable reactions (Lilly and Woodley, 1985), (6) The partition of a water-insoluble product into the organic phase may also protect it from any further breakdown by the biocatalyst, as well as minimising any product inhibition.
There are however problems associated with using a two-liquid phase system. The presence of an organic phase is known to affect the activity and/or stability of the biocatalyst, bringing about total inactivation in some cases, for example benzene and toluene (Playne and Smith, 1983).

The second phase may also introduce mass transfer problems. It is important to consider the potential fire and health risks that would be encountered when using volatile and flammable solvents on a large scale. It is well known that many organic solvents inactivate biocatalysts, however, study of the exact mechanisms of inactivation by the various organic solvents has received little attention.

It is important to be aware of physiological phenomena, and in most cases bacteria can only be studied under well-specified conditions. Pseudomonads are particularly suitable for physiological observations; their growth requirements are in most cases extremely simple and their generation time is relatively short even in mineral media. The genetics and biochemistry of pseudomonads is also well known, as is their behaviour in large scale fermentations.

This thesis sets out to look at the interaction of organic solvents with Pseudomonas putida, and in particular with the hydroxylation of naphthalene by the enzyme toluene dioxygenase.

The effects of organic solvents upon the Gram-positive bacterium Arthrobacter simplex have been documented, (Hocknull, 1989; Hocknull and Lilly, 1987). In the present study P. putida UV4 was employed to determine the response of a Gram-negative bacterium to organic solvents. Gram-negative bacteria have an extra membrane, the outer membrane, a structure which is not present in
Gram-positive bacteria. The effects of organic solvents upon this outer membrane and its role in shielding the cytoplasmic membrane from the inhibitory effects of organic solvents are detailed.

The bioconversion of naphthalene by the enzyme toluene dioxygenase was chosen for study in a two liquid phase system for the following reasons: (1) The double hydroxylation of naphthalene by toluene dioxygenase, as with other aromatic oxygenases, can be carried out in intact cells only. It is not possible to obtain active preparations of toluene or naphthalene dioxygenases due to the instability of the enzyme complexes (Catterall and Williams, 1971; Yeh et al., 1977; Geary et al., 1984). It is therefore important to study the behaviour of such cellular biocatalysts in two-liquid phase systems.

(2) The toluene and naphthalene dioxygenase enzyme complexes have been well characterised which will hopefully assist in understanding the possible interaction of organic solvents with the complexes in a two-liquid phase system.

(3) Toluene dioxygenase has been shown to be a soluble enzyme, (Geary et al., 1984), with a requirement for the co-factor NADH. It is therefore possible to study the effect of organic solvents upon co-factor regeneration and the effect of disrupting membrane organisation on enzyme activity.

(4) Toluene dioxygenase and other aromatic dioxygenases are of great interest to industry, and it is likely that findings obtained from this system will be directly applicable to other industrially important systems.
Toluene and naphthalene dioxygenases are multicomponent enzyme systems (Ensley et al., 1982; Ensley and Gibson, 1983; Yeh et al., 1977; Geary et al., 1984) which catalyse the hydroxylation of naphthalene, toluene and related compounds, forming naphthalene-1,2-dihydro-1,2-diol from naphthalene. (see Fig. 1.1).

Fig. 1.1 Hydroxylation of naphthalene by toluene and naphthalene dioxygenases forming cis-1,2-dihydro-1,2-diol

In Pseudomonas strains which have been isolated by selective enrichment on naphthalene as a sole carbon source (Brilon et al., 1981), the genes coding for the early enzymes of naphthalene metabolism including naphthalene dioxygenase are usually carried on plasmids (Cane and Williams, 1982; Connors and Barnsley, 1982). The enzyme toluene dioxygenase, of P. putida UV4, is however thought to be coded for on the chromosome and not on a plasmid (Taylor, 1987). The hydroxylation of many aromatic compounds such as toluene, benzene, anthracene and phenanthrene, can be performed by the enzymes toluene and naphthalene dioxygenase. There are
difficulties in obtaining purified preparations of such enzyme complexes and any observed activity is low (Catterall & Williams, 1971). This necessitates the use of whole cells in biotransformations involving aromatic dioxygenases. It was therefore considered to be of great interest to investigate the interaction of organic solvents with the Gram-negative bacterium Pseudomonas putida in a range of two-liquid phase systems, especially with the hydroxylation of naphthalene by the enzyme toluene dioxygenase and the regeneration of the co-factor NADH.

1.2 The microbial hydroxylation of naphthalene.

Many strains of Pseudomonas are capable of growing on naphthalene as a sole carbon source, usually via a plasmid encoded naphthalene degradation pathway. Naphthalene is first hydroxylated at positions C-1 and C-2 to form the dihydrodiol by the enzymes naphthalene and toluene dioxygenase (Fig.1.1). The dihydrodiol is then converted to salicylate with the release of pyruvate via five further enzymic steps, See fig.1.2. Salicylate is then catabolised to central metabolites by the meta (plasmid encoded) or ortho (chromosomally encoded) pathways (Cane and Williams, 1986).

The Pseudomonas testosteroni A3 Naphthalene dioxygenase is an exceptionally non-specific enzyme capable of hydroxylating many analogues of naphthalene (Knackmuss, 1976; Brilon et al, 1981). The dihydrodiols formed are then further degraded through the naphthalene degradation pathway as for naphthalene. However 2-naphthoic acid after being hydroxylated to form 1,2-dihydroxy-
THE UTILISATION OF NAPHTHALENE BY PSEUDOMONAS PUTIDA

Naphthalene oxidation

Salicylate oxidation

Fig. 1.2 Pathway for the catabolism of naphthalene. Enzymes (genes) shown are: 1, naphthalene dioxygenase (*nahA*); 2, naphthalene cis-dihydrodiol dehydrogenase (*nahB*); 3, 1,2-dihydroxynaphthalene dioxygenase (*nahC*); 4, 2-hydroxychromene-2-carboxylate isomerase (*nahD*); 5, 2-hydroxybenzalpyruvate aldolase (*nahE*); 6, salicylaldehyde dehydrogenase (*nahF*); 7, salicylate hydroxylase (*nahG*); 8, catechol 2,3-oxygenase (*nahH*); 9, 2-hydroxymuconic semialdehyde dehydrogenase (*nahI*); 10, 4-oxalocrotonate tautomerase (*nahJ*); 11, 4-oxalocrotonate decarboxylase (*nahK*); 12, 2-oxo-4-pentenoate hydratase (*nahL*); 13, 2-oxo-4-hydroxypentanoate aldolase (*nahM*); 14, catechol 1,2-oxygenase (*catA*); 15, *cis,cis*-muconate lactonizing enzyme (*catB*).

*Taken from Cane, 1986*
1,2-dihydronaphthalene-2-carboxylic acid accumulates in the culture medium as a "dead-end" product and can be used as a measure of dioxygenase activity. (Knackmuss, 1976).

It is also possible to study the activity of naphthalene and toluene dioxygenases by using mutant strains in which dihydriodiol dehydrogenase, the second enzyme in the degradative sequence, has been inactivated. The dihydriodiol therefore accumulates and can be used as a measure of the dioxygenase activity. This was the case for *P. putida* UV4 used in this study.

1.2.1 **Reaction mechanism.**

Naphthalene is converted to (+)-cis-1(R),2(S)-dihydroxy-1,2-dihydro-naphthalene by both naphthalene and toluene dioxygenases, (see Fig. 1.1). The reaction requires NADH as an electron donor as well as molecular oxygen (Jeffrey *et al.*, 1975; Jerina *et al.*, 1971). Gibson and his colleagues using a mutant strain of *P. putida* revealed that both atoms of the molecular oxygen involved in the hydroxylation of naphthalene and other aromatics are stereospecifically incorporated into the naphthalene nucleus to form cis-naphthalene dihydrodiol (Gibson *et al.*, 1970; Jeffrey *et al.*, 1975; Jerina *et al.*, 1971). Hayaishi (1964) studied naphthalene degradation in *P. putida* 119, and using radiolabelled oxygen also found that both atoms of the oxygen incorporated into the naphthalene dihydrodiol arose from the same molecule of molecular oxygen. The above findings confirm that the enzymes naphthalene and toluene dioxygenase are true dioxygenases in character.

NADPH has been shown to act as an electron donor for naphthalene
oxidation, however, its activity was less than 50% of that observed with NADH in *Pseudomonas putida* NCIB 9816 (Ensley *et al.*, 1982). Similar results have been reported by Griffiths and Evans, (1965), and Catterall and Williams, (1971).

Naphthalene after being hydroxylated to form the dihydrodiol is dehydrogenated by the enzyme *cis*-dihydrodiol dehydrogenase to form the diol, which is further degraded to central metabolites. The dihydrodiol can be accumulated as a "dead-end" product if a mutant strain of *Pseudomonas* is used, as mentioned earlier, in which the *cis*-dihydrodiol dehydrogenase has been inactivated.

The analogue of naphthalene, 2-naphthoic acid, is also hydroxylated by naphthalene dioxygenase at positions C1 and C2, as for naphthalene, to form (+)-*cis*-1,2-dihydroxy-1,2-dihydronaphthalene-2-carboxylic acid which accumulates as a "dead-end" product. The dihydrodiol of 2-naphthoic acid accumulates due to the fact that naphthalene *cis*-dihydrodiol dehydrogenase has no affinity for it, this being due to the presence of a carboxyl group at C-2. It is therefore possible to study the activity of naphthalene dioxygenases from pseudomonads containing fully intact naphthalene degradation pathways by using 2-naphthoic acid as a substrate. The enzyme toluene dioxygenase from *P. putida* UV4 used in this study was unable to hydroxylate 2-naphthoic acid.

It should be noted that the double hydroxylation required the presence of NADH, and the amount of 2-naphthoic acid co-oxidised by resting cells was limited. Constitutively utilisable substrates providing NADH, such as succinate, malate or salicylate, raised the yield of the co-oxidation products (Knackmuss, 1976).
1.2.2 The involvement of co-factors and co-factor regeneration.

As mentioned in section 1.2.1 the double hydroxylation of naphthalene and its analogues requires the presence of NADH as a co-factor. NADH and to a lesser extent NADPH provide the reducing power required. In order that the hydroxylation may proceed at a favourable rate, the cells must have high levels of NADH. The NAD\textsuperscript* created from the hydroxylation of naphthalene must therefore be reduced back to NADH. The addition of substrates such as glucose, ethanol, succinate, malate, salicylate etc. can provide the reducing power needed to regenerate NADH from NAD\textsuperscript* as they are metabolised by the cell.

It has been shown that resting cells, in which the level of co-factor regeneration is low, show only limited dioxygenase activity (Knackmuss, 1976; Brilon et al, 1981). Enzyme activity is also significantly stimulated by FMN and FAD suggesting that a flavoprotein similar to those reported to participate in benzene dioxygenase is present (Axcell and Geary, 1975).

1.2.3 Properties of naphthalene and toluene dioxygenases.

The naphthalene and toluene dioxygenases of *Pseudomonas putida* have been studied by several groups, in an attempt to elucidate their structure and functioning.

Naphthalene and toluene dioxygenases are multicomponent enzyme systems which can be resolved into three protein components by DEAE-cellulose chromatography (Geary *et al*, 1984; Ensley *et al*, 1982). The enzyme systems rapidly lose activity upon exposure to air and are therefore difficult to purify to any great extent.
in an active form. Laborde (1979) however observed that
dioxygenase activity was stabilised by incorporating ethanol,
glycerol and dithiothreitol into the buffer used to prepare cell
extracts. Ensley et al (1982) used this observation to resolve
naphthalene dioxygenase into three protein components which were
found to be essential for dihydrodiol formation. The components
were named A, B, and C by Ensley. Component A was found to be the
NADH binding protein which would reduce cytochrome c in the
presence of NADH or NADPH. Cytochrome c reduction was enhanced by
the addition of FAD and FMN, and a similar enhancement of
dioxygenase activity by the addition of flavin nucleotides was
observed. Components B and C were found to be iron-sulphur
proteins (Geary et al, 1984; Ensley et al, 1982; Ensley and
Gibson, 1983). Component B bound naphthalene which was oxidised
to product only upon the addition of components A and C, NADH and
oxygen. These results, together with the detection of 6.0g-atoms
of iron and 4.0g-atoms of acid labile sulphur per mole of the
purified enzyme suggest that component B is an iron-sulphur
protein which functions in the terminal step of naphthalene
oxidation. Ensley and Gibson (1983) upon purifying component B
found it to have an Mr ≈ 158,000 which could be resolved into two
subunits with molecular weights of ≈ 55,000 and 20,000, indicative
of an α₂β₂ quaternary structure.
These observations suggest that naphthalene dioxygenase is similar
to the three component benzene / toluene and pyrazon dioxygenases
(Axcell and Geary, 1975; Subramanian et al, 1981; Sauber et al,
1977). Each of these multicomponent systems contains a
flavoprotein that transfers electrons from reduced pyridine
nucleotides to a small non-haem iron protein which in turn reduces
a terminal non-haem iron-containing oxygenase. Naphthalene
dioxygenase, however, has some additional properties which suggest
that this enzyme system has characteristics in common with a wider
range of oxygenases, for example, the ability of component A to
reduce cytochrome c in the absence of components B and C, its
ability to use NADH and NADPH as an electron donor, and the
stimulation by FAD and FMN suggest that this protein may be an
iron-containing flavoprotein similar to those involved in the
benzoate dioxygenase (Yamaguchi and Fugisawa, 1978). It should
also be noted that the addition of exogenous iron to the purified
naphthalene dioxygenase enzyme system resulted in little increase
in activity (Ensley et al., 1982). The purified toluene and
pyrazon dioxygenases mentioned above require the addition of
exogenous iron for maximal activity.
No cross-reactivity is observed between the components of the
toluene and naphthalene dioxygenase subunits, indicating that the
enzymes although similar in make-up are not one and the same,
(Zamanian and Mason, 1978).
The flow of electrons in the toluene and naphthalene dioxygenase
systems is thought to be from NADH → component A → component C →
component B (Ensley and Gibson, 1983; Geary et al., 1984) see fig.1.3.
The most successful purification of naphthalene dioxygenase
reported in the literature is that of Dua and Meera (1981). Dua
and Meera used Corynebacterium renale and achieved a 420-fold
purification. The enzyme followed Michaelis-Menten kinetics with
K_m values of 2.9mM and 1.42mM for naphthalene and NADH
respectively. Maximal activity was found at 30°C and pH 6.5.
IETP = Intermediate electron transport protein

Fig. 1.3  The organisation of proteins in naphthalene dioxygenase.

(adapted from Geary, 1984)
No substrate or co-enzyme inhibition or activation was observed in this system.

The naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816, when purified, was inactivated upon exposure to air and could be reactivated by reducing agents. This loss of activity and subsequent reactivation has been attributed to oxidation and reduction of the Fe²⁺ ions contained within the iron-sulphur proteins (Catterall and Williams, 1971). Catterall and Williams (1971) when studying the specificity of the naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816 found that cell-free extracts would utilise anthracene, phenanthrene, 2-methylnaphthalene and others, but no oxygen uptake was observed for benzene. Jeffery *et al.* (1975) however found that cell extracts of *P. putida* could utilise both benzene and toluene but were unable to support growth. This may be due, however, to the presence of other oxygenases in the crude extracts.

One other point which must be considered is the affect of freezing the cells at -40°C upon the enzyme activity, stability and response to organic solvents. It is known that freezing the cells reduces the dioxygenase activity, (Brazier, 1989), and that continued freeze-thawing can drastically reduce this activity. All activity was lost on heating at 55°C for 5 minutes (Catterall and Williams, 1971).

It is important to note, that when measuring the activity of toluene and naphthalene dioxygenases, the rate of product formation (dihydrodiol), and not the rate of naphthalene disappearance should be taken. It has been shown (Shamsuzzaman and Barnsley, 1974) that naphthalene is concentrated within the cells of *Pseudomonas* even in the absence of any dioxygenase
activity. The rate of naphthalene disappearance could therefore appear to be greater than the rate of dihydrodiol formation. To monitor naphthalene disappearance could therefore give a false representation of enzyme activity. It is preferable therefore to measure product formation to gain an insight into the true level of biocatalyst activity. A similar phenomenon was reported to occur in experiments on cholesterol oxidation using *Nocardia rhodochrous* (Lilly, 1982) in which substantial amounts of cholesterol were found to adhere to the cells.

1.2.4 *Induction of naphthalene and toluene dioxygenases.*

The genes coding for the breakdown of naphthalene, toluene and other aromatics, including *nahA* and *tolA*, which code for naphthalene dioxygenase and toluene dioxygenase respectively are usually carried on plasmids (Dunn and Gunsalus, 1973; Williams and Murray, 1974; Boronin et al, 1977, 1980; Cane and Williams, 1982). The toluene dioxygenase produced by *P. putida* UV4 used in this study is however thought to be chromosomally encoded (Taylor, 1987).

*Pseudomonas putida* NCIB 9816, also called *Pseudomonas* P$_e$ (Williams et al, 1975) contains two plasmids, the larger of which, (87Kbp), encodes the enzymes for the conversion of naphthalene as far as catechol, which is then further metabolised by the enzymes of the chromosomally encoded *ortho* pathway, see figure 1.2, (Cane and Williams, 1986). The plasmid is designated NAH2 by Connors (1982). Naphthalene dioxygenase is found to be induced in several Pseudomonads when grown upon salicylate as a sole carbon and
energy source, or when salicylate is added to cultures growing on succinate, during early log phase and allowed to grow until the population has increased two-fold. The activity of the naphthalene dioxygenase achieved using succinate and salicylate is however low when compared to the level of activity achieved from growing cells on salicylate alone (Shamsuzzaman, 1974). 2-aminobenzoic acid and 2-hydroxybenzyl alcohol, analogues of salicylate, have been observed to induce naphthalene dioxygenase gratuitously. The current view is that salicylate is the inducer and induces all of the enzymes from naphthalene dioxygenase to salicylate dehydrogenase co-ordinately. Enzymes involved in the breakdown of salicylate via the meta pathway to form pyruvate and acetaldehyde have also been shown to be encoded upon nah plasmids, however these genes are usually not active and salicylate breakdown to form succinate and acetyl CoA is brought about through the ortho pathway which is chromosomally encoded (Cane and Williams, 1986).

Toluene dioxygenases have been shown to be induced by toluate and toluene, (Zeyer et al, 1985; Yeh et al, 1977).

The expression of toluene dioxygenase by P. putida UV4 in this study was constitutive, being catabolite repressed at high glucose concentrations, no inducer was therefore required.

1.2.5 The influence of oxygen concentration on the induction of naphthalene and toluene dioxygenases.

As oxygen is one of the substrates for both naphthalene and toluene dioxygenases, it is likely that it may exert an influence on the induction of the enzyme. Buckland et al (1976) observed
that the cholesterol oxidase of *Nocardia* was influenced by the dissolved oxygen tension and that maximal cholesterol oxidase production was obtained at 30% - 40% of air saturation. Hanisch et al (1980) also studied the effect of dissolved oxygen tension and found the induction of progesterone 11 α-hydroxylase in *Rhizopus nigricans* to be maximal at 10 - 15% of air saturation. Russel (1981) also observed that the synthesis of salicylate hydroxylase may be influenced by oxygen concentration. The highest rates of induction being observed at lower oxygen transfer rates. It has been suggested by Russel that salicylate hydroxylase synthesis is controlled by catabolite repression. As the rate of salicylate turnover per mole of enzyme falls, so intracellular catabolite concentration is reduced and enzyme synthesis is derepressed. It is likely that a reduction in the dissolved oxygen tension would bring about such a decrease in salicylate turnover per mole of enzyme, resulting in the synthesis of more salicylate hydroxylase.

It therefore seems likely that careful control of dissolved oxygen tension during the induction of naphthalene and toluene dioxygenases may result in enhanced levels of induction and more efficient biocatalysts.

1.3 Two-liquid phase biocatalysis.

A two-liquid phase system in this context is one in which both an organic phase and aqueous phase are present. The organic phase acts as a reservoir of substrates which partition into the aqueous phase, as required. A number of advantages can be gained from the use of such a
two-liquid phase system when carrying out the bioconversion of compounds of low aqueous solubility. It is possible using a two-liquid phase system to obtain high substrate and/or product concentrations in the reactor, and possibly reduce any substrate/product inhibition which may be occurring. The use of a two phase system may also ease the process of biocatalyst and product recovery. The partitioning of products into the organic phase can also reduce the possibility of any undesirable side reactions occurring in the aqueous phase eg. hydrolysis.

There are however potential disadvantages in using a two phase system. Many organic solvents are known to inactivate or denature biocatalysts. This inactivation by the organic solvent could be due to solvent dissolved in the aqueous phase, contact between the biocatalyst and the aqueous-organic interface, or a combination of the two. The presence of a second phase may also bring about mass transfer problems between the two phases, this being related to solvent viscosity, affinity for substrates, temperature etc.

It is useful to classify two phase systems in order to assist in the design of new industrial processes and to further our understanding of these systems.

The classification of these two phase systems has been carried out by Lilly (1982); Lilly (1983), based upon phase ratio, the site of the bioconversion ie. at the liquid-liquid interface or in the bulk aqueous phase, the presence or absence of organic solvent and the type of biocatalyst used ie. free in solution or immobilised.

A more recent study by Lilly & Woodley (1985) based its classification initially on the theoretical concentration profiles for the transfer of a water-insoluble reactant from the organic phase to the biocatalyst, and upon the relative phase volumes
which determines whether the aqueous phase is continuous or discontinuous. Classification was also carried out in relation to catalyst type (soluble or insoluble-suspension or fixed bed), phase distributions of reactants and products were also used in classification.

Three theoretical concentration profiles were depicted by Lilly & Woodley (1985) (See Fig.1.4). Profile A depicts the profile observed when a soluble biocatalyst such as an enzyme is used. It can be seen from profile A that there are changes in reactant concentration at the interface because of film resistances to mass transfer and partitioning of the reactant between the phases. Profile B depicts the profile observed when an insoluble biocatalyst such as a cell is used. This system has an extra set of concentration gradients and partition coefficients which have to be taken into consideration. When the volume of the aqueous phase is reduced relative to the organic phase, a situation is reached where there is no longer a discreet aqueous phase between the organic phase and the insoluble biocatalyst. The biocatalyst in this case is surrounded by a "shell-like" aqueous film and is depicted by profile C.

The transfer of components across the interface does affect the performance of the reactions. This transfer will in part be governed by the interfacial area per unit volume, between the aqueous and organic phases as well as solvent viscosity, stirrer speed and phase ratio. The affinity of the solvent for the substrate should also be considered as this will affect the rate of partitioning into the aqueous phase.

Lilly & Woodley (1985) finally distinguished five alternative
Fig. 1.4 Theoretical concentration profiles for transfer of a water-insoluble reactant from an organic phase to a biocatalyst. The films at liquid/liquid and liquid/solid interfaces are shown by dashed lines.
reaction systems (See Fig.1.5) based upon phase ratio and biocatalyst form.

1.3.1 Optimisation of organic solvent in a two-liquid phase system.

Although the use of organic solvents has its advantages, for example, increasing substrate concentrations in the reactor, reducing possible substrate/product inhibition and easing the recovery of biocatalyst and product, there are limitations. Many organic solvents are known to inactivate biocatalysts. Brink and Tramper (1985) have tried to characterise the effects of solvents upon activity by relating solvent polarity, determined by the Hildebrand solubility-parameter ($\delta$), to the rate of microbial epoxidation of propene and 1-butene. They concluded that high biocatalytic rates can be expected when the polarity of the organic solvents is low, and its molecular weight above 150. Laane et al (1985) is however of the opinion that $\delta$ is a poor measure of solvent polarity, and that a better correlation between the rate of epoxidation and solvent polarity can be obtained using the partition coefficient, log P. Laane observed that epoxidation activity was low in relatively polar solvents of log P < 2, variable between log P 2 and 4 and high in apolar solvents of log P > 4 (Partition was measured in a standard water-octanol two-phase system).

Laane et al (1987a) has suggested that the solvents are having their deleterious effects due to their ability to distort the essential water layer around the biocatalysts. Laane et al (1987b) also demonstrates that further optimisation in a two phase system
Fig. 1.5 Diagramatic representation of the five alternative reaction systems
(Taken from Lilly and Woodley, 1985)
can be achieved by tuning the polarity of the organic phase and the interphase between the biocatalyst and the organic solvent to that of the substrate and product. Boeren et al. (1987) have shown that microbial viability, when required for biocatalytic activity, places further constraints upon the optimisation procedure and on the choice of solvents used.

Other factors which influence solvent choice are:

- solvent capacity for substrates and products,
- substrate and product log P,
- solvent volatility,
- health aspects such as toxicity and flammability.

1.3.2 Solvent affinity for substrate(s) and product(s).

The use of an organic solvent in a bioconversion involving a water insoluble substrate and/or product allows the bioconversion to operate at high substrate and product concentrations, thus improving the recovery of any products formed. It is possible to obtain greater concentrations of reactants in a two-liquid phase system because of the greater solubility of the relatively aqueous insoluble substrate(s) in organic solvents.

The organic phase acts as a reservoir of substrates which partition into the aqueous phase to saturate it. As the substrate is utilised by the biocatalyst, more substrate must partition into the aqueous phase. The affinity of the solvent for the substrate may greatly affect this partitioning rate. A solvent of high log P, i.e. more hydrophobic in nature, is likely to have a higher affinity for a non-polar substrate, this high affinity may limit the rate of substrate partitioning into the aqueous phase and
create mass transfer problems. It is necessary therefore to select a solvent system taking solvent, substrate and product polarities into account, so that mass transfer of substrates into the aqueous phase and products out into the organic phase do not become limiting.

Buckland (1975) found that the concentration of cholesterol could be increased by two orders of magnitude, by using organic solvents, over the concentration achieved in water.

1.3.3 The solubility of organic solvent in the aqueous phase.

It is not clear whether the deleterious effects of many organic solvents upon biocatalysts are due to the solvent dissolved in the aqueous phase or to contact between the biocatalyst and the aqueous / organic interface. If the deleterious effects are found to be due to solvent dissolved in the aqueous phase, then the need to immobilise would be reduced and the solubility of the solvent in the aqueous phase would become a main point of concern. It is of use to know the solubilities of solvent in the aqueous phase when selecting a solvent system. It is also important to note that the solubility of organic solvent can be influenced by the presence of free enzyme or microorganisms in the aqueous phase, eg. the solubility of hexadecane in spent growth medium increases in proportion to the concentration of protein in the medium (Kappelli & Finnerty 1980).
1.3.4 **Biocatalyst activity and stability in a two-liquid phase system.**

One of the potential disadvantages of using a two phase system is the possibility that the organic solvent may have deleterious effects upon the biocatalysts used.

It is well known that many organic solvents affect biocatalyst activity and stability, but it is still unclear as to the exact mechanisms of this inactivation.

A number of groups have attempted to correlate the properties of the organic solvents used in two phase systems to their affects upon biocatalyst activity and stability, in an attempt to lay down a set of rules to assist in the optimisation of two-liquid phase reaction systems.

Brink & Tramper (1985) and Laane et al (1985) have shown that biocatalyst inactivation, in the case of cells, is related to the polarity of the solvent used, which in the opinion of Laane et al (1985) is best represented by log P. It was found that solvents of low log P < 2, ie. the most polar solvents, inactivated cells rapidly, and that solvents of log P > 4, ie. less polar and more hydrophobic, were less likely to bring about rapid inactivation of the cells.

It has been reported by Lilly (1982) that reasonably good stabilities have been observed in two-liquid phase systems.

Omata et al (1980) has reported that the immobilisation of cells confers a greater operational stability upon cells catalysing menthyl ester hydrolysis in water saturated n-heptane. Similar results of immobilisation were obtained by Yamane et al (1979). It
is not clear however if this was a result of increased stability alone, or due to the removal of any direct contact of the cells with the organic/aqueous interface.

Many of the biotransformations carried out using microorganisms in a two phase system are performed in the absence of nutrients, in a buffer system eg. 50mM Tris.HCl or phosphate buffer. A carbon source may be supplied, such as ethanol or malate, in cases where reducing power in the form of NADH is required for the bioconversion under investigation to occur. It has been shown that *Pseudomonas* can hydroxylate only limited amounts of 2-naphthoic acid in a resting state, due to the lack of reducing power in the form of NADH (Knackmuss *et al*, 1976). The addition of malate, succinate or salicylate raised the yield of the co-oxidation product considerably, this being due to regeneration of the co-factor NADH (Brilon *et al*, 1981).

The addition of a carbon source may enhance co-factor regeneration as well as providing energy to fuel some cell maintenance, however the absence of a nitrogen source limits the amount of protein synthesis the cells can undertake in the two phase system. The addition of a nitrogen source may increase the observed operational stability of the biocatalyst by allowing protein synthesis to replace enzymes lost due to solvent denaturation or natural protein turnover. It has been shown that the addition of an inhibitor of protein synthesis such as chloramphenicol results in a dramatic decrease in operational stability (Kloosterman & Lilly, 1985), indicating that even in the absence of exogenous nutrients *de novo* protein synthesis is in some cases beneficial to maintaining a good level of operational stability. The addition of exogenous nutrients including a nitrogen source may increase
operational stability still further.
A reduction in cell energy levels can enhance the rate of cell
proteolysis resulting in a decreased operational stability
( Goldberg and St.John, 1976 ). This increased level of
proteolysis brought on by poor nutritional conditions is likely to
occur in a two phase system in which the cells are suspended in
buffer alone. Kloosterman and Lilly (1985) found that the addition
of D-glucose enhanced operational stability. The D-glucose was
found not only to maintain sufficient energy levels to prevent
triggering of cell proteolysis, it also supplied the energy
required for the continuous de novo synthesis of enzymes which
enabled the cells to utilise the available D-glucose. If an
inducer of the enzyme systems involved in the bioconversion could
be added to allow their preferential synthesis, then operational
stability may benefit from the addition of a nitrogen and carbon
source.

1.3.5 The immobilisation of biocatalysts and the importance of
immobilisation in increasing biocatalyst stability and in
reducing solvent damage.

Immobilisation of the biocatalyst can confer a number of
advantages to both aqueous and two-liquid phase systems. Product /
biocatalyst separation in an aqueous system, or a two-liquid phase
system in which the product is water soluble, can be greatly
enhanced if the biocatalyst is immobilised either in or on a
support matrix. Immobilisation is also frequently found to
increase operational stability in both aqueous and two-liquid
phase systems.
If the deleterious effects of many organic solvents are found to be due to direct contact between the biocatalyst and the organic/aqueous interface, then entrapment within a gel matrix such as calcium alginate, preventing direct contact with the interface may increase operational stability. This was found to be the case for the Δ¹-dehydrogenation of hydrocortisone by the Gram-positive bacterium *Arthrobacter simplex* (Hocknull, 1989).

There are however a number of disadvantages which have to be considered when determining the value of immobilisation to any particular system which include the following.

1. Entrapment in a network of small pore size reduces the access of high molecular weight molecules into the cells. This would reduce the reaction rate if the substrate was of a high molecular weight.

2. Hydrophilic gels, for example calcium alginate, limit the rate of diffusion of hydrophobic substances into and out of the gel matrix. It is possible to promote the penetration of a hydrophobic substrate into a hydrophilic gel in order to increase the reaction rate by using a highly polar organic solvent. This however also results in a more rapid inactivation of the entrapped cells (Omata *et al*., 1980).

3. Biocatalyst contact with the gel monomers before polymerisation has been shown to be detrimental to the viability of the cells and to biocatalyst activity (Ohlson *et al*., 1978).

4. The presence of organic solvent in the aqueous phase can lead to irreversible bead shrinkage (Ohlson *et al*., 1978; Carrea *et al*., 1979).

5. Oxygen transfer into the beads may also be limiting if oxygen is required as a substrate or for cell maintenance.
Fukui *et al* (1980; 1984) observed that the conversion of highly hydrophobic steroids in non-polar organic solvents could only be carried out using cells entrapped in a hydrophobic support matrix. Transformation activities were closely correlated to the partition coefficients of substrates, and in some cases co-factors, between the gel matrix and the organic solvent system used.

One of the major problems associated with using hydrophobic supports arises when either one of the substrates or co-factors is water soluble, for example PMS the artificial electron acceptor in its oxidised form is insoluble in organic solvent, showing a low partition coefficient towards hydrophobic gels leading to low activity (*Fukui et al* 1980; *Fukui†Tanaka* 1984).

Although the partitioning of hydrophobic substrates into hydrophilic gels may reduce the specific activity of the biocatalyst, the stability of the cells is increased and good levels of conversion have been observed (*Duarte & Lilly*, 1980). It is therefore important to consider all the above mentioned factors which may influence the reaction rate and operational stability when determining the value of immobilisation.

One other point which must be considered is the stability of the gel matrix itself. Calcium alginate gels have been found to be unstable in the presence of various complexing anions, such as phosphate, citrate, EDTA and lactate. The use of alginate gels would therefore be of little use in phosphate buffer. It is however possible to use Tris.HCl buffer which does not have such a drastic affect upon the gel stability, although Tris buffer has been shown to increase the permeability of the outer membrane of some Gram-negative bacteria (*Hancock*, 1984). This increased permeability may increase the rate of biocatalyst inactivation in...
a two-liquid phase system by allowing easier access of organic solvents into the cell, and by enhancing any co-factor leakage which may be occurring.

Birnbaum et al (1981) has developed a method of covalently stabilising calcium alginate so that the physical and chemical stability in buffer containing phosphate can be enhanced. Preparations were found to remain stable in phosphate buffer for at least ten days without substantial release of cells. The physical stability of calcium alginate beads has also been reported to be significantly improved by the use of an air lift loop reactor ( Kloosterman & Lilly, 1985 ).

1.4 Organic solvent - biocatalyst interactions

It is well known that the presence of an organic solvent in a two-liquid phase system can bring about biocatalyst inactivation, resulting in loss of activity and / or stability.

Although attempts have been made to characterise these organic solvent - biocatalyst interactions with respect to organic phase polarity ( Brink & Tramper, 1985; Laane et al, 1985 ) and other physical characteristics of two-liquid phase systems, little attention has been paid to the actual mechanisms of biological inactivation by organic solvents, except for some reports using whole cells with toluene ( Jackson & DeMoss, 1965; DeSmet et al, 1978; Woldringh, 1973 ) and n-alkanes ( Teh & Lee, 1974; Gill & Ratledge, 1973 ).

It is hoped that the elucidation of these mechanisms of biocatalyst inactivation coupled with the knowledge gained from the effects of changing physical parameters within a two phase...
system will greatly enhance the observed biocatalyst operational activity and stability.

It is unclear whether the deleterious effects of organic solvents that have been observed in some cases are due to the solvent dissolved in the aqueous phase, to contact between the biocatalyst and the aqueous / organic interface, or a combination of the two. Brink & Tramper (1985) observed that the introduction of a water-immiscible organic solvent phase brought about inactivation of the cell, as well as clotting of biomass and aggregation of the cells at the liquid-liquid interface. The cells were immobilised in calcium alginate to prevent direct cell-organic solvent contact, however, gel entrapment did not seem to provide any additional protection against the organic solvent and many organic solvents still caused rapid loss of activity.

The immobilisation of cells in hydrophobic gels has been observed to maintain high operational stabilities in two phase systems (Fukui et al, 1980; Fukui and Tanaka, 1984), however, whether this is due to the protection from the organic-aqueous interface provided by the gel matrix is unclear, as it is likely that cell division occurred within the gel due to reactivation with nutrients.

Organic solvents have been reported in the literature to bring about loss of cell viability. This loss of viability could be due to the observed changes in membrane structure, permeability, fluidity and functioning, alterations in cell morphology as well as alterations in cell metabolism. The following sections review the available literature on the potential effects of organic solvents upon microorganisms.
1.4.1 Effects upon membrane permeability and fluidity

The cytoplasmic membrane is a metabolically active, mobile, semipermeable structure upon which many enzyme mediated reactions occur. The electron transport chain, responsible for the generation of energy in the form of ATP and recycling of co-factors, is also an integrated part of the cytoplasmic membrane structure.

The electron transport chain relies upon the generation of a proton gradient over the cytoplasmic membrane, which is dependent upon its semipermeable nature to provide energy for normal cell activity and the proton motive force required for active bacterial motility. Active transport of molecules, for example sugars, over the cytoplasmic membrane is an energy requiring process and is also dependent upon an active electron transport chain.

Damage to the cytoplasmic membrane resulting in loss of selective permeability is likely to result in an inability to regenerate energy and co-factors via the electron transport chain and eventually to loss of cell viability / "death".

Toluene has been shown to render bacteria permeable to low molecular weight compounds and results in leakage of soluble protein and RNA (mainly rRNA) into the environment (Jackson & DeMoss, 1965; DeSmet et al, 1978). The leaked proteins have been characterised by DeSmet et al (1978) and have been found to be from the cytoplasmic and outer membranes. Only in the presence of EDTA did toluene treatment bring about the release of significant levels of cytoplasmic proteins, malate dehydrogenase in particular.
One of the most interesting aspects in the alteration of the outer membrane permeability of Gram-negative bacteria by various agents is that the addition of exogenous Mg$^{2+}$ can reverse these permeability changes (Hancock, 1984). Both Schindler & Osborn (1979) and Coughlin et al (1981) have demonstrated that the lipopolysaccharide (LPS), see figure 1.6, which forms a continuous layer within the outer leaflet of the outer membrane, has a strong divalent cation-binding site. This provides strong evidence that a single class of outer membrane site is involved in all of these permeability alterations, presumably a Mg$^{2+}$ binding site.

It is the belief of Hancock (1984) that the majority of the outer membrane permeability alterations brought about by permeabilising agents are due to interactions with the LPS cation-binding sites which non-covalently cross-bridge adjacent LPS molecules in the presence of Mg$^{2+}$. EDTA treatment of cells brings about the secretion of copious amounts of LPS, presumably due to the removal or displacement of Mg$^{2+}$ from the LPS cation binding sites, resulting in membrane destabilisation. This LPS release increases membrane fluidity (Rottem and Leive, 1977) and may allow an easier passage of hydrophobic molecules through the outer membrane due to this membrane disruption.

The level of protein released by cells in the presence of toluene seems to be dependent on the aqueous concentration of toluene present, and upon the temperature of the incubation (DeSmet et al, 1978; Jackson & DeMoss, 1965). Lower incubation temperatures probably result in a lower solubility of toluene in the aqueous phase, cellular lipid and lipoprotein, resulting in a reduced level of toxicity. It should be noted that the destruction
Fig. 1.6 Lipopolysaccharide molecule showing the three regions (Re-drawn from Stryer, 1981)
of galactoside permease by toluene occurs at 4°C, thus, not all the effects of toluene are temperature dependent.

Hexane has also been observed to remove membrane selective permeability from *Cladosporium resinae*, resulting in a considerable loss of potassium and protein from the cells, possibly due to limited disorganisation of the cell membrane (Tee & Lee, 1974).

The presence of linoleic acid and other long chain unsaturated fatty acids has been shown to increase membrane permeability as evidenced by the measurement of the leakage of 260nm absorbing material and an increase in membrane fluidity (Greenway and Dyke, 1979; Raychowdhury *et al.*, 1985). This increase in membrane fluidity with alteration of the membrane permeability in the presence of fatty acids, due to their surfactant action, may be the cause of the growth inhibitory effects of various fatty acids. It has been shown that Gram-negative bacteria are much more resistant to the inhibitory effects of fatty acids than Gram-positives (Sheu & Freese, 1973). This is possibly due to the presence of the outer membrane, a structure not found in Gram-positive bacteria, preventing fatty acids from reaching the inner fatty acid sensitive cytoplasmic membrane. The impermeability of the outer membrane appears to be related to the structure and distribution of the LPS molecules. "Deep rough" mutants which have lost 80% to 90% of their polysaccharide moiety from their LPS molecules, see figure 1.6, have greatly increased sensitivity towards certain hydrophobic antibiotics and dyes (Roantree *et al.*, 1969).

Gram-negative cells upon EDTA treatment to partially remove the LPS layer are also found to be more sensitive to actinomycin,
lysosyme and other hydrophobic compounds (Leive, 1968). It therefore seems likely that the intact LPS layer of Gram-negative bacteria screens the cells against many hydrophobic compounds, preventing their accumulation in the inner cytoplasmic membrane at inhibitory concentrations.

Growth of *E. coli* in the presence of alcohols of various chain lengths has been shown to radically change their fatty acid composition (Ingram, 1976). These changes represent an adaptive membrane alteration compensating for the direct physiochemical interaction of alcohols with the membrane. Benzyl alcohol (Hubbel et al., 1970; Paterson et al., 1972), as well as ethanol and other alcohols (Grisham, 1973; Hui and Barton, 1973), have been shown to directly affect membrane fluidity. These changes in fluidity are analogous in many ways to those induced by changes in growth temperature (Fulco, 1974; Olson and Ingram, 1975).

Grisham (1973) has shown a close correlation between the ability of the alcohol to disrupt lipid structure and the hydrophobicity of the alcohol, measured by their octanol-water partition coefficients. The use of log partition coefficients (log P) to predict the effect of a particular solvent system on biocatalyst activity and stability is useful and has been used by Laane et al. (1985) in the classification of many organic solvents.

The cytoplasmic membrane bilayer is in a fluid state under normal physiological conditions. Changes in reaction temperature and the intercalation of various hydrophobic molecules into this membrane may result in a changed membrane fluidity. An intrinsic membrane protein in an imperfect analogy can be thought of as a solute molecule in a lipid solvent - its resident lipid bilayer, as only a fraction of such intrinsic membrane proteins are exposed to bulk
water or even the interfacial regions between bulk water and the bilayer itself. Thus it is important to consider how alterations in the lipid environment of a membrane protein across the bilayer affects the activity of the membrane protein.

In certain cases specific membrane enzymes may perturb or interact with surrounding lipids in specific ways. Thus the regulation of membrane enzyme activity by membrane lipids might be specific to particular enzymes and specific lipids (Carruthers and Melchior, 1986).

Carruthers and Melchior (1986) while studying the properties of sugar transport in synthetic bilayers made up of one lipid species, and on an ATPase system, found that no consistent relationship between fluidity and activity was observed. However these bilayers were artificial and may not give an accurate indication of the situation in a native membrane where the intrinsic membrane proteins may interact with more than one type of lipid species and with other membrane proteins. The effect of changes in membrane fluidity upon permeability was not considered by Carruthers and Melchior.

Organic solvents which are found to alter biocatalyst activity and stability, in the case of *Arthrobacter simplex*, seem to have their primary affect upon the electron transport chain (Hocknull and Lilly, 1987; 1988). Selective permeability seems to be perturbed resulting in the disruption of the electron transport chain, which relies upon an intact semi-permeable membrane across which a proton gradient can exist, in order to function.

The Δ¹-dehydrogenation of hydrocortisone by *Arthrobacter simplex* requires the presence of an active electron transport chain in order to regenerate the co-factor FADH from FAD⁺. The addition of
an artificial reducing agent, Phenazine methosulphate (PMS), to
cells which have been permeabilised by certain organic solvents,
showing low enzyme activity, restores the enzyme activity
indicating that the enzyme itself is not the site of initial
biocatalyst inactivation and suggests that the primary site of
biocatalyst inactivation is the electron transport chain
(Hocknull and Lilly, 1987; 1988).

It is interesting to note that motile bacteria, for example
Pseudomonads, possessing flagella require a proton motive force
(pmF) in order to bring about flagella rotation. The disruption
of membrane selective permeability is also likely to result in
loss of bacterial motility. The study of the ability of organic
solvents to halt bacterial motility may also serve as an indirect
method of observing the disruption of the electron transport
chain.

1.4.2 Inhibition of cell transport mechanisms

The uptake of many nutrients into a bacterial cell involves the
expenditure of energy supplied under aerobic conditions by
oxidative phosphorylation. It has been observed that some organic
solvents can have an effect upon nutrient uptake (Sheu & Freese,
1972a+b; Jackson & DeMoss, 1965; Teb & Lee, 1974; Gill &
Rateledge, 1973). This effect may be due to a direct interaction
with the transport proteins involved in uptake, or an indirect
effect via disruption of oxidative phosphorylation, the process
required to power the active transport of such nutrients into the
cell.

Jackson & DeMoss (1965) have shown that toluene brings about
inhibition of amino acid uptake in E. coli, however it is unclear if this is due to a direct interaction with the amino acid permease, or due to disruption of oxidative phosphorylation. Amino acid uptake is also disrupted by various fatty acids. This inhibition of amino acid uptake has been observed in isolated membranes in the presence of an active electron transport chain, indicating that fatty acids have a direct effect upon the amino acid permease, probably by uncoupling the amino acid carrier proteins from the cytochrome-linked electron transport system, to which they may be coupled via protein interaction or via a cation gradient (Sheu et al., 1972b).

Inhibition of glucose assimilation and transport by n-decane and other n-alkanes has been observed in Candida 107 by Gill & Ratledge (1973). n-Decane rapidly inhibited both glucose assimilation and transport. It was also found that inhibition only occurs with metabolisable alkanes, and yeasts which are unable to metabolise n-alkanes are not inhibited by them. The n-alkanes are oxidised via n-alcohols to fatty acids. Fatty acids of chain length C₁₃ or less are not incorporated into cell lipids and significantly, it is alkanes of such chain lengths which are found to be the most potent inhibitors of glucose incorporation. This inhibition of glucose transport cannot be due to inhibition of energy production, to which transport is linked, as the process of fatty acid oxidation is itself an energy yielding process.

Gill & Ratledge (1973) concluded that the effective inhibition of glucose uptake and catabolism was probably due to an accumulation of some intermediate or intermediates of alkane metabolism. Possible intermediates include: (1) The build up of ATP during alkane oxidation and degradation which may lead to inhibition of
the enzymes of glycolysis and the TCA cycle. Such inhibition would bring about accumulation of glucose-6-phosphate which according to Azam & Katyk (1969) would then inhibit the process of glucose transport. However, octane and nonane were most effective inhibitors and were oxidised slowly to generate low levels of ATP. (2) Free fatty acids or their corresponding acetyl CoA esters could themselves be inhibitors of glucose transport. Morgan & Kornberg (1969) have suggested that acetyl CoA brings about the inhibition of glucose uptake in E. coli. Feedback inhibition, if operating in Candida 107 when oxidising alkanes, would lead to the accumulation of acetyl CoA. This would then be reinforced by de novo production of acetyl CoA by β-oxidation of the appropriate fatty acyl CoA esters (see Fig. 1.7).

1.4.3 Effects upon cell morphology and ultrastructure.

Apart from the clotting of biomass (Brink & Tramper, 1985), light microscopy of biocatalysts in the presence of organic solvents does not seem to reveal any morphological changes. Electron micrographs of toluene treated E. coli have revealed that cell lysis does not occur, although the cytoplasm does appear to collapse to the interior of the cell (Jackson & DeMoss, 1965) and partial dissolution of the cytoplasmic membrane coupled with the displacement of nuclear material towards the cell periphery was observed by Woldringh (1973) upon treatment with 0.25% toluene. Freeze-fracture electron microscopy has revealed that toluene treatment of E. coli results in the loss of a fracture plane in the cytoplasmic membrane and in the formation on an extra fracture
Fig. 1.7 A possible sequential feedback inhibition pattern in *Candida* 107 to account for inhibition of glucose transport and metabolism by alkanes (redrawn from Gill & Ratledge, 1973).
plane in the outer membrane. It has been suggested by DeSmet et al (1978) that the loss of this fracture plane is probably due to disorganisation of the bilayer by removing phospholipids from the cytoplasmic membrane. Toluene treatment has also been observed to cause phospholipid vesicles to appear extracellularly in Tris / EDTA treated cells and intracellularly in Tris / Mg²⁺ cells (DeSmet et al, 1978). The formation of an extra fracture plane in the outer membrane upon treatment with toluene is probably due to disorganisation of the outer membrane by removal of LPS from the outer membrane, possibly allowing phospholipid bilayer regions to form.

The outer membrane is less susceptible to the formation of fracture planes due to the lack of phospholipid bilayer regions. Rough mutants have a higher phospholipid content than the smooth wildtype and have more phospholipid bilayer regions (Smit, 1975). The occurrence of fracture planes in the outer membranes of these rough mutants, as revealed by freeze-fracture electron microscopy, is therefore increased (see Fig. 1.8).

The outer membrane of Gram-negative bacteria is found to be much less sensitive to the effects of organic solvents, despite the fact that toluene treatment can result in the appearance of a fracture plane within it, as shown in freeze-fracture electron micrographs. This is probably due to the shielding effect of the continuous layer of hydrophilic lipopolysaccharide within the outer membrane. It would therefore be of interest to see if the shielding provided by the outer membrane LPS, protects the inner membrane from the potentially damaging effects of organic solvents. This may be achieved using rough mutants which lack the polysaccharide moiety of their LPS molecules, see figure 1.6,
Figure 1.8 Schematic representation of the outer membrane of both a wildtype and rough mutant Gram-negative bacterium.

Wildtype outer membrane note the absence of a phospholipid bilayer.

Rough mutant note the presence of phospholipid bilayer regions which enable a fracture plane to be formed during freeze-fracture electron microscopy.
making their surface more hydrophobic in nature. It is likely that these rough mutants will be more rapidly inactivated by organic solvents due to outer membrane disorganisation caused by the removal of the polysaccharide moiety of the LPS.

1.4.4 Effects upon cell metabolism.

It is likely that inhibition of intermediary metabolism by the exposure to organic solvents is due to a number of factors.

(1) Direct interaction of the solvent with the enzyme, resulting in protein unfolding and therefore denaturation.

(2) Loss of cytoplasmic membrane selective permeability, resulting in the inactivation of the electron transport chain. This inactivation would result in a reduction in cell ATP levels and in an inability to regenerate co-factors. The active transport of nutrients into the cell would also be reduced by the inactivation of the electron transport chain.

(3) Inactivation of the cytoplasmic permease systems. Many ions and nutrients, including amino acids, glucose etc., are taken into the cell via specific permease systems.

(4) Inhibition of protein synthesis. Jackson & DeMoss (1965) have observed that treatment of *E. coli* with toluene brings about the inhibition of protein synthesis due to ribosome dissociation. This dissociation occurs in intact cells, with no dissociation being observed *in vitro*. This dissociation in intact cells could be due to a reduction in the *in vivo* levels of magnesium due to loss of selective permeability. Magnesium is required to allow protein synthesis to occur.

The inhibition of protein synthesis halts protein turnover as well
as any enzyme induction.

It is still unclear as to which stage the inhibition of protein synthesis occurs and whether this is due to ribosome dissociation, ATP and magnesium depletion, or inhibition of amino acid uptake mechanisms. The lack of a nitrogen and carbon source during the biotransformation may also reduce the level of protein synthesis observed. (5) Accumulation of toxic intermediates intracellularly.

It has been suggested that the inhibition of glucose transport and assimilation in *Candida 107* by alkanes is due to the accumulation of acetyl CoA. This Acetyl CoA is thought to feedback inhibit the uptake of glucose (see section 1.4.2 and Fig. 1.7).

The inhibition of biocatalysis as well as alterations in intermediary metabolism monitored by oxygen uptake, ATP synthesis, loss of viability, product formation, gas production etc. (Sheu & Freese, 1972; Playne & Smith, 1983; Catterall & Williams, 1971), are due to one or more of the above listed points.

Hocknull and Lilly, (1987; 1988) have demonstrated for the Δ¹-dehydrogenation of hydrocortisone by *Arthrobacter simplex*, that the initial site of inactivation by organic solvents is the electron transport chain and not the enzyme itself.

Replication and repair of DNA in cells of *E. coli* treated with toluene has been observed to occur, although the cells are non-viable. There is however a requirement for ATP. Cells treated with 1% toluene require the addition of both ATP and magnesium, indicating the requirement of an intact electron transport chain and a sufficient level of magnesium within the cell, both of which rely upon the semipermeable nature of the cytoplasmic membrane (Moses & Richardson, 1970).
1.5. Objectives of study

This study set out to investigate the effects of exposure of *Pseudomonas putida* to a selection of organic solvents in two-liquid phase systems, on viability, cell growth, permeability to hydrophobic compounds, motility, and in particular the interactions of organic solvents with the toluene dioxygenase system performing the hydroxylation of naphthalene. It is hoped that the characterisation of the inhibitory effects of many organic solvents upon the microbial physiology of *Pseudomonas putida* will increase our understanding of biocatalyst-solvent interactions with the ultimate aim of increasing biocatalyst stability and activity, as well as providing a set of guidelines by which other two-liquid phase systems may be optimised. The hydroxylation of naphthalene by *Pseudomonas putida* UV4 was also characterised.

The effects of cell freezing upon the activity of the naphthalene dioxygenase, membrane permeability, and the behaviour of these cells in two-liquid phase systems was investigated, in order to assess the value of cell freezing to biocatalyst storage.

The relationship between log P and biocatalyst activity and stability was determined, and a comparison made with the Δ1'-dehydrogenation of hydrocortisone by the Gram-positive bacterium *Arthrobacter simplex* in the presence of organic solvents, to determine the role of the outer membrane of *Pseudomonas putida*, a Gram-negative bacterium, in increasing solvent resistance. The effect of outer membrane disruption on biocatalyst activity and stability was also investigated.
The effects of organic solvents upon the loss of selective permeability / inhibition of the electron transport chain, and the interaction of solvents with toluene dioxygenase complex received particular attention. The effects of organic solvents upon the inhibition of cell motility was also studied. Bacterial motility requires a proton motive force, this also relies upon the intact semipermeable nature of the cytoplasmic membrane. Loss of selective permeability is likely therefore to result in the inhibition of bacterial motility.

Studies were also performed to determine whether the observed toxic effects of organic solvents are due to the solvent dissolved in the aqueous phase, contact between the biocatalyst and the aqueous-organic liquid-liquid interface or a combination of the two. The resolution of the above will determine whether or not biocatalyst immobilisation is of value in cell stabilisation.

The role of immobilisation in increasing biocatalyst stability in the presence of organic solvents was also investigated.
2. MATERIALS, EQUIPMENT AND METHODS

2.1 MATERIALS

2.1.1 Microorganisms

Pseudomonas putida UV 4, supplied by ICI Biological Products, is able to hydroxylate a wide range of aromatic compounds. The enzyme responsible, toluene dioxygenase, is constitutively produced by this mutant, although it is catabolite repressed by glucose. The wild type organism is able to grow on many aromatics as its sole carbon source.

The mutant UV 4 is, however, unable to utilise aromatic compounds as sole carbon source, as it is defective in the dehydrogenase enzyme required for the breakdown of aromatic dihydrodiols which consequently accumulate. Toluene dioxygenase activity was found in cells grown at 28°C with pulsed glucose. No activity is observed above 32°C. P. putida, a Gram-negative bacterium, metabolises glucose via the Entner-Doudoroff pathway (Entner & Doudoroff, 1952), the Embden-Meyerhof pathway being completely absent in Pseudomonads.

Arthrobacter simplex NCIB 8929 was used to investigate the effect of organic solvents upon cell growth, for a Gram-positive bacterium. The Δ¹-dehydrogenation of hydrocortisone performed by this organism in organic-aqueous two-liquid phase has been characterised (Hocknull, 1989).
## 2.1.2 Chemicals

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* denotes a chemical constituent of the defined salts medium.

Figures in (brackets) are solvent log P values.

Log P is defined as the logarithm of the solvent partition coefficient in a standard octanol-water two-phase system.

$$\text{Log} \, P = \log \frac{[\text{Solvent}]_{\text{octanol}}}{[\text{Solvent}]_{\text{water}}}$$

### SOLVENTS

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<td>Dimethyl phthalate (2.3)</td>
<td></td>
</tr>
<tr>
<td>Dichloroethane (1.3)</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td></td>
</tr>
<tr>
<td>Di butylamine (2.7)</td>
<td></td>
</tr>
<tr>
<td>Di-iso octyl phthalate (9.8)</td>
<td></td>
</tr>
</tbody>
</table>
Di-isopentyl ether (3.9) BDH
Ethyl benzene (3.1) Sigma
Methyl cyclohexane (3.7) Aldrich
Phthalic acid diethyl ester (3.3) Sigma
Propyl benzene (3.6) Aldrich
Tetradecene (7.5) Sigma
Toluene (2.5) BDH

2.1.3 Fermentation media

A defined salts medium was used for growth in both shaken flasks and fermentations, based upon Evans et al. 1970. The medium was prepared for carbon limiting growth.

The amount of each component required to make up 1 L is detailed below:

<table>
<thead>
<tr>
<th>Source</th>
<th>Formula / stock molarity</th>
<th>ml required</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus</td>
<td>NaH$_2$PO$_4$.2H$_2$O / 2M</td>
<td>5.0</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>NH$_4$Cl / 4M</td>
<td>20.5</td>
</tr>
<tr>
<td>Potassium</td>
<td>KCl / 2M</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium</td>
<td>Na$_2$SO$_4$.10H$_2$O / 1M</td>
<td>2.0</td>
</tr>
<tr>
<td>Chelating agent</td>
<td>Citric acid / 1M</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>MgCl$_2$ / 0.25M</td>
<td>5.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>CaCl$_2$ / 0.02M</td>
<td>1.0</td>
</tr>
<tr>
<td>Trace metals solution</td>
<td>* *</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Molybdenum \( \text{Na}_2\text{MoO}_4 \) / 0.001M 0.1
 Buffer (shake flasks only) \( \text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} \) / 0.2M 15.0

### Trace metals per 5 L:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl concentrated</td>
<td>5ml</td>
</tr>
<tr>
<td>ZnO</td>
<td>2.04g</td>
</tr>
<tr>
<td>( \text{FeCl}_3 \cdot 6\text{H}_2\text{O} )</td>
<td>27.00g</td>
</tr>
<tr>
<td>( \text{MnCl}_2 \cdot 4\text{H}_2\text{O} )</td>
<td>10.00g</td>
</tr>
<tr>
<td>( \text{CuCl}_2 \cdot 2\text{H}_2\text{O} )</td>
<td>0.85g</td>
</tr>
<tr>
<td>( \text{CoCl}_2 \cdot 6\text{H}_2\text{O} )</td>
<td>2.38g</td>
</tr>
<tr>
<td>( \text{H}_3\text{BO}_4 )</td>
<td>0.31g</td>
</tr>
</tbody>
</table>

Shaken flasks required the addition of phosphate buffer, to control pH, at 15 ml per litre. The final pH adjustments were made with KOH to pH 7.0.

#### 2.1.4 Buffer media

Biotransformations were carried out in both K-phosphate and Tris-HCl buffer at a pH of 7.0 and a molarity of 50mM.

Phosphate buffer was made up using 50mM solutions of dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate. The salts were dissolved to 50mM and the dihydrogen salt added to the dipotassium salt to the desired pH of 7.0.
Tris-HCl was made up by dissolving Tris buffer to 50mM and bringing to the desired pH with dilute HCl. Deionised water was used throughout.

pH was measured using a Pye Unicam pH meter.

2.2 EQUIPMENT

2.2.1 2 litre culture vessel.

_Pseudomonas putida_ was routinely grown in an MBR mini bioreactor (1.9 L working volume, 2.5 L total volume). The glass tank measured 26cm high by 13cm wide, withstanding a gauge pressure of 2.5 Bar. Agitation was provided by two 6 bladed-turbine impellers of diameter 4.5 cm. Stirrer speeds of 700 rpm and 1000 rpm were used for cell growth and sterilisation respectively. The fermenter and medium were sterilised in-situ at 121°C for 20 min, followed by cooling to 28°C prior to inoculation. Heating was provided by two elements within the fermenter, cooling was achieved by circulating water through an internal cold finger. The pH was controlled at 7.0 by the addition of 3M KOH using a Watson Marlow peristaltic pump. Aeration was provided via a Charles Austen air pump (Charles Austen Pumps Ltd. Ordnance Street, Blackburn, Lancs.) at a flow rate of 40 L / hr during cell growth. The air exit line contained a water cooled condenser. Glucose (12.5% w/v) was pulsed, 5 s on 20 s off providing a pulse rate of 2g glucose / hr, using a Watson Marlow peristaltic pump. pH, temperature and rpm were monitored and controlled using
a BCS BioPC package (BCS Software) operated on a Tandon PC. DOT was monitored but not controlled.

2.2.2 75ml aerated stirred tank reactors

Biotransformations were performed in 75ml glass stirred tank reactors, working volume 60ml (see figure 2.1). Reactors were maintained at 28°C in a heated water bath (Grant Instruments Cambridge Ltd.), with stirring via a constant torque Citenco motor (Park Products Ltd. Ordnance Street, Blackburn, Lancs.) which powered a pack of 6 reactors at \( \approx 800 \) rpm. Aeration was provided using a Charles Austen air pump (Charles Austen Pumps Ltd. 100 Royston Road, Byfleet, Weybridge, Surrey.), the flow rate being controlled using 6 in-line Platon needle valve flow meters (flow range 0 - 100 cc/min.).

2.2.3 High Pressure Liquid Chromatography

High pressure liquid chromatography was used to quantify the levels of naphthalene (substrate) and naphthalene-1,2-dihydro-1,2-diol (product) present during biotransformation, and to determine saturation concentrations of naphthalene in various organic solvents. Analysis was performed using an LDC/Milton Roy CM 4000 multiple solvent delivery pump in line with an LDC/Milton Roy Spectro Monitor 3000 variable wavelength detector. Substrate and product peaks were detected at a wavelength of 254nm. A Techsil 10 CN 25cm
Figure 2.1 The 75 ml aerated stirred tank reactor
x 4.6mm column, purchased from HPLC Technology (Wellington House, Waterloo Street, West Macclesfield, Cheshire.), was operated as a normal phase system, the mobile phase being 1,2-Dichloro-ethane HiperSolv for HPLC (BDH) with 2% Methanol (v/v) HPLC solvent (Fisons). Solvent was degassed in a sonication bath. The mobile phase operated at a flow rate of 1ml/min. giving a pressure of approximately 450 psi.

Samples were loaded via a Promis II autosampler, capable of processing 96 samples. The dedicated integrator, LDC/Milton Roy CL-10B, calculated product formed using an external standard maintained within the integrator program (Appendix I).

Samples were placed in sealed vials (LDC/Milton Roy - 2ml), 20μl being injected onto the column via the Promis II autosampler. A flush volume of 90μl was used to prevent carry over from the previous sample. An analysis time of 6 min was employed, naphthalene having a peak retention time of ≈ 3.2 min. and naphthalene-1,2-dihydro-1,2-diol of ≈ 4.1 min. (see figure 2.2).

2.3 **ANALYTICAL METHODS**

2.3.1 **Identification of naphthalene-1,2-dihydro-1,2-diol.**

The hydroxylation of naphthalene by *Pseudomonas putida* UV4, a blocked mutant known to lack the dehydrogenase enzyme necessary for the further breakdown of many aromatic compounds, was expected to accumulate naphthalene-1,2-dihydro-1,2-diol. The product, not being commercially available, was analysed to confirm that the
single peak detected by High Pressure Liquid Chromatography was in fact naphthalene-1,2-dihydro-1,2-diol, and not some further breakdown product.

Crystals of pure naphthalene-1,2-dihydro-1,2-diol were obtained as detailed in section 2.4.4 from dichloro-ethane. These crystals were observed to be needle like in structure (plate 1).

2.3.1.1 C:H:N and C:N:O analysis.

Img samples of crystalised product were analysed to determine the ratio of C:H:N:O by the Dept. of Chemistry, University College London. Theoretical values for the % C:H:N:O were calculated to be C - 74.07, H - 6.17, N - 0.00, O - 19.75.

Observed values were found to be C - 74.52, H - 6.20, N - 0.00, O - 19.28. These values indicate that C, H & O are present in the correct ratio for the product to be naphthalene-1,2-dihydro-1,2-diol. The product does appear to contain a low level of naphthalene resulting in a slightly higher % C and % H and a slightly lower % O. The results however do not yield information on the structure of the product, although it is unlikely that the product is anything other than naphthalene-1,2-dihydro-1,2-diol, as breakdown products and other intermediates of the metabolism of naphthalene by Pseudomonas putida differ in their C:H:O ratio eg. α- naphthol C - 83.33, H - 5.55, O - 11.11. The substrate naphthalene has a C:H:O ratio C - 93.75, H - 6.25, O - 0.00.
Plate 1. Micrograph of naphthalene-1,2-dihydro-1,2-diol crystalised from dichloroethane. Magnification x 400.
2.3.1.2 Mass spectrometry.

Samples of product were analysed using mass spectrometry by the Dept. of Chemistry, University College London, to determine product molecular weight. The theoretical molecular weight of the dihydrodiol was calculated to be 162. Peaks were observed at 162, 144, 116 and 115. The peak at 162 confirmed the product to be of the correct molecular weight to be dihydrodiol. The base peak at 144 was formed due to the elimination of H₂O. The further elimination of CO and CHO resulted in the peaks detected at 116 and 115.

2.3.1.3 Nuclear Magnetic Resonance Spectroscopy to confirm cis conformation of dihydrodiol.

2 mg of the dihydrodiol was dissolved in 0.7 ml deuterated chloroform (99.5 % D, Sigma) and its proton NMR spectrum obtained at 500 MHz on a Bruker AM-500 instrument (Department of Pharmaceutical Chemistry, School of Pharmacy, University of London). After a 90° pulse, each free induction decay was accumulated over 1.67 s using 16,384 data points and a sweep width of 4902 Hz. Eight scans were averaged and Fourier-transformed without weighting. Spectra were acquired at 295 K.
2.3.2 Determination of naphthalene and naphthalene-1,2-dihydro-1,2-diol by High Pressure Liquid Chromatography (HPLC).

Both naphthalene and naphthalene-1,2-dihydro-1,2-diol were monitored during biotransformation using HPLC, as described in section 2.2.3.

Column retention times were found to be \( \approx 3.2 \) min. and \( \approx 4.1 \) min. for naphthalene and naphthalene-1,2-dihydrodiol respectively. An example trace showing the chromatography obtained and peak retention times is shown in figure 2.2.

Standard curves were constructed for both naphthalene and naphthalene-1,2-dihydro-1,2-diol in dichloro-ethane (DCE) and are shown in figures 2.3 and 2.4. Product concentrations calculated by the integrator CL-10B program were derived from this product standard curve.

**Figure 2.2** HPLC trace showing substrate and product retention times.
Figure 2.3 Naphthalene standard curve.
Figure 2.4 Naphthalene-1,2-dihydro-1,2-diol standard curve.
2.3.2.1 Sample preparation for HPLC.

Samples (200µl) were taken from the stirred tanks into 28 ml solvent-resistant glass screw-cap vials containing 5ml of 1,2-dichloro-ethane, and vortexed for 10 seconds to allow both substrate and product to partition into the dichloro-ethane. The samples, dissolved in dichloro-ethane, were then placed in 2ml sealed glass vials for HPLC analysis. Samples not immediately processed were stored at 4°C. No product breakdown was observed.

It is important to note that in the aerated stirred tanks, air could on occasion be taken up as part of the sample. Samples observed to contain air were placed back into the reactors and a second sample taken. In reactors containing solid naphthalene as substrate, Guilson P200 sampling tips were found to block, resulting in low sample volumes being taken up. This problem was overcome by increasing the bore of the sample tips by cutting off a 2mm section from the end. The sample uptake volume of these modified tips was not adversely affected.

2.3.3 Optical density determination.

The optical densities at 670nm of culture samples were measured in a 1cm path length cuvette, against a water blank, using a Pye Unicam PU 8600 UV/VIS spectrophotometer (Pye Unicam Ltd., Cambridge). Samples were diluted into the range 0 - 0.5 AU. using 50mM K-phosphate buffer, pH 7.0. Optical density measurements upon samples containing dissolved solvent
were carried out in quartz cuvettes, the plastic cuvettes becoming opaque upon contact with solvent.

2.3.4 **Dry weight determination**

A correlation between bacterial wet weight and dry weight was sought in order to calculate specific bacterial enzyme activities in grams product per gram dry cell weight per hour. Bacteria were harvested as detailed in section 2.4.3.6 at 12,000 rpm; 30 minutes. The bacterial paste was weighed (0.4 - 0.5g wet wt.) into pre-dried (24hr at 95°C), pre-weighed, porcelain crucibles and the wet weight noted. Crucibles containing cell paste were dried at 95°C to constant weight.

Dry weight was found to be ≈ 25% of wet weight. This value is used throughout to calculate dry weight from a known wet weight. A relationship between optical density and grams dry cell wt. per litre has been determined, Brazier (1989), dry cell wt. in grams per litre being equivalent to:

\[
\frac{\text{Abs.670nm} + 0.129}{2.371}
\]

This relationship was used in the determination of biocatalyst dioxygenase activity during growth, section 2.4.3.7.
A major component of Gram-negative bacterial outer membranes has been shown to be lipopolysaccharide (LPS) Nikaido & Vaara (1985). This LPS contains rhamnose within the internal core region of the polysaccharide moiety. Rhamnose was detected using the cysteine-\( \text{H}_2\text{SO}_4 \) reaction, described by Dische (1955) and Osborn (1963).

**Reagents**

A solution containing 6 vol concentrated \( \text{H}_2\text{SO}_4 \) : 1 vol \( \text{H}_2\text{O} \).

A 3% (w/v) solution of cysteine-HCl.

Rhamnose standards of 20\( \mu \)g and 100\( \mu \)g/ml.

2.25ml of ice-cold \( \text{H}_2\text{SO}_4 \) (6 vol concentrated \( \text{H}_2\text{SO}_4 \) : 1 vol \( \text{H}_2\text{O} \) ) were slowly added to duplicate samples (0.5 ml) in an ice-water bath with mixing. Samples were equilibrated at 28°C for 3 min, and then heated in a vigorously boiling water bath for exactly 10 min. After cooling, 0.05ml of 3% cysteine-HCl was added to one sample, the other serving as a blank.

Absorbancy was measured over the range 380nm to 420nm exactly 2 hr after addition of cysteine, and corrected for nonspecific absorbance by scanning against a duplicate sample minus cysteine (see figure 2.5). Rhamnose standards in the range 20\( \mu \)g to 200\( \mu \)g/ml were run alongside the samples, see figure 2.6 for standard curve. Under these conditions \( \Delta \text{A} 400 - 412\text{nm} \) when related to the \( \Delta \text{A} 400 - 412\text{nm} \) of the rhamnose standards, yields the
quantity of rhamnose released per ml of sample.

Samples were prepared by centrifugation (MSE Micro Centaur ultracentrifuge; 2 minutes) or by filtration (0.2μm) to remove cells and the supernatants assayed for Rhamnose. Samples were stored at 4°C before assaying and at -40°C for long term storage.

Figure 2.5 LPS assay, example absorbance profile for rhamnose at 100 μg / ml of buffer.
Figure 2.6 LPS assay, rhamnose standard curve.
2.4 EXPERIMENTAL METHODS

2.4.1 Strain storage

The master culture was maintained at - 60°C in 50mM Phosphate buffer at pH 7.0 containing 10% w/v glycerol, in 1ml ampoules. Working cultures were maintained on nutrient agar plates at 4°C, after 48hrs growth at 28°C. Working cultures were replated every 4 weeks using colonies shown to be active upon indole plates, see 2.4.2. The strain was observed to lose some activity with time. Active colonies were reselected using agar plates supplemented with 10g/L glucose. Colonies observed to be active in the presence of glucose were then replated and stored at 4°C.

2.4.2 Selection of active colonies using indole.

*Pseudomonas putida* UV4 was plated onto nutrient agar containing 0.01% (w/w) indole, and incubated at 28°C for 24 - 48hrs. Active colonies producing toluene dioxygenase were able to hydroxylate the indole resulting in the formation of indigo. Active colonies were therefore observed to turn dark blue in colour (see plate 2). These colonies were then taken into shaken flasks for subsequent inoculation into the fermenter. Indole plates were not used for long term storage of cells, as storage on indole plates for over 2 weeks resulted in activity loss in some colonies when replated, i.e. a single colony when plated out may produce
Plate 2  *Pseudomonas putida* UV4 on indole plate showing active colonies.
both active and non-active colonies. Colonies showing activity upon indole plates should not therefore be assumed to be at full activity on plates over 1 week old. Active colonies on indole plates were normally used within 48hrs. Nutrient agar plates containing 0.01% indole were on occasion supplemented with 10g/L glucose for reselection of cells with higher activity in the presence of glucose. Plates incubated at 32°C and above produced no indigo, when the temperature was dropped to 28°C the colonies turned blue.

2.4.3 Cultivation of Pseudomonas putida.

2.4.3.1 2 L fermenter instrumentation and operation.

A 2L MBR mini bioreactor was used throughout for cell growth, see 2.2.1. The pH was controlled to 7.0 using 3M KOH pulsed peristaltically. RPM was maintained at 700 rpm. Air flow was controlled to 40 L/hr through a needle valve flow meter. Glucose was pulsed peristaltically from a pre-sterilised 125g/L stock, 5s on, 20s off, to supply ~2g glucose/hr to the fermenter. The fermenter was operated throughout cell growth under sterile conditions. A typical fermentation lasted for 18hrs, with pH, DOT, RPM and Temperature being monitored via a BCS BioPC package (BCS Software).

Sterilisation Procedure:
The fermenter containing medium was taken to 100°C at which point
the air exit line on the condenser was closed. All other ports were sealed by rubber septa and blanking screws. The temperature was maintained at 121°C for 20 minutes. The fermenter was then cooled to 60°C at which point the air outlet valve on the condenser was opened, and the condenser turned on. The temperature was then controlled to 28°C. The pH of the medium after sterilisation was 1.9 and was raised to 7.0 by KOH addition before inoculation.

2.4.3.2 Preparation of inoculum.

An active colony of *P. putida* was inoculated from an indole plate into 50ml of sterile defined salts medium at pH 7.0 (see section 2.1.3), containing 1g glucose (20g/L). The inoculated 250ml unbaffled shake flasks were placed in a rotary incubator (LH Fermentation Mk X incubator shaker) at 28°C and 150 rpm. The flasks were incubated for ~24 hrs to give an optical density A670nm of ~2. One shake flask containing 50ml of inoculum was then used to inoculate the 2 L fermenter.

2.4.3.3 Growth for the production of active cells.

Toluene dioxygenase although constitutive in *P. putida* UV4, is not produced in the presence of high levels of glucose, i.e. it is catabolite repressed. It is therefore necessary, for the production of *P. putida* containing high levels of toluene dioxygenase, to grow under glucose limiting conditions. This is
achieved by pulsing glucose into the fermentation at a fixed rate of 2g/hr.

The fermenter was inoculated with one 50ml shaken flask to give an initial optical density of \( \approx 0.07 \) at A670nm in the fermenter, see section 2.4.3.2. The initial volume of the fermenter after inoculation was 1.55 L, the addition of glucose and KOH for pH control resulting in a final volume of 1.9 L at 18 hours. Antifoam was added (5ml polypropylene glycol MW 2000) to prevent foaming during growth. Temperature was controlled to 28°C, with an agitation speed of 700rpm. PH was maintained above 7.0 by the addition of KOH. Aeration was supplied at 40L/hr. DOT, PH, RPM and TEMP. were monitored throughout, DOT ranging from 100% to 0% and PH ranging from 7.0 to 7.6. Temperature and pH remained constant. Cells were typically harvested after 18hrs at an optical density of \( \approx 20 \).

Samples were taken from the fermenter at the time of harvesting and plated onto indole plates to ensure that all the cells grown up had the ability to produce toluene dioxygenase. This procedure also served as a screen for any potential contaminants which would not turn blue on the indole plates.

2.4.3.4 Determination of glucose levels during growth.

The concentration of glucose in the fermenter was monitored using the Technicon GLU-CINET enzymatic colourimetric assay (Technicon Chemicals co. SpA. Siena, Italy). Samples were taken from the fermenter at 30 minute intervals and the cells removed by
centrifugation in a MSE microcentrifuge. 20µl samples were added to cuvettes containing 2.5 ml Glucinet reagent. The samples were mixed and left to stand for 30 minutes for the colour to develop. Samples were measured at a wavelength of 510nm. Glucose concentrations in g/L were obtained by referring to a standard curve, see figure 2.7. The reconstituted GLU-CINET reagent was stored at 4°C for up to 30 days. Samples containing more than 5g/L were diluted before being assayed.

2.4.3.5 Determination of gluconate levels during growth.

Gluconate levels present during cell growth were monitored using an enzymatic UV colourimetric kit (Boeringer Mannheim). Samples were taken at 30 min intervals from the fermenter and cells removed by centrifugation in an MSE microcentrifuge. The supernatants obtained were frozen at -60°C before analysis. The assay was performed as follows:

100µl samples were added to 3ml cuvettes containing 1ml solution 1 + 20µl suspension 2 + 1.9ml deionised water with mixing. Absorbance at A340nm was taken at ≈ 5 min. (A₁). The reaction was started by the addition of 20µl of suspension 3. The cuvettes were mixed and allowed to stand for 30 min to allow the reaction to run to completion. The absorbance at A340nm was taken (A₂). A blank was run as above with no sample and 2.0ml of deionised water. The ΔA (A₂ - A₁) was determined for both sample and blank.
Figure 2.7 Glucose standard curve.
The $\Delta A$ blank was subtracted from the $\Delta A$ sample values.

$$\Delta A = \Delta A_{sample} - \Delta A_{blank}$$

The absorbance differences measured should be at least 0.100 absorbance units to achieve sufficiently accurate results.

Gluconate concentration can be calculated from the following equation:

$$C = \frac{5.961}{6.3} \times \Delta A \text{ [g D-gluconic acid/L sample solution]}$$

If the sample has been diluted during preparation, the result should be multiplied by the dilution factor $F$.

Sample solutions were diluted as detailed below:

<table>
<thead>
<tr>
<th>Estimated amount of D-gluconic acid</th>
<th>dilution factor ($F$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.6 g/L</td>
<td>1</td>
</tr>
<tr>
<td>0.6 - 6.0 g/L</td>
<td>10</td>
</tr>
<tr>
<td>6.0 - 60 g/L</td>
<td>100</td>
</tr>
</tbody>
</table>

Theoretical values for the levels of gluconate present were calculated from the measured glucose concentrations and optical densities, using a yield on glucose value of 0.55g dry cell wt./g glucose.

2.4.3.6 Cell harvesting and storage.

Bacteria were harvested from the fermenter typically when the optical density was 20. Cells were centrifuged down in 250 ml pots
at 12,000 rpm for 15 min at 4°C, using an MSE High Speed 18 centrifuge. The supernatant was removed and the cell paste stored at 4°C until required. Cells were normally stored for up to 24-48hrs, beyond which activity was found to be greatly reduced. Cells were also frozen in liquid nitrogen and stored at -60°C to determine storage ability.

2.4.3.7 Determination of dioxygenase activity during growth.

Cells were taken from the fermenter at various stages of growth and optical density measured at 670nm. Cells were assayed for dioxygenase activity in 75 ml stirred tank reactors, using either a dodecane : buffer two-liquid phase system, containing 2.5 g naphthalene per reactor, or an aqueous system containing solid naphthalene, as described in section 2.4.5. Activities were measured over 1 hr with sampling. Specific activities were calculated from the optical density and volume of the cells added, see section 2.3.4. Cell concentration in the 75 ml reactors was kept below 0.5g dry cell wt. / L of aqueous phase, to avoid oxygen limitation.

2.4.3.8 Growth in the presence of organic solvents.

*P. putida* (Gram-negative) and *Arthrobacter simplex* (Gram-positive) were grown in the presence of a number of solvents to determine the solvent log P values beyond which no growth occurred. Cells from overnight cultures were inoculated (100μl) into 28 ml
glass screwcap bottles containing 10 ml of sterile nutrient broth and 5 ml of solvent. Initial optical density was noted. Samples were incubated with shaking at 28°C, 150rpm. Controls containing no solvent were also performed. Samples were examined after 20 hrs for signs of growth. After 25 hrs of growth, tubes were centrifuged to pellet the cells. Solvent and buffer were now poured off and 10 ml of 50mM phosphate buffer; pH 7.0 added. Cells were resuspended in the buffer and optical density measurements taken at A670nm. Growth curves were performed for a selected range of solvents in 250 ml shake flasks containing 25 ml medium and 5 ml solvent, at 28°C with shaking at 150rpm.

2.4.4 The production, extraction and purification of naphthalene-1,2-dihydro-1,2-diol.

Biotransformation for the production of naphthalene-1,2-dihydro-1,2-diol from naphthalene was performed on a 1 L scale in 50mM phosphate buffer at pH 7.0. *P. putida* at 10g wet wt./L, grown as detailed in section 2.4.3.3, was added to 1 L of phosphate buffer containing 10 g/L glucose and 10g/L particulate naphthalene (<850μm diameter). The biotransformation took place at 28°C, 800 rpm with an air flow rate of 60 L/Hr. The conversion was allowed to proceed for 5 hrs, after which time the particulate naphthalene was removed with filtration. Centrifugation followed at 4°C to remove cells and remaining solid naphthalene. The supernatant containing product and a low level of
dissolved naphthalene ( < 30 mg/L at 20°C ) was then placed in a 5 L volumetric flask with 2.5 L of dichloromethane and mixed vigorously. The phases were allowed to separate and the two phases analysed by HPLC for substrate and product. Product was observed at high levels in the dichloromethane with a small trace of naphthalene.

The dichloromethane containing product was separated and rotary evaporated under vacuum at 45°C. Evaporation yielded gram quantities of product, which when recrystallised from dichloroethane furnished needle-like crystals, see plate 1. Analysis revealed these crystals to be naphthalene-1,2-dihydro-1,2-diol, see section 2.3.1.

2.4.5 Operation of stirred tank reactors in the determination of dioxygenase activity and stability of free cells.

Biotransformations were performed using 75 ml aerated stirred tanks in groups of 6, see section 2.2.2. Reactors were typically operated at 800 rpm with an air flow of 60 - 80 cc/min. and a temperature of 28°C. Cell loadings were maintained below 1.7 g wet wt./ L of reactor volume ( 0.1 g wet wt. per reactor ), to avoid oxygen limitation.

Samples ( 200µl ) were taken into 28 ml glass vials containing 5 ml dichloroethane, effecting a 25-fold dilution. Vials were vortexed to stop the reaction and to allow substrate and product to partition into the dichloroethane. Samples were then analysed by HPLC, as detailed in section 2.3.2.
2.4.5.1 Single phase aqueous system.

Biotransformations in the absence of organic solvent were performed with either 50mM phosphate or 50mM Tris buffer at pH 7.0. Each reactor contained 8 g/L glucose and 1 g particulate naphthalene. Reactors containing 55 ml of buffer were equilibrated with stirring at 28°C for 10 minutes, before the addition of cells in 5 ml of buffer producing a final working volume of 60 ml. Sample tips were modified to prevent blockage by naphthalene during sampling, see section 2.3.2.1.

At high air flow rates particulate naphthalene had a tendency to be removed from the reaction system, being displaced onto the sides of the reactor. This could result in a reduced reaction rate and was overcome by limiting the air flow rate to a maximum of 80 cc/min.

2.4.5.2 Two liquid phase system.

Biotransformations were normally carried out at a phase ratio of 1:1, 30 ml of organic solvent : 30 ml phosphate or Tris buffer. Glucose was present at a concentration of 8 g/L in the reactor, 16 g/L of buffer. Naphthalene was provided at a concentration of ≈ 80 - 100 g/L of solvent, 40 - 50 g/L in the reactor. This level of naphthalene dissolved in the organic phase allowed hydroxylation to occur at the maximal rate.

Reactors containing 25 ml of buffer and 30 ml organic solvent were equilibrated with stirring at 28°C for 10 minutes, before the
addition of cells in 5 ml of buffer producing a final working volume of 60 ml.
It should be noted when using extremely volatile solvents, at or near to saturation with naphthalene, that the air sparge tubes were found to block due to the crystallisation of naphthalene. This in some cases limited the amount of time aeration could be effectively provided.

2.4.5.3 Effect of carbon source on productivity.

The hydroxylation of toluene by \textit{P. putida} UV 4 has been performed using ethanol as co-substrate, Brazier (1989). It was undesirable however, when investigating the tolerance of \textit{P. putida} to organic solvents, to have ethanol present, ethanol itself being potentially toxic to the biocatalyst.
Aqueous phase biotransformations were performed with either ethanol or glucose as co-substrate, as described in section 2.4.5.1. Biocatalyst activity and stability were compared over 5 hrs. Cells with no carbon source were also run to demonstrate the need for co-substrate.

2.4.6 Adhesion of cells to the organic-aqueous liquid-liquid interface.

5 ml alliquots of \textit{Pseudomonas putida} suspension in 50 mM phosphate buffer; pH7.0, were placed in test tubes (1cm internal dia.) containing 1 ml of a range of organic solvents with log P values
of 1.8 - 8.8. The contents were mixed vigorously for 1 minute using a whirlimixer (Fisons Scientific Equipment), followed by a 15 minute separation period to allow the phases to separate. The optical density of the aqueous phase was then measured against the corresponding control, which contained buffer and solvent but no cells. Quartz cuvettes were used throughout. The procedure was repeated at various initial cell concentrations, the reduction in optical density being expressed as a % of cells adhered to the organic-aqueous interface. This method is adapted from Rosenberg, E (1980) and Rosenberg, M (1984).

2.4.7 Determination of cell viability following solvent exposure.

100μl samples of cell suspension, optical density = 20, were placed into 5 ml of sterile Tris buffer; pH 7.0, containing 0.5 ml of organic solvent. Solvents with log P ranging from 2.5 to 8.8 were employed. Cells were exposed to solvent without mixing at 28°C for 2 hrs. Samples were taken at 1hr and 2hr, and after dilution were plated onto indole plates to determine viability. Dilutions in the range 10° to 10° were employed. Indole plates were incubated for 48hrs at 28°C, at which point colonies were counted and the percentage viability calculated.
2.4.8 Solvent exposure for the determination of changes in membrane permeability.

*P. putida* (0.2g wet wt. / 0.05g dry wt.) was suspended in 10 ml of 50 mM phosphate buffer, pH 7.0, in 30 ml glass screw cap bottles, containing 0.5 ml of organic solvent. Solvents ranged in log P from 0.8 to 7.5. The contents were mixed vigorously using a whirlimixer (Fisons Scientific equipment) for 30 s, followed by centrifugation at 4200 rpm for 6 min. to pellet down the cells. Buffer and solvent were poured off, and any coagulated / lysed cell debris washed out with phosphate buffer, leaving a pellet of intact cells.

Cells were resuspended in 10 ml of phosphate buffer, 5 ml of which was centrifuged down for dry weight determination. Optical densities were measured at A670nm. 1 ml samples of solvent treated cells were taken to determine their ability to take up gentian violet, a hydrophobic dye, as described in section 2.4.9.

Some solvents of low log P value were found to coagulate / lyse all the cells so that no cell pellet resulted. This problem was overcome to some extent by doubling the cell loadings used.

2.4.9 Gentian violet uptake as a measure of outer membrane permeability.

1 ml of cell suspension of known optical density and dry wt. was added to 9 ml gentian violet solution (10 mg gentian violet per litre of phosphate buffer; pH 7.0) in 30 ml glass screw cap
bottles. Cells were exposed to gentian violet for 30 min. with
shaking (150 rpm) at 28°C. After 30 minutes exposure the cells
were centrifuged down at 4200 rpm; 15 minutes. The absorbance of
the supernatant was now measured at A590 and compared to the
initial stock gentian violet solution. The % gentian violet taken
up could be determined knowing the cell concentration and the
amount of gentian violet removed.

This method was used to determine the effect of solvent exposure
upon permeability, as well as to investigate the effect of
lipopolysaccharide removal from the outer membrane upon
permeability.

Adapted from Gustafsson (1973).

2.4.10 Removal of lipopolysaccharide from the outer membrane using
ethylene diaminetetraacetate (EDTA).

P. putida was suspended in 50 mM Tris-HCl buffer (pH 7.0)
containing EDTA at concentrations ranging from 0 - 1 mM. Higher
concentrations were observed to bring about lysis. Cells at a
concentration of 0.4 g wet wt per 20 ml of buffer were exposed to
EDTA at 28°C with shaking (150 rpm) in 30 ml glass bottles.
After 5 min, 1.5 ml samples were taken, and the cells rapidly
removed (< 2 min) in a microcentrifuge (MSE). Supernatants
were taken and assayed for rhamnose as detailed in section 2.3.5.
Supernatants not immediately screened for rhamnose were frozen at
-60°C until required.
5 ml portions of EDTA treated cells were assayed for their ability to hydroxylate naphthalene, both in aqueous and organic two-liquid phase systems using phosphate buffer as detailed in section 2.4.5. 1 ml portions were exposed to gentian violet as detailed in section 2.4.9 to determine the effect of lipopolysaccharide release upon membrane permeability to a hydrophobic dye.

2.4.11 Observation of cell motility using the hanging drop method.

*Pseudomonas putida* is actively motile in phosphate buffer when observed in an inverted hanging drop. Cell suspensions dropped onto cover slips were inverted and supported as shown in figure 2.8.

Cells exposed to organic solvents as detailed in sections 2.4.7 and 2.4.8 were examined for motility microscopically using the hanging drop method, at magnifications of x 400 and x 1000. All motility was compared to unexposed cells in phosphate buffer. Motility was scored: ++ = motility as in control, + = some motility observed, - = no motility observed.

2.4.12 Immobilisation of *P. putida* in calcium alginate beads.

Cells were suspended in 50 mM Tris-HCl buffer; pH 7.0 and mixed with 4% sodium alginate, in the ratio 1 part cell suspension : 3 parts sodium alginate. Sodium alginate was prepared using 50 mM Tris-HCl buffer at pH 7.0.

For small scale production, sodium alginate + cells were placed in
Figure 2.8. Hanging drop for the observation of cell motility
10 ml syringes. 5 ml volumes were dropped through a fine bore needle into stirred chilled 50 mM Tris-HCl + 0.1 M CaCl₂. This created small droplets, which upon contact with the CaCl₂/Tris buffer, formed solid beads of calcium alginate containing entrapped cells. Beads were left to harden with stirring for 20 min. before being washed in Tris buffer to remove CaCl₂. Entrapped cells were then transferred to 75 ml stirred tanks for the determination of activity and stability in the presence and absence of organic solvents.

Large scale production was performed as above, using a peristaltic pump to drive the sodium alginate + cells through a fine bore needle. Beads were typically 1.5 - 2.0 mm in diameter.

2.4.13 Determination of the dioxygenase activity of immobilised cells.

5 ml batches of immobilised *P. putida* were placed in 75 ml stirred tanks as described in section 2.4.5. Tris-HCl buffer was used throughout, as phosphate buffer dissolved the beads forming calcium phosphate. Beads were added to 60 ml buffer in the case of an aqueous system, and 30 ml for an organic two-liquid phase system, creating a samplable volume of 60 ml in both cases. To determine the effects of oxygen limitation cells were loaded into beads at concentrations ranging from 0.005g - 0.1g wet wt. per 5 ml of beads (0.25g - 5g dry cell wt. per litre of beads). A cell loading of 0.03g wet wt. per 5 ml of sodium alginate was employed for the study of solvent effects upon immobilised cells.
3. RESULTS

3.1 Cultivation of *Pseudomonas putida*.

*Pseudomonas putida* UV4, a constitutive mutant for the production of toluene dioxygenase, was grown routinely on a 2 L scale to produce cells with the ability to hydroxylate naphthalene, forming the corresponding dihydrodiol, as described in section 2.4.3. The mutant strain, although constitutive for the production of toluene dioxygenase, did not produce this enzyme in the presence of high levels of glucose due to catabolite repression. Glucose was therefore added continually during growth to ensure that growth was carbon limited during enzyme production.

*P. putida* after inoculation into the 2 L vessel had a typical lag period of 3 to 4 hours, followed by exponential growth between 4 and 10 hours. A reduced growth rate was observed between 10 and 18 hours due to carbon limitation, see fig.3.1 and 3.2.

3.1.1 Standard pH and DOT profiles.

During the initial 3 to 4 hour lag period, pH was observed to increase from pH 7 to 7.5, with a corresponding decrease in dot from 100% to 85% of saturation. The increase in pH is thought to be due to the utilisation of gluconate produced during growth of the shake flask inoculum. As growth became exponential from 4 hours onwards, the pH returned to pH 7 and was maintained at this level by the controlled addition of alkali. Dot decreased
during exponential growth to reach a minimum value of ≈ 6% of saturation at 9 - 10 hours. pH and dot increased sharply at 9.5 hours, dot remaining constant at 73% of saturation from 10 hours onwards, while pH after an increase to 7.4 dropped back and remained constant at pH 7 from 12 hours onwards. The sharp changes in dot and pH at 9.5 hours correspond to a reduction in observed growth rate. The profiles shown in fig.3.2 are typical of a standard 2 L run for the production of active cells.

3.1.2 Glucose and gluconate concentrations during growth. When glucose concentration during growth was measured, it was observed that glucose became limiting at 7 - 8 hours, whereas exponential growth continued to 10 hours, see fig.3.3. Pseudomonas aeruginosa has been shown to produce gluconate during growth in the presence of glucose (Midgley and Dawes, 1973). A gluconate assay was therefore performed alongside the glucose assays, showing that gluconate was in fact produced. Glucose was found to peak at 5 g/L in 5.5 hours and had decreased to 0 g/L in 8 hours. Gluconate was produced as the glucose level reached 3.5 - 4 g/L and peaked at 5.3 g/L in 7.5 hours. Gluconate was depleted at 10 - 10.5 hours, at this point the growth rate reduced, as cells grew on the pulsed glucose alone. The cells were therefore subjected to carbon limited growth from 10 - 18 hours.
Figure 3.1 Growth of *Pseudomonas putida* UV4 on a 2 L scale with pulsed glucose.
Dry cell weight versus time from inoculation.

Figure 3.2 Standard pH, dot and log dry cell weight profiles for cell growth on a 2 L scale.

Figure 3.3 Glucose and gluconate levels present in the fermenter during growth with pulsed glucose.
Figure 3.1
Figure 3.3

GLUCONATE (1/6)

Growth Time (hr.)

Log Dry Cell Weight

GLUCOSE (1/6)
3.1.3 Observed cell yield on glucose.

Cell yields during growth were calculated knowing the levels of glucose added and remaining at any point during growth, as well as the dry cell weight. It can be seen from figs. 3.4 and 3.5 that cell yields during the first 10 hours of growth were unusually low, reaching the expected cell yield and remaining constant at 0.55g dry cell weight / g glucose from 10 hours onwards. The low cell yields observed were due to glucose being converted only as far as gluconate, a sugar not detected by the glucose assay. The observed cell yields over the first 10 hours are therefore due to a build up of gluconate, fig. 3.5. The cell yield reached its expected level, from 10 hours onwards, when all the gluconate had been utilised to form biomass. A mass balance was performed during growth to predict the levels of gluconate that were likely to be formed. It can be seen from fig. 3.6 that the predicted and observed gluconate levels are in agreement. Predicted gluconate levels were calculated using a cell yield value of 0.55g dry cell weight / g glucose.

3.1.4 Toluene dioxygenase production during growth.

Toluene dioxygenase production was monitored during growth using its ability to hydroxylate naphthalene, forming the corresponding dihydrodiol, see section 2.4.3.7. Figure 3.7 shows both the total and specific activities observed. Specific activities do not vary significantly during growth. It should be noted from the total
Figure 3.4 Observed cell yields (g dry cell weight / g glucose) for growth on glucose.
Figure 3.5  Observed cell yields on glucose compared with the concentrations of glucose and gluconate present during growth.

Figure 3.6  Predicted and measured gluconate levels during growth on a 2 L scale.

Figure 3.7  Measured total and specific toluene dioxygenase activities during growth.
Figure 3.7

Specific Activity (mg/g H2O)

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Growth Time (Hr-a)

7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

Total activity (g product/Hr)
activity profile, that the vast majority of enzyme production occurs during exponential growth (7 - 11 hrs) in the absence of excess glucose. The slight decrease in specific activity observed from 12 hours onward could be due to carbon limited growth, or oxygen limitation during the activity assays due to increased cell density. It is interesting to note that the presence of gluconate does not appear to inhibit enzyme production. Enzyme production is, however, inhibited if glucose does not become limiting during growth, due to a longer than usual lag period.

3.2 Biocatalyst storage.

After harvesting, cells were centrifuged down at 12 000 rpm for 15 minutes at 4°C. The cell paste was observed to be pink in colour when the cells were active. The cell paste was stored at 4°C in a fridge for up to two days. Cell pastes over two days old showed little activity and turned black in colour.

3.3 Characterisation of toluene dioxygenase activity and stability in the aqueous stirred tank reactor.

It can be seen from figures 3.8 and 3.9 that the hydroxylation of naphthalene by \textit{P. putida}, forming the dihydrodiol, occurs at a rate of \(\approx 1\) g product / g dry cell weight / hour over the first 6 hours. The profiles observed were typical for the biotransformation of naphthalene in phosphate and Tris buffers. Cell activities observed were in the range \(\approx 0.5 - 1.5\) g / g dry
cell weight / hour. Variations in activity, resulting from the need to grow fresh cells each week, did not affect biocatalyst stability.

Solid naphthalene was present at a concentration of 17 g / L. This concentration was used throughout for biotransformations in aqueous stirred tanks and shaken flasks. Naphthalene concentrations of 8, 25, 34 and 42 g / L did not alter the rate of hydroxylation. The particle size of solid naphthalene used was in the range ≈ 500 - 900μm, particle sizes smaller than this were found to be removed from the reaction system during active aeration, resulting in a reduction in product formation rate.

Cells frozen in liquid nitrogen and stored at -40°C for one week, showed little retention of activity after thawing, see figure 3.8, indicating that long term storage of *P. putida* was not feasible.

The addition of KCN in an attempt to destroy the electron transport chain was found to result in a total loss of activity at concentrations of 10mM and above, as seen in figure 3.8.

3.3.1 The requirement for a carbon source.

The hydroxylation of aromatic compounds such as naphthalene and toluene by *P. putida* requires the co-factor NADH₂. This co-factor can be regenerated using, for example, ethanol or glucose as an energy source.

The hydroxylation of toluene by *P. putida* UV 4 has been performed using ethanol as the carbon source (Brazier, 1989). The use of ethanol in this study was however undesirable, ethanol itself
Figure 3.8  Typical product - time profiles for the hydroxylation of naphthalene in an aqueous environment (△), with the addition of 10, 25 and 50 mM KCN (●), and for cells stored frozen at - 40°C for 1 week (■).  
Biotransformations were performed in 75 ml stirred tank reactors as described in section 2.4.5.1  
Cell concentration 0.42 g dw / L.
3.9 Typical product-time profile for the hydroxylation of naphthalene in an aqueous stirred tank reactor, extended run showing biocatalyst stability.

Cell concentration 0.42g dcw / L.
being a toxic solvent (Naylor, 1988).

Figure 3.10 shows a comparison of product formed for both ethanol and glucose in aqueous stirred tanks. Glucose was found to give an equivalent rate of reaction to ethanol and was therefore used as carbon source throughout this study. Little product formation was observed in the absence of an added carbon source.

3.3.2 The requirement for oxygen.

The enzyme toluene dioxygenase as the name suggests requires oxygen to perform the hydroxylation of naphthalene, utilising both atoms of the molecular oxygen. *P. putida* also requires oxygen for aerobic respiration, pseudomonads being strict aerobes.

Figures 3.11 and 3.12 show the effect of cell loading, in 75 ml stirred tanks, upon product formed and specific product formed at constant air flow rate and stirrer speed. Oxygen becomes noticeably limiting above 0.5 g dry cell weight per litre. Cell loadings used in this study were therefore operated in the range 0.21 - 0.42 g dry cell weight per litre, unless otherwise stated, to avoid oxygen limitation. Shaken flasks were operated at 0.21 g dcw / L.

Figure 3.13 shows the effect of air flow rate upon product formed for 0.83 g dcw / L cells. Cells with no active aeration were found to produce product at \( \approx 12 \% \) of the maximum rate obtained with active aeration. Cells aerated at 40, 80 and 100 cc/min were found to produce product at the maximal rate. At air flow rates above
Figure 3.10 The effect of carbon source on the hydroxylation of naphthalene in an aqueous stirred tank reactor. Glucose (4.2 g/L, ▲), ethanol (3.3% v/v, ■) and no added carbon source (▼). Cell concentration 0.83 g dcw/L.
3.11 Product – time profiles for various cell loadings in aqueous stirred tank reactors. Cell loadings 0.21 (●), 0.42 (△), 0.83 (□), 2.1 (▽), and 2.5 g dw / L (○).

Air flow 80cc / min. Stirrer speed 800rpm.
3.12 Product formation rate (g/g dcw/hr) versus cell loading in aqueous stirred tank reactors. Air flow 80cc/min.
3.13 Product - time profiles in aqueous stirred tank reactors for various air flow rates. Air flow rates of 0 (●), 40 (▲), 80 (□) and 100cc/min (▽) were employed. Cell loading 0.83g dcw / L.
100 cc/mln product formation rates were reduced due to displacement of the solid naphthalene from the system.

All 75 ml stirred tanks were operated at an air flow rate of \( \approx 80 \) cc/min in this study, ensuring adequate aeration.

3.3.3 The effect of buffer molarity and pH.

It can be seen from fig.3.14 that buffer molarity affects the rate of product formation, being most marked in 500 mM phosphate buffer which allowed bioconversion to proceed at less than 20% of that obtained in 50 mM phosphate buffer. 500 mM phosphate buffer also appeared to affect biocatalyst stability, producing a non-linear product-time profile.

50 mM phosphate and Tris buffers of pH 7.0 were used throughout this study.

Initial pH appeared to have little affect upon the observed product-time profiles, as seen in fig.3.15. Biotransformations were carried out at pH 7.0 to avoid possible breakdown of the dihydrodiol to form \( \alpha \)-naphthol at low pH.

3.3.4 The effect of initial product concentration.

The production of naphthalene-1,2-dihydrodiol as observed in figure 3.9 stops at around 8 hours after forming 2.7 g/L product. In order to determine whether this was due to product inhibition or biocatalyst stability, cells were removed by centrifugation. The buffer containing product at 2.7 g/L, after
3.14 Product-time profiles at various buffer molarities. Phosphate buffer (pH 7) was used at 50 (〇), 100 (△) and 500mM (□). Cell loading 0.42g dcw / L.
3.15 Product-time profiles for aqueous stirred tank reactors in phosphate buffer of varying pH; 5.5 (○), 6.0 (△), 6.5 (▽), 7.0, 7.5 and 8.0 (□). Cell loading 0.42g dw / L.
being re-adjusted to pH 7.0 and the addition of naphthalene, was re-used with fresh cells for a further 6 hours. The rate of product formation was unaffected and remained linear over the 6 hour period, producing ≈ 6 g/L product.

When a cell loading of 1.67 g dcw/L was employed in an aerated shaken flask, product was found to crystallise out as the product saturation level of ≈ 7.5 g/L was exceeded, forming needle-like crystals, see plate 3.

Product inhibition, at the product concentrations achieved in this study, did not appear to be taking place.

3.4 Verification of product as being 

(+)-cis-naphthalene-1,2-dihydro-1,2-diol.

It can be seen from the C:H:N and C:N:O measurements that Carbon, Hydrogen and Oxygen are in the correct proportions to be naphthalene dihydrodiol. The mass spectrometry results also indicate that the product formed is of the correct molecular weight. These results together with the observation of one product peak during HPLC analysis would tend to suggest that the product is indeed the dihydrodiol.

Nuclear magnetic resonance spectroscopy was performed in order to determine if the diol was cis or trans in conformation. The aliphatic protons at positions 1 and 2 were found to share a common peak splitting; the three bond coupling constant 3J was found to be 4.9 Hz which corresponded to a dihedral bond angle for the two protons of 45°. The bond angles
Plate 3 Formation of needle-like crystals of naphthalene-1,2-dihydrodiol in phosphate buffer as the product concentration exceeded 7.5 g / L.
predicted using molecular models were: trans = 60° & 170°

\[ \text{cis} = 45° \text{ & } 60° \]

Two bond angles were possible due to the two possible conformations of the dihydrodiol ring.

The measured value of 45° suggests that the dihydrodiol was in the cis conformation. The literature concerning the hydroxylation of aromatics by bacteria also confirms the above findings (Jeffrey et al, 1975., Jerina et al, 1971.).

3.5 Characterisation of toluene dioxygenase activity and stability in the presence of organic solvents.

The hydroxylation of naphthalene in two-liquid phase stirred tanks was performed at a working volume of 60 ml, 30 ml organic solvent: 30 ml buffer. Figure 3.16 shows a typical product-time profile for the hydroxylation of naphthalene at 83 grams per litre of undecanol (log P 4.5). It can be seen that the rate of product formation remains constant over the first 5 - 6 hours, as seen for the aqueous system, fig.3.9.

The 75 ml reactors were aerated at 80 cc/min as for the aqueous system. Active aeration was found to result in solvent evaporation for some of the more volatile solvents eg. pentane and hexane. The level of solvent loss for the vast majority of solvents was minimal over the period of sampling.

Naphthalene saturated volatile solvents upon aeration were found to block the air-line sparge tubes due to the crystallisation of naphthalene within them. The concentration of naphthalene was
3.16 Typical Product-time profile for the hydroxylation of naphthalene in a two-liquid phase stirred tank reactor, see section 2.4.5.2, containing 30 ml undecanol:

30 ml phosphate buffer pH 7, 50 mM.
Undecanol contained 83 g / L dissolved naphthalene.
therefore operated at 83 g / L of solvent, below solvent saturation unless otherwise stated.

3.5.1 The effect of initial naphthalene concentration.

Figures 3.17 and 3.18 show the product-time profiles for the hydroxylation of naphthalene at varying initial concentrations in the non-toxic solvent dodecane (log P 6.6) for cells at 0.83 g / L of emulsion. Product-time profiles were observed to be linear above 12 g / L, over a two hour period. Naphthalene concentrations below this level resulted in product formation rates which decreased with time.

When the rate of product formation (g/g/h) is plotted against [naphthalene] as in fig.3.19, naphthalene is shown to become limiting below 40 g / L of solvent, ≈ 30 % of solvent saturation (see table 3.1).

Naphthalene was used at a concentration of 83 g / L of solvent throughout this study unless otherwise stated. Most of the solvents used were operated at ≈ 55 % of solvent saturation (see table 3.1).

Cell loadings did not exceed 0.42 g dcw / L of emulsion ensuring that naphthalene did not become limiting over the period of sampling.
Figure 3.17 and 3.18  Product-time profiles in two-liquid phase stirred tanks, with varying [naphthalene].
Dodecane loaded with 2 - 120g/L naphthalene.
30ml dodecane: 30ml phosphate buffer.
Cell loading  0.83g dcw / L emulsion.
Figure 3.17

Product Formed (g/g) vs. Bioconversion Time (min)

- 2 g/L naphthalene (▼)
- 4 g/L naphthalene (□)
- 8 g/L naphthalene (●)
- 12 g/L naphthalene (▼)

Figure 3.17

Product Formed (g/g) vs. Bioconversion Time (min)

- 12 g/L naphthalene (▼)
- 20 g/L naphthalene (□)
- 25 g/L naphthalene (○)
- 33 g/L naphthalene (△)
Product formed (g/g dcw/hr) in a dodecane: buffer two-liquid phase system versus naphthalene g / L solvent.

Cell loading 0.83g dcw / L emulsion.
### NAPHTHALENE SOLUBILITY IN ORGANIC SOLVENTS

<table>
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<th>SOLVENT</th>
<th>LOG P</th>
<th>NAPHTHALENE (g/L)</th>
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<tr>
<td>Tetradecene</td>
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<td>175</td>
</tr>
<tr>
<td>Dodecane</td>
<td>6.6</td>
<td>140</td>
</tr>
<tr>
<td>Undecane</td>
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<td>152</td>
</tr>
<tr>
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<tr>
<td>Octanol</td>
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<td>142</td>
</tr>
</tbody>
</table>

Table 3.1 Naphthalene solubility in organic solvents measured at 28°C.
3.5.2 The effect of stirrer speed.

Increasing the stirrer speed, and hence the amount of interface available for the cells to contact, in a decanol (log P 4.0): buffer two-liquid phase system, resulted in a reduction in the rate of product formation, indicating that interface is of importance in bringing about biocatalyst inactivation by decanol. The increases in stirrer speed were shown to reduce biocatalyst activity but not operational stability as seen in fig.3.20. When product formed, as a percentage of the decanol saturated aqueous control, was plotted against stirrer speed (fig.3.21), it could be seen that an increase in stirrer speed from 500 rpm to 750 rpm resulted in a 30% decrease in activity. An increase from 750 rpm to 1000 rpm resulted in a decrease of only 7%. The extra interface generated at stirrer speeds above 750 rpm does not appear to be of great importance in bringing about further biocatalyst inactivation. Decanol was observed to bring about a maximum reduction in product formation rate of ≈ 50% at stirrer speeds of 750 rpm and above (fig.3.21).

When the product formed, as a percentage of the aqueous control, was plotted against stirrer speed for the non-toxic solvent dodecane, a totally different profile from that of decanol was obtained (fig.3.22). The results show that an increase in stirrer speed resulted in an increase in product formed up to 750 rpm above which the level remained constant. Product-time profiles were linear over the 2 hours of measurement. Increasing
3.20 Product – time profiles for the hydroxylation of naphthalene in a decanol (log P 4) two-liquid phase system at various stirrer speeds; 500rpm (●), 750rpm (△), 1000rpm (▽).
Controls – 150rpm shake flask with 200μl decanol (○), 750rpm aqueous stirred tank (□).
Cell loading 0.42 g dcw / L
3.21 Product formed in 2 hours as a percentage of the decanol shaken flask control versus stirrer speed.
Control - 150rpm shake flask with 200μl decanol.
Cell loading 0.42 g dw / L
Product formed in a dodecane (log P 6.6) two-liquid phase system over 2 hours, as a percentage of aqueous control, versus stirrer speed.
Control - 750 rpm aqueous stirred tank.
Cell loading 0.42 g dcw / L
the level of available interface did not appear to be detrimental to biocatalyst activity or stability.

3.5.3 The effect of phase ratio.

It can be seen from fig.3.23 that the percentage organic solvent present (v/v) does not alter the level of product formation in relation to the aqueous control for dodecane, being ≈ 97% of the aqueous control throughout. However, increasing the percentage organic solvent present for decanol could be seen to have a dramatic effect upon product formation, bringing about a reduction to 40% of control at 10% (v/v) decanol and a further reduction to 25% of control above 20% (v/v) decanol. All product-time profiles were linear over the two hour period.

3.6 The effect of solvent log P upon biocatalyst activity and stability.

3.6.1 Stirred tanks.

Figures 3.24, 3.25 and 3.26 show the product-time profiles obtained in two-liquid phase stirred tank reactors for a selection of organic solvents, over a range of log P values. It is interesting to note that the profiles were linear over the three hour period measured. Product formation stopped in the hexane two-liquid phase stirred tank above 3 hours. When the levels of product formed, as a percentage of the aqueous
3.23 Product formed in 2 hours, as a percentage of aqueous control, versus percentage organic solvent in reactor for dodecane (□) and decanol (▲).
Control - 750 rpm aqueous stirred tank.
Cell loading 0.42 g dcw / L
Solvents were saturated with naphthalene = 140 g / L.
3.24 Product - time profiles in two-liquid phase stirred tank reactors with solvents of various log P values; Di-iso-octyl phthalate (△), Tetradecene (□), Dodecane (▽), Methyl cyclohexane (■), Hexanol, Heptanol, Dichloroethane, Octanol, Nonanol, Toluene (○), Aqueous control (●). For log P values see section 2.1.2.

30ml solvent: 30ml buffer. 800 rpm, 80cc/min air flow.
Solvents were saturated with naphthalene.
Cell loading 0.42 g dcw / L
3.25 Product - time profiles in two-liquid phase stirred tank reactors with solvents of various log P values; Nonane (△), Dodecanol (□), Ethyl decanoate (▽), Decanol (■), Hexane (●), Aqueous control (○). For log P values see section 2.1.2.

30ml solvent: 30ml buffer. 800 rpm, 80cc/min air flow. Solvents were saturated with naphthalene.

Cell loading 0.42 g dw / L.
3.26 Product - time profiles in two-liquid phase stirred tank reactors with solvents of various log P values; Di-iso-pentyl ether (△), Pentane (□), Aqueous control (○). For log P values see section 2.1.2.

30ml solvent: 30ml buffer. 800 rpm, 80cc/min air flow.
Solvents were saturated with naphthalene.
Cell loading 0.42 g dw / L.
controls, for the various organic solvents were plotted against the solvent log P (see section 2.1.2 for definition) a sigmoidal curve was obtained (fig.3.27), showing a sharp cut off point between log P 3.5 and 4.0, solvents above log P 4.0 were found to allow high levels of activity comparable with the aqueous control. Solvents in the range 3.5 to 4.0 were shown to allow intermediary levels of activity, and below log P 3.5 little or no activity was observed. The sigmoidal curve obtained after 2 hours of bioconversion, could also have been obtained at 1 hour and 3 hours due to the linear nature of the product-time profiles.

The level of product formed for the solvent di-iso-octyl phthalate (log P 9.8) appeared slightly low, ~ 90% of the aqueous control. It is thought to be due to the high viscosity of the solvent, resulting in mass transfer problems, especially oxygen transfer.

3.6.2 Shaken flasks.

Hydroxylation of naphthalene was carried out in 250 ml shaken flasks in the presence of organic solvents, 30 ml buffer: 30ml organic solvent. Product-time profiles were found to be linear as in the stirred tank, producing a sigmoidal curve when product formed was plotted against log P (fig.3.29). It is interesting to note, however, that the level of product formed in shaken flasks, for the solvents di-iso-pentyl ether (log P 3.9) and decanol (log P 4.0) is far greater than in the equivalent stirred tank (fig.3.28), indicating that the increased amount of interface available in the stirred tank is responsible for a
proportion of biocatalyst inactivation for these two solvents. Di-iso-pentyl ether allowed product formation in the shaken flask as in the aqueous control. Interface appears to be of greatest significance for di-iso-pentyl ether, the shaken flask producing product at 740% of that in the stirred tank.

It should be pointed out that the biocatalyst has been shown to exhibit a variable nature in the presence of decanol and di-iso-pentyl ether in the stirred tank. Figure 3.28 shows that decanol removed all biocatalyst activity in the stirred tank, while di-iso-pentyl ether allowed product to be formed at ≈ 13% of the aqueous control. These levels represent the worst case. It can be seen from figs. 3.25 and 3.26 that decanol and di-iso-pentyl ether produced product at ≈ 25% and 55% of the aqueous controls respectively. Figures 3.20 and 3.21 show product formation to occur, for a decanol two-liquid phase system, at ≈ 50% of the aqueous control.

The variable nature observed with these two solvents was not seen for other solvents of either higher or lower log P.

The differing behaviour of the biocatalyst observed is likely to be due to batch variations in growth conditions, a different batch of cells being employed each week.

3.7 Adhesion of cells to the organic-aqueous interface.

Solvents of log P above 4 were able to remove ≈ 20% of the cells to the interface (fig. 3.30), resulting in little organic-aqueous emulsion after 15 minutes separation. Solvents of log P < 4.0 were
observed to be much more effective at removing cells from the aqueous phase, forming large amounts of stable emulsion, which in some cases could be observed to remain for several days. An initial absorbance of 0.5 - 1.0 at A 670 nm was used throughout. Plate 4 shows the emulsions formed after 30 seconds of mixing for dodecane, pentane and heptanol. It is interesting to note the much smaller size of the emulsion droplets formed upon the mixing of heptanol (log P 2.4).

The stability of the organic-aqueous emulsions formed in two-liquid phase stirred tanks were found to increase with time, stable emulsions being formed in many cases during the first hour of biotransformation.

It has been reported that pseudomonads are capable of producing rhamnolipid surfactants under conditions of nitrogen limitation (Venkata Ramana and Karanth, 1989).

The strain of *P. putida* used in this study was also found to produce a rhamnose containing surfactant in the aqueous stirred tank, as detected using the rhamnose assay in section 2.3.5. It is unclear however if the rhamnose containing surfactant and the rhamnose containing lipopolysaccharide released by exposure to EDTA (section 3.12) are one and the same. The level of rhamnose containing surfactant produced was found to be only a small fraction of the level of that detected when LPS was removed from the outer membrane using EDTA.

When the supernatant of 0.9 mM EDTA treated cells, containing released lipopolysaccharide, was mixed with a range of organic solvents, emulsions were formed which were stable for many days.
Plate 4

**Dodecane log P**
- 6.6
- 30 s mixing
- X 40 magnification

**Pentane log P**
- 3.0
- 30 s mixing
- X 20 magnification

**Heptanol log P**
- 2.4
- 30 s mixing
- X 100 magnification
3.27 Product formed in two-liquid phase stirred tank reactors over 2 hours as a percentage of aqueous controls, against solvent log P.

Cell loading 0.42 g dcw / L
3.28 Product – time profiles in two-liquid phase stirred tank and two-liquid phase shaken flask systems.

30ml solvent : 30ml buffer.
Shaken flasks: Di-iso-pentyl ether (□), Decanol (△).
Stirred tanks: Di-iso-pentyl ether (■), Decanol (▲).
Stirred tanks 800rpm, Shaken flasks 150 rpm.
Cell loading 0.21 g dw / L.
Product formed in two-liquid phase stirred tank and two-liquid phase shaken flask systems in 3 hours, against solvent log $P$. 30ml solvent : 30ml buffer.

Shaken flasks (△), Stirred tanks (▲).

Stirred tanks 800rpm, Shaken flasks 150 rpm.

Cell loading 0.21 g dcw / L.
3.30 Cell adhesion to the organic-aqueous interface as measured by the percentage decrease in aqueous phase optical density, versus solvent log P. 5ml cell suspension + 1ml solvent were mixed vigorously for 1 min followed by 15 min separation, before measuring the optical density of the aqueous phase. Data is an average of five runs.

See section 2.4.6 for details of controls.
and in some cases for many weeks. Solutions of EDTA when mixed with organic solvents did not produce stable emulsions.

3.8 Biocatalyst immobilisation in calcium alginate.

3.8.1 Optimisation of cell loading.

\textit{P. putida} was immobilised in calcium alginate beads as detailed in section 2.4.12. Product-time profiles for various cell loadings in aqueous stirred tanks are shown in fig.3.31 and are observed to be linear over the initial 5.5 hours. Figure 3.32 shows the specific product formed per hour over a range of bead cell loadings. The product formation rate fell above a cell loading of 1 g dcw / L of beads, indicating mass transfer limitation. Cell loadings below 1 g dcw / L of beads were found to have a product formation rate as for the free cell control, of \( \approx 0.8 \) g/g/hr.

3.8.2 Operation in the presence of organic solvents.

Immobilised and free cells were placed in aqueous and decanol two-liquid phase stirred tanks and the product-time profiles measured over 2.5 hours (fig.3.33). The profiles obtained were linear in all cases.

Free cells in decanol were found to bring about the hydroxylation of naphthalene at \( \approx 26 \% \) of the rate of free cells in the aqueous stirred tank. Immobilised cells in decanol brought about the
hydroxylation at ≈ 81% of the rate of immobilised cells in the aqueous stirred tank.

Immobilised cells placed in a di-iso-pentyl ether two-liquid phase stirred tank produced product at the rate of the immobilised aqueous control. Free cells in the presence of di-iso-pentyl ether had a production rate of ≈ 55% of the free cell aqueous control (data not shown). Immobilisation conferred no advantage upon product formation rates for solvents above log P 4.0. Immobilisation was found to be ineffective for octanol (log P 2.9), ethyl benzene (log P 3.1), cyclohexane (log P 3.2) and hexane (log P 3.5).

3.9 The effect of solvent exposure upon biocatalyst viability.

Cells were exposed to organic solvents for two hours as described in section 2.4.7. Cells over the two hour period experienced organic solvent saturated sterile buffer. Little organic interface was present as the bottles were not mixed to avoid cell removal to the interface.

Cells were plated out, after serial dilution into sterile ice cold buffer, onto indole containing plates. Cell counts were performed after incubation, values for the percentage viability when compared to the aqueous control were calculated and plotted against solvent log P (fig. 3.34).

A sigmoidal curve was observed, solvents of log P above 4.0 having no detrimental effect upon cell viability. Cells exposed to hexane
(log P 3.5) were found to have retained = 40% viability after two hours. Cells exposed to solvents below log P 3.2 lost all viability within two hours. Cells exposed to solvents of log P 2.9 to 3.2 for 30 minutes were found to have a small proportion of viable cells, < 5%.

3.10 The effect of solvent exposure upon cell motility.

*P. putida* is found to be actively motile in phosphate buffer when observed using a microscope, see section 2.4.11. This motility is by means of polar flagella which allow rapid bacterial movement. Motility relies upon a proton motive force maintained by the electron transport chain. Motility is therefore an indirect method of monitoring the state of the electron transport chain.

Motility was observed when cells were exposed to organic solvents without mixing as in section 2.4.7. Figure 3.35 shows the motility observed after two hours of solvent exposure related to solvent log P. A sigmoidal curve was observed, cells exposed to solvents above log P 3.9 showing relatively normal motility as in the aqueous control. Cells exposed to solvents of log P < 3.3 showed no motility. Hexane (log P 3.5) and phthalic acid diethyl ester (log P 3.3) showed intermediary levels of motility. Nonanol (log P 3.4) was found to be toxic allowing no motility. A similar profile to that observed in fig.3.35 could be observed
3.31 Cell immobilisation

Product-time profiles in aqueous stirred tank reactors at various cell loadings in 3% (w/v) calcium alginate beads. Cell loadings g dw/L beads, 0.5 (●), 1.0 (■), 1.67 (▲), 1.75 (△), 2.5 (□). Free cells 0.21 g dw/L (○).

5 ml beads added per reactor.
3.32 Cell immobilisation

Product formed (g dcw/g/hr) in aqueous stirred tank reactors, versus bead cell loading (g dcw/L beads).

Activity of free cells 0.8 g/g/h.

5 ml beads added per reactor.
3.33 Cell immobilisation

Product-time profiles for aqueous and two-liquid phase stirred tank reactors, for free and immobilised cells.
Free cells, Decanol (△), Aqueous (□).
Immobilised cells, Decanol (▲), Aqueous (■).
Bead cell loading 1.75 g dry / L beads.
Free cells 0.146 g dry / L.
5 ml beads added per reactor
3.34 Cell viability as a percentage of aqueous control after 2 hours solvent exposure, versus solvent log P.

See section 2.4.7 for method of solvent exposure.
Motility (2 Hrs solvent exposure)

3.35 Cell motility after 2 hours solvent exposure, versus solvent log P.

++ = motility similar to that of aqueous control.
+  = some motility observed.
-  = no motility observed.

Results are qualitative, (+) levels of motility being related to cell viability at 2 hours, fig. 3.34.
when cells were allowed to contact the organic-aqueous interface with mixing.

3.11 The effect of solvent exposure upon membrane permeability.

The effect of solvent exposure upon membrane permeability was determined by measuring the uptake of the hydrophobic dye gentian violet (log P 1.16, Nikaido, 1976). Details of the method can be found in section 2.4.8 and 2.4.9. Untreated cells were found to take up ≈ 5 μg gentian violet / mg dry cell weight.

Figure 3.36 shows the results obtained for dye uptake verses log P for cells exposed to a range of solvents, excluding alcohols. Exposure to solvents of log P > 4.0 resulted in the same level of dye uptake as the untreated control ≈ 5 μg / mg dcw. Dye uptake increased when cells were exposed to solvents of log P < 4.0 reaching a level of ≈ 95 μg / mg dcw at a log P of 2.5 (toluene).

It is important to note that none of the solvents used in fig.3.36 had any great affinity for the dye gentian violet. When a solution of gentian violet was mixed with organic solvent, gentian violet remained in the aqueous phase upon separation of the phases.

The dashed line at 13 μg / mg dcw represents the maximum level of dye uptake due to the removal of lipopolysaccharide from the outer membrane by EDTA treatment, see sections 3.12.1 and 3.12.3.

Figure 3.37 shows the results obtained for cells exposed to alcohols. Only dodecanol (log P 5.0) brought about no increase
in cell permeability to gentian violet. As log P decreased the level of dye uptake increased in a linear fashion reaching a value of \( \approx 140 \mu g / mg \) dw at a log P of 0.8 (butanol).

The alcohols in contrast to the non-alcohol series of Fig. 3.36 had a great affinity for the dye gentian violet. When a solution of gentian violet was mixed with organic solvent, gentian violet partitioned into the organic phase upon separation of the phases. No organic phase was present during measurement of dye uptake, see section 2.4.9.

Figure 3.38 gives a comparison of the alcohol and non-alcohol series. Exposure to alcohols in the log P range 2.9 to 4.5 brought about a greater increase in dye uptake than non-alcohols of the same log P. Toluene (log P 2.5) and dibutylamine (log P 2.7) were found to fit the linear profile obtained for the alcohol series.

3.12 Selective removal of lipopolysaccharide from the outer membrane.

EDTA in Tris buffer has been shown to remove about one-half of the LPS but little else from \textit{E. coli} (Leive, 1965a).

3.12.1 Effects upon outer membrane permeability.

Cells were treated with varying concentrations of EDTA in 50 mM Tris buffer. See sections 2.4.9 and 2.4.10 for details of method. The level of LPS released and the amount of hydrophobic dye taken
The effect of solvent exposure upon uptake of the hydrophobic dye gentian violet.

Gentian violet uptake, versus solvent log P.

Alcohols not included.

--- = Maximum level of uptake due to removal of lipopolysaccharide from the outer membrane by EDTA treatment.
3.37 The effect of solvent exposure upon uptake of the hydrophobic dye gentian violet.

Gentian violet uptake, versus solvent log P.

Alcohol series.
3.38 The effect of solvent exposure upon uptake of the hydrophobic dye gentian violet.

Gentian violet uptake, versus solvent log P,

(●) alcohol series, (○) non-alcohol series.
up after EDTA treatment were measured and are shown in fig. 3.39. EDTA was shown to be very effective in bringing about the release of LPS, 0.4 mM bringing about the release of ≈ 90% of the releasable LPS. EDTA at 0.65 mM and above brought about the release of 100% of the releasable LPS. Tris buffer 50 mM pH 7.0 alone was found to release ≈ 10% LPS.

It is interesting to note that the level of gentian violet uptake increased as the amount of LPS released from the outer membrane increased. Gentian violet uptake rose from ≈ 5 µg / mg dw at 0 mM EDTA to a maximum level of ≈ 12 µg / mg dw at 0.65 mM EDTA and above.

3.12.2 Effects upon biocatalyst activity and stability in aqueous and two-liquid phase reactors.

Product-time profiles in aqueous stirred tanks for cells treated with a range of EDTA concentrations were found to be linear, fig. 3.40. EDTA treatment in the range 0.4 to 0.9 mM brought about a reduction in product formation rate, dropping to ≈ 84% of the aqueous control at 0.4 mM EDTA (90% LPS released) and ≈ 80% at 0.8 mM EDTA (100% LPS released). The linear nature of the product-time profiles indicates that biocatalyst stability is unaffected by EDTA treatment.

Product-time profiles in octane (log P 4.5) two-liquid phase stirred tanks over a range of EDTA concentrations were found to be non-linear in nature, fig. 3.41. The presence of octane, a solvent shown to be non-toxic for untreated cells, was found to bring
about a reduction in cell activity and stability, the rate of product formation falling off with time.

The removal of LPS by EDTA treatment can be seen to bring about biocatalyst inactivation by octane.

Figure 3.42 compares the levels of product formed in aqueous and octane two-liquid phase stirred tanks against EDTA concentration. The level of product formation in the aqueous stirred tank drops to ≈ 84% of the control at 0.4 mM EDTA and levels out decreasing by only a few percent above 0.4 mM.

The level of product formation in the octane two-liquid phase system, however, decreased in an almost linear fashion as EDTA concentration increased to 0.8 mM.

Figure 3.43 shows the levels of product formed in octane two-liquid phase stirred tanks at various EDTA concentrations, as a percentage of the corresponding aqueous EDTA treated controls.

The profiles for 1 hour and 2 hours bioconversion show the effect of increasing the concentration of EDTA during treatment. At 0.8 mM EDTA, product formed in 1 hour was observed to be ≈ 50% of the aqueous control and at 2 hours ≈ 35% indicating a reduction in biocatalyst stability. The greatest reductions in biocatalyst stability were observed for EDTA treatments of 0.4 to 0.6 mM.

When cells pretreated with 0.8 mM EDTA were placed in two-liquid phase stirred tanks containing a range of solvents the log P profile seen in figure 3.45 was obtained, the product-time profiles of which can be seen in fig.3.44. The product-time profiles for all solvents tested were found to be non-linear, indicating a reduction in biocatalyst stability.
Gentian violet uptake and percentage LPS released, against [EDTA] mM of pre-treatment.

Gentian violet uptake (○), LPS released, % of maximum (▲).

Maximum level of releasable LPS due to EDTA treatment

= 20 mg / g dry cell wt., = 2% of dry cell wt.
3.40 Product-time profiles in aqueous stirred tanks in the presence of varying concentrations of EDTA.

[EDTA]; 0mM (●), 0.4mM (△), 0.6mM (○), 0.7mM (□), 0.8mM (◇), 0.9mM (◇). Cell concentration 0.42g dw / L.
3.41 Product-time profiles for octane : buffer (1:1) two-liquid phase stirred tanks in the presence of varying concentrations of EDTA. 
[EDTA]; 0mM (▲), 0.4mM (△), 0.6mM (○), 0.7mM (●), 0.8mM (■). Cell concentration 0.42g dcw / L.
3.42 Product formed in 1 hour for octane two-liquid phase and aqueous stirred tanks, versus [EDTA] mM.

Octane two-liquid phase (□), aqueous stirred tank (▽).
3.43 Product formed as a percentage of aqueous controls for octane two-liquid phase stirred tanks, versus [EDTA] mM. Product in 1 hour (▼), Product in 2 hours (□).
3.44 Product-time profiles in two-liquid phase stirred tanks for cells pre-treated with 0.8mM EDTA (5 min), with solvents of various log P values.

Solvent log P: 4.5 (○), 5.6 (△), 6.1 (▽), 7.5 (▱), 8.8 (□), Aq (□).
3.45 Product formed in two-liquid phase stirred tank reactors over 2 hours as a percentage of the aqueous control, against solvent log P. 0.8mM EDTA pre-treated cells (▲), non-treated cells (△). Cell loading 0.42g dw / L emulsion.
The log P profile obtained confirmed that the removal of LPS from the outer membrane resulted in a reduction in biocatalyst activity, the level of inactivation decreasing in a linear fashion as log P increased.

3.12.3 Effects upon outer membrane permeability - Indole adapted strain.

P. putida when repeatedly grown and stored upon indole containing plates over a period of several weeks was found to have an increased resistance to removal of LPS by EDTA. Figure 3.46 shows that no LPS could be removed below 0.6 mM EDTA. Above 0.6 mM EDTA LPS was rapidly removed to a maximum level of ≈ 20 mg / g dcw, ≈ 2% of dry cell weight, as for the unadapted strain (fig.3.39). It is interesting to note that the level of gentian violet uptake was identical for both indole-adapted and non-adapted cells. Gentian violet uptake for the adapted strain only increased as LPS was released above 0.6 mM EDTA, reaching a maximum level of ≈ 13 μg / mg dcw when 100% of the releasable LPS was removed, as for the unadapted strain (fig.3.39).

3.12.4 Effects upon biocatalyst activity and stability in aqueous and two-liquid phase reactors - Indole adapted strain.

It is interesting to observe that the release of LPS, brought about by EDTA treatment, resulted in an increase in reaction rate in the aqueous stirred tank, fig.3.47. The reaction rate was in
fact found to increase below the concentration of EDTA required to bring about LPS release. Figure 3.48 shows the levels of product formed per hour at various EDTA concentrations for an aqueous system. EDTA treatment can be seen to result in an ≈ 20 % increase in reaction rate.

When cells were pretreated with 0.5 mM EDTA, below the level required to remove LPS, and placed in two-liquid phase stirred larks, the product-time profiles shown in fig.3.49 were obtained. Reaction rates were again observed to be above the level of the untreated aqueous control.

When cells were pretreated with 0.75 mM EDTA, resulting in the removal of ≈ 40 % of LPS, the cells became less resistant to the presence of organic solvents. Figure 3.50 shows the product formed by 0.75 mM EDTA treated cells in organic solvents of various log P. No activity was observed in solvents up to a log P of 5.6. In the three solvents tested above a log P of 5.6, productivity increased with increasing log P, reaching ≈ 90 % of the untreated two-liquid phase control at a log P of 8.8. Product-time profiles were of a similar nature to those observed in fig.3.44.

The log P profile obtained, when compared to the untreated log P profile, can be found to be similar to that for the Δ¹-dehydrogenation of steroids by the Gram-positive bacterium Arthrobacter simplex in the presence of organic solvents, ( Harrop et al, 1989 ).
3.46 Gentian violet uptake and percentage LPS released, against [EDTA] mM of pre-treatment.

Strain adapted to growth in the presence of indole.

Gentian violet uptake (○), LPS released, % of maximum (▲).

Maximum level of releasable LPS due to EDTA treatment

= 20 mg / g dry cell wt., = 2% of dry cell wt.
3.47 Product-time profiles in aqueous stirred tanks in the presence of varying concentrations of EDTA.

Strain adapted to growth in the presence of indole.

[EDTA]; 0mM (●), 0.25mM (■), 0.5mM (▲), 0.6mM (▼), 0.7mM (□), 1.0mM (○). Cell concentration 0.42g dw / L.
3.48 Product formed (g/g/hr) in aqueous stirred tank reactors, versus [EDTA] mM.

Strain adapted to growth in the presence of indole.

Cell loading 0.42g dw / L emulsion.
3.49 Product-time profiles in two-liquid phase stirred tanks after pre-treatment with 0.5mM EDTA (5 min), with solvents of various log P values.

Strain adapted to growth in the presence of indole.

Log P value; 7.5 (■), 6.6 (○), 5.1 (▼), 5.0 (△), Aq (◆).
3.50 Product formed in two-liquid phase stirred tank reactors over 2 hours, against solvent log P.

0.75mM EDTA pre-treated cells, % of untreated controls (■), non-treated cells, % of aqueous control (△).

Cell loading 0.42g dw/L emulsion.

Strain adapted to growth in the presence of indole.
3.13 Growth in the presence of organic solvents.

*P. putida* and *A. simplex* were grown in the presence of organic solvents as described in section 2.4.3.8.

3.13.1 Effect of solvent log P.

When the level of growth obtained after 25 hours was plotted against solvent log P, the profiles seen in fig.3.51 were obtained. *P. putida* appeared to grow well in solvents of log P 3.2 and above. No growth was observed below a log P of 2.9. *A. simplex* was able to grow well in tetradecene (log P 7.5), solvents of lower log P were found to be detrimental to growth. No growth occurred below a log P of 5.1.

Figure 3.52 shows the growth profiles obtained for growth in 250 ml shaken flasks containing 25 ml medium and 5 ml organic solvent. The growth profiles obtained for solvents of log P > 3.2 were as for the control containing no solvent. Growth did occur in solvents of log P 3.2 to 2.9 with a longer lag period. Octanol (log P 2.9) allowed some initial growth after a long lag period.
3.13.2 **Effect upon motility and permeability.**

*P. putida* grown in the presence of cyclohexane (log P 3.2) was found to be actively motile. Cells when transferred to solvents of lower log P were found to be non-motile. Cells grown in the presence of octanol were found to be motile during growth. When growth stopped at ≈ 10 hours (fig.3.52) motility ceased.

Growth in the presence of organic solvents of low log P was not found to alter the level of gentian violet uptake.
3.51 Growth of *P. putida* and *Arthrobacter simplex* in the presence of organic solvents, versus solvent log P.

(+++) = Growth as in control, (++) = 60% of control,

(+) = 30% of control, (-) = No cell growth.

*P. putida* (○), *A. simplex* (△).

See section 2.4.3.8 for details of method.
3.52 Growth of *P. putida* in the presence of organic solvents.

Absorbance 670 nm against growth time.

250 ml shaken flasks - 25 ml growth medium + 5 ml solvent.
Log P 2.9 (▼), 3.2 (●), Control with no solvent (△).
Solvents of log P > 3.3, with the exception of nonanol, grew at a similar rate to the control.

See section 2.4.3.8 for details of method.
4. DISCUSSION

There are many biotransformations of industrial importance which require the regeneration of co-factors e.g., hydroxylations and dehydrogenations. It is this type of reaction which usually necessitates the use of cellular biocatalysts, as they have the ability to regenerate co-factors cheaply and efficiently. The cost of biocatalyst production is also likely to be a key factor in choosing the biocatalyst form, enzymes being expensive to isolate and purify, a point in favour of using whole cells.

The hydroxylation of naphthalene by \textit{P. putida} described in this study is one such reaction which requires the use of intact cells. The enzyme involved, toluene dioxygenase, is difficult to purify being made up of three sub-units. The purified enzyme is also inactivated in air, and requires the presence and regeneration of the co-factor NADH₂ for activity.

\textit{P. putida} has been reported widely in the literature to be able to hydroxylate naphthalene (Yen and Serdar, 1988; Ensley \textit{et al}, 1982; Barnsley, 1975; Hsieh and Wang, 1980). Most of these strains possess the plasmid encoded enzyme naphthalene hydroxylase. \textit{P. putida} UV4, used here in this study, is able to hydroxylate naphthalene using the chromosomally encoded enzyme toluene dioxygenase, which can hydroxylate a range of poly-aromatics. The enzymes are similar in make-up consisting of 3 sub-units. Toluene dioxygenase upon purification does, however, require the addition of Fe²⁺ for activity \textit{in vitro} (Geary \textit{et al},
Most of the studies in chemical transformations by intact cells have been carried out in aqueous solution. There is now considerable interest in the use of two-liquid phase bioreactors in which compounds only sparingly soluble in water can be dissolved to high concentrations in water-immiscible organic solvents so as to bring about a bioconversion in a two-liquid phase environment (Lilly, 1983).

There are many potential advantages of operating in the presence of a second liquid phase (Lilly and Woodley, 1985); however, there are drawbacks, including the possible inactivation of the biocatalyst by the organic solvent (Lilly et al, 1987). With the development of this new technology there comes an increasing need to understand how the catalyst interacts with its environment during biotransformation and in particular the effect of the potentially toxic organic phase on catalytic activity.

This study describes how *P. putida*, a Gram-negative bacterium, behaves in the presence of organic solvents during the hydroxylation of naphthalene.

### 4.1 Production of toluene dioxygenase in *Pseudomonas putida* during growth.

Growth on glucose yielded ~10 g dry cell weight per litre after an 18 hour growth period (fig.3.1). Glucose became limiting at ~7 h (fig.3.3), and growth from 7 h onwards was under glucose limiting conditions. It is interesting to note that the majority
of enzyme production occurred from 7 h onwards (fig. 3.7) in the absence of high levels of glucose.

When bacteria were grown in the presence of high levels of glucose throughout, either in shaken flasks or when excess glucose was added to the 2 L fermenter due to a longer than usual lag period, cells were found to have little or no toluene dioxygenase activity. The results indicate that the enzyme toluene dioxygenase is subject to catabolite repression with growth in the presence of excess glucose. Hsieh and Wang (1980), observed that increasing the glucose concentration above 1.5 g/L during growth reduced the specific product formation rate, indicating that excess glucose inhibits the induction of naphthalene dioxygenase by catabolite repression. The results obtained in shaken flasks are also confirmed by the work of Naylor (1988), using the same strain of *Pseudomonas putida*. Naylor on the basis of this finding wrongly concluded that growth for the production of active cells on glucose was not possible and resorted to the use of sodium pyruvate, obtaining a maximum optical density of 4.6 after 18 h growth on a 10 L scale.

The results in fig. 3.3 and 3.7 show that it is possible to produce active cells capable of hydroxylating naphthalene, if glucose is pulsed and becomes limiting from 7 h of growth onwards. The optical density achieved in 18 h of growth was found to be ≈ 20, four times the level achieved by Naylor in 18 h with growth on sodium pyruvate.

The pH and DOT profiles observed during growth (fig. 3.2) were typical for the production of active cells. The increase in DOT at
10 h corresponded to a reduction in growth rate as the cells entered carbon limited growth (fig.3.2 and 3.3).

A large proportion of the active cells were produced between 7 and 10 h of growth (fig.3.7). The dot during this period decreased from ≈ 50 % to a minimum of ≈ 5 % of air saturation at 9.5 h.

DOT level has been shown to be critical for the induction of many enzymes which utilise oxygen as one of the substrates eg. cholesterol oxidase in *Nocardia rhodochrous*, was found to be maximal between 35 and 45 % of air saturation (Buckland et al, 1976). A DOT level of 10 % to 15 % being optimal for the induction of progesterone 11α-hydroxylase in *Rhizopus nigricans* (Hanisch et al, 1980). The induction of salicylate hydroxylase in *P. putida* was also found to be increased when the oxygen transfer rate was reduced at lower stirrer speeds (Russell, 1981).

It is possible that the level of toluene dioxygenase produced during growth may also be affected by the DOT level. It was observed that when dot increased to ≈ 70 % of air saturation (fig.3.2), the specific enzyme activity declined. It is unclear, however, if this was due to the increase in DOT, or the onset of carbon limited growth. Further study of the effect of DOT upon toluene dioxygenase production, during exponential and carbon limited growth, may allow the production of more active cells.

Although glucose became limiting from 7 h onwards, exponential growth continued for a further 3 h (fig.3.3). This exponential growth was due to the utilisation of gluconate which had been
produced from 4 h onwards during rapid growth upon glucose. The presence of high levels of gluconate (≈ 5 g/L, fig. 3.3) did not appear to inhibit enzyme production (fig. 3.7). The production of gluconate by *P. putida* accounts for the observed cell yield upon glucose seen in figs. 3.4 and 3.5. The predicted and measured levels of gluconate shown in fig. 3.6 indicate that glucose was converted solely to gluconate.

The levels of toluene dioxygenase produced throughout this study were typically able to bring about the hydroxylation of 1 g of naphthalene per 1 g dry cell weight per h. The level varied however reaching levels as high as 1.4 g/g dcw. Cells were not used if the activity was found to be below 0.5 g/g dcw.

It is important to note that cells grown up fresh each week, including those with lower and higher than average activities, performed in the same manner when exposed to organic solvents, except that variable responses were observed for the solvents di-iso-pentyl ether and decanol, see section 3.6.2. This variation in biocatalyst solvent tolerance is likely to be due to variations in growth conditions from batch to batch. Growth conditions have been shown to alter membrane composition and fluidity, discussed in section 4.9, and are likely to be responsible for the above mentioned variable response of different batches to di-iso-pentyl ether and decanol.

Cells in the form of a cell paste were found to remain active for a maximum of 2 days. Freezing the cells in liquid nitrogen resulted in an immediate loss of activity; activity after one weeks storage at -40°C was ≈ 16% of that for freshly harvested
cells. A 90% reduction in activity upon freezing in liquid nitrogen was observed for this same strain by Brazier (1989). Long term storage is therefore not possible and necessitates the growth of fresh cells for each biotransformation.

Growth of *P. putida* UV4 with pulsed glucose as described above was found to be adequate for the purposes of this study. However, the problems encountered with catabolite repression, resulting in varying levels of enzyme production, coupled with the fact that no enzyme production was observed above 32°C, do not make *P. putida* UV4 the ideal strain to study further the effects of growth conditions upon biocatalyst behaviour in the presence of organic solvents. This problem is discussed in sections 4.9 and 4.10.

4.2 Production of (+)-cis-naphthalene-1,2-dihydroxy-1,2-diol.

*P. putida* UV4 performed the hydroxylation of naphthalene producing a single product, confirmed to be (+)-cis-naphthalene-1,2-dihydroxy-1,2-diol as detailed in section 3.4. The literature supports the cis conformation, the trans isomer being formed by eukaryotic organisms only (Gibson and Subramanian, 1984).

*P. putida* UV4 was able to hydroxylate naphthalene forming the dihydrodiol in an aqueous stirred tank reactor with solid naphthalene (fig.3.8), requiring the presence of a co-substrate to regenerate reducing power (fig.3.10), and the substrate oxygen via active aeration (fig.3.13). Ethanol and glucose were found to be good sources of reducing power, and for the reasons mentioned in
3.3.1 glucose was used in preference to ethanol in this study. The small amount of product produced by cells with no added carbon source most probably resulted from utilisation of the cells remaining internal energy reserves, once depleted no further product was formed.

The hydroxylation of naphthalene occurred over a wide pH range (fig.3.15); however, the biotransformation was performed at pH 7.0 to avoid possible product breakdown to α-naphthol, shown to occur under acidic conditions (Hsieh and Wang, 1980).

It was interesting to note that buffer molarity had an influence upon product formation, an increase in molarity bringing about a reduction in product formation rate. In the case of 500 mM phosphate buffer, stability as well as activity seemed to be affected resulting in a reduction in product formation rate with time (fig.3.14). Ionic strength has also been observed to be important in the action of 4-methoxybenzoate monoxygenase of *P. putida* (Eich et al., 1985), 50–100 mM NaCl being optimal for activity, with concentrations above 100 mM resulting in a reduction in specific activity. 500 mM NaCl resulted in a specific reaction rate 15% of the maximum rate achieved with 50 mM NaCl. Eich proposed that this inactivation was due to direct interaction of the ions with the reductase sub-unit of the enzyme system.

Dihydrodiol was formed at the rate of \( \approx 1 \text{ g per g dww per h} \), and production was found to be linear over the initial 5 to 6 h of biotransformation. The reaction rate decreased from 6 h onwards, with no further product being formed after 7 h (fig.3.9). This loss of activity was shown not to be the result of product
inhibition, as fresh cells produced product at the rate of 1 g/g dcw/h over a 6 h period, after being transferred into buffer containing high levels of product. It was even possible to produce concentrations above the saturation level in buffer, resulting in crystal formation, plate 3 (section 3.3.4). It is likely that the cells when placed in phosphate buffer, in the presence of glucose, lost activity due to enzyme breakdown, enzyme synthesis not being able to occur in the presence of the high levels of added glucose due to catabolite repression.

The levels of aeration required to provide adequate oxygen transfer were determined (section 3.3.3, Fig.3.11 and 3.12). All further biotransformations were performed under non-limiting conditions at cell concentrations ranging from 0.21 - 0.42 g dcw per litre. Cell concentrations in 250 ml shaken flasks did not exceed 0.21 g dcw per litre.

The hydroxylation of naphthalene in the presence of organic solvents has also been characterised and a typical product-time profile can be seen for the non-toxic solvent undecanol in fig.3.16. The product formation rate and product-time profile are identical to those seen in fig.3.8 for the aqueous stirred tank system, indicating that the presence of undecanol is not detrimental to the hydroxylation of naphthalene by \textit{P. putida}.

The product formation rate in the organic-aqueous stirred tank was dependent upon the concentration of naphthalene in the organic phase (figures 3.17, 3.18 and 3.19). For a cell loading of 0.83 g dcw/L of emulsion, a naphthalene concentration of > 45 g/L of solvent was required to allow the maximal rate of product
formation of \( \approx 1 \text{ g/g dcw / h} \), and below this concentration the rate of product formation fell rapidly.

Biotransformations were usually carried out at a naphthalene concentration of 83 \( \text{g/L} \) of solvent, and a cell loading of 0.42 \( \text{g dcw / L of emulsion} \), ensuring that both naphthalene and oxygen transfer were not limiting during the period of sampling.

As can be seen from the naphthalene solubility data in table 3.1 most of the solvents were operated at \( \approx 55 \% \) of saturation. Concentrations above this level were not used with the more volatile solvents e.g. pentane and hexane, as solvent evaporation during active aeration brought about the crystallisation of naphthalene blocking the aeration tubes.

The behaviour of the biocatalyst was found to be dependent upon the nature of the organic solvent used. A good correlation between biocatalyst activity and solvent log P was demonstrated (fig.3.27). The use of log P in predicting biocatalyst performance is discussed fully in the following sections.

4.3 **The use of solvent log P for the prediction of biocatalyst activity and stability.**

The log P concept giving an indication of solvent polarity was first applied to biocatalysis in the presence of organic solvents by Laane et al (1985). A good correlation between activity and solvent polarity was observed when log P was used as a measure of polarity. Another indicator of solvent polarity, the Hildebrand solubility-parameter, \( \delta \), was applied to biotransformations in
organic media by Brink and Tramper (1985), however, a poor correlation between δ and activity was observed. Other methods of quantifying solvent polarity have been described in relation to biocatalyst activity retention by Laane et al (1987), who observed poor correlations with biocatalyst activity retention.

The log P index has proved to be the best single indicator of biocatalyst-organic solvent interaction and has been adopted by many workers in the field of two-liquid phase biocatalysis in recent years. When Laane plotted the results of Brink and Tramper (1985), for epoxidation-activity retention against log P, a clear sigmoidal-shaped correlation was observed. Activities were observed to be low in solvents of log P < 2, variable between log P 2 and 4 and high in solvents with log P > 4 (Laane et al, 1985, 1987).

The results presented in this thesis showing product formation, as a percentage of the aqueous control, against log P, fig.3.27, agree strongly with the results of Laane et al (1987), a sigmoidal curve being observed. High activities were observed above log P = 4 and below log P = 3 no activity was observed. A sharp transition in productivity was observed between log P values of 3 and 4, although productivity increased as log P increased through this range, with the exception of nonanol (log P 3.4) and decanol (log P 4.0).

Due to the linear nature of the product-time profiles obtained in all the solvents tested, figures 3.24, 3.25 and 3.26, the log P plot observed in fig.3.27 taken after 2 hours of product formation could also have been obtained at 0.5, 1, 2 or 3 hours, indicating
that after the initial loss of activity produced by exposure to some solvents eg. decanol, no further loss is brought about due to solvent exposure.

Product-time profiles obtained for the Δ1-dehydrogenation of hydrocortisone by the Gram-positive bacterium *Arthrobacter simplex* in various two-liquid phase systems, in contrast to those mentioned above for *P. putida*, were observed to be curved in nature, indicating a time dependent loss of activity (Hocknull, 1989).

The log P concept has been shown to break down when applied to free enzymes (Klibanov, 1986), and no correlation between solvent log P and enzyme activity retention was observed. Laane *et al* (1987), however, states that the enzyme activities of cholesterol oxidase, xanthine oxidase and enoate oxidase do correlate with log P producing a sigmoidal relationship, although the correlations were not shown.

4.3.1 Limitations of using log P alone.

The correlation between biocatalyst activity and log P was found to be sigmoidal in this study, in the data of Laane *et al* (1985, 1987), and in the data of Playne and Smith (1983), however, the Δ1-dehydrogenation of hydrocortisone in a two-liquid phase stirred tank by free *Arthrobacter simplex*, a Gram-positive bacterium, in the presence of organic solvents, only occurred at the rate of the aqueous control in di-iso-octyl phthalate (log P 9.8). As the log P decreased below 9.8, the level of
activity retention also decreased down to a log P of 3, below which no activity was observed (Hocknull and Lilly, 1987). In this case the log P concept can be seen to break down, there being no justification for the statement that organic solvents of log P greater than 4.0 do not result in biocatalyst damage.

The enzyme responsible for the Δ1-dehydrogenation by *A. simplex* was found to be stable down to a log P of 4, the addition of the artificial electron acceptor phenazine methosulphate (PMS) resulting in a sigmoidal curve (Hocknull and Lilly, 1987), of a similar nature to that of *P. putida*, fig. 3.27.

When *A. simplex* was prevented from contacting the organic-aqueous interface by immobilisation in calcium alginate beads, (Hocknull, 1989), a sigmoidal correlation between activity retention and log P was also observed. These results show the behaviour of *A. simplex*, in relation to log P, to be dependent upon whether the cells were free or immobilised.

Solvent log P values in most cases are obtained from the 'hydrophobic fragmental constant' system of calculation introduced by Rekker (1977, 1979). These values usually give good agreement with the experimental data, but there are however some deviations eg. pentane; 3.0 calculated, 3.31 measured and pentanol; 1.3 calculated, 1.56 measured (Halling, 1989).

It can be argued that theoretical correlations reflect an intrinsic property of the solvent polarity that affects both octanol-water partitioning and biocatalyst activity, and that deviations of experimental values from the trend may reflect some specific interactions with octanol or water that were not mirrored.
In effects on the biocatalyst (Halling, 1989).

The slight discrepancies between calculated and measured values are not thought to be of great importance, as the current correlations with biocatalyst behaviour are in many cases not sufficiently good enough for the differences in log P values to be very significant.

Duarte and Lilly (1980) found that the oxidation of cholesterol forming cholestenone by *Nocardia rhodochrous* could take place in organic solvents of log P < 2. Dichloroethane (log P 1.3) was shown to allow maximal activity along with trichloroethane.

The enzyme chymotrypsin adsorbed to porous glass beads was found to have high a reaction rate and operational stability over the log P range 1.5 to 2.1 (Reslow *et al.*, 1987). Activity was observed down to a log P of 0.65. Dichloroethane (log P 1.3) allowed the esterification to occur at the maximal rate. Log P values above 2.5 were found to result in a reduction in reaction rate due to the low solubility of the substrate, N-acetyl phenylalanine, in these solvents.

The dehydrogenation of testosterone by a free enzyme system, coupled to the reduction of pyruvate to regenerate co-factor, was observed to function well down to a log P of 0.68 (Cremonesi *et al.*, 1974).

The oxidation of steroid hormones by fungal laccase in the presence of organic solvents was found to function well down to a log P of 0.68 (Lugaro *et al.*, 1973). Solvents of high log P were found to be less efficient because of the low steroid solubility in them.
Boeren et al (1987), observed that the growth stage of the microorganism at the moment of solvent addition could itself have an effect upon biocatalyst behaviour in the presence of organic solvents.

The water content of the organic solvent has also been observed to affect the reaction rate (Reslow et al, 1987; Halling, 1987).

Despite the limitations mentioned above, log P remains the best index for predicting the behaviour of biocatalysts in the presence of organic solvents. As more data becomes available for the various types of biocatalyst in relation to solvent log P, a clearer picture of the true value of log P as a predictor of biocatalyst behaviour will appear.

When using whole cells, it is likely that the biocatalyst type, e.g. Gram-positive or Gram-negative bacterium, yeast or animal cell, immobilised or free, as well as the reaction type, should be taken into consideration alongside log P, allowing for a more educated prediction of biocatalyst performance in organic solvents.

4.3.2 Solvent selection using a combination of log P, biocatalyst form and reaction type.

While log P alone has been shown to be a good indicator of biocatalyst behavior, it is likely that other factors which have been shown to influence biocatalytic behaviour should also be considered.

Since this thesis is primarily concerned with the way in which
whole cells interact with the organic phase, the examples will be confined mainly to whole cell systems.

When the productivities of *P. putida*, a Gram-negative bacterium, and *A. simplex*, a Gram-positive bacterium, were compared in the presence of a range of organic solvents in the stirred tank, a striking difference in the log P profiles was observed, fig.4.1. This striking difference is thought to be due to differences in bacterial cell wall structure and composition (Harrop et al, 1989). The hydroxylation of naphthalene by *P. putida*, and the Δ1-dehydrogenation of hydrocortisone by *A. simplex*, require the regeneration of a co-factor to allow the reactions to proceed, and both systems relying upon the presence of an intact electron transport chain for this regeneration to occur (Hocknull and Lilly, 1987; sections 4.6 and 4.7 of this thesis). It is thought that the presence of the outer membrane in *P. putida*, a structure lacking in Gram-positive bacteria, confers increased resistance to the potentially toxic organic solvents, by excluding their entry to the more solvent sensitive inner membrane, which contains the electron transport chain. When this outer membrane in disorganised by treatment with EDTA, fig.3.45, the log P profile obtained becomes more like that for the Gram-positive *A. simplex*. This finding, together with the extensive literature concerning the shielding function of the outer membrane against hydrophobic compounds, discussed fully in section 4.5, adds weight to the argument that this is an observed Gram-positive - Gram-negative difference (Harrop et al, 1989).
Product formed by *P. putida* (△) and *A. simplex* (●) in two-liquid phase stirred tank reactors (50% v/v solvent) over 2 hours as a percentage of aqueous control, against organic solvent log P. Biotransformations were done in 100 ml stirred tank reactors (working volumes 60 ml and 70 ml and stirrer speeds 800 rpm and 750 rpm for *P. putida* and *A. simplex* respectively). Both systems were operated under non-oxygen limiting conditions. For *P. putida* the rates of product formation were linear over the 2 hour period, even with the more toxic low log P solvents. Biotransformations took place using non-growing bacteria in phosphate buffer (50mM, pH 7) and Tris-HCl buffer (50mM, pH 7.8) for *P. putida* and *A. simplex* respectively. Organic solvents in both cases were saturated with substrate. Log P is defined as the logarithm of the solvent partition coefficient in a standard octanol-water two-phase system.
It is interesting to note that where sigmoidal correlations using log P have been reported for free cells in two-liquid phase stirred tanks, no Gram-positive organisms have been noted. When \textit{A. simplex}, a Gram-positive bacterium, was immobilised in calcium alginate beads, however, a sigmoidal log P profile was obtained in the two-liquid phase stirred tank (Hocknull, 1989). It is likely that immobilisation in hydrophilic calcium alginate beads protects the solvent sensitive cytoplasmic membrane from direct contact with the organic solvent in an analogous way to the outer membrane of Gram-negative bacteria.

The biocatalyst type eg. Gram-positive or Gram-negative, as well as the form eg. immobilised of free, alongside log P, would seem to be of great importance in predicting behaviour in the presence of organic solvents.

The type of enzyme reaction being performed can also be observed to affect the type of log P profile obtained. Reactions in which co-factor regeneration is not involved, or not electron transport linked, have been shown to function in solvents of log P < 2 (Lugaro \textit{et al}, 1973; Cremonesi \textit{et al}, 1974; Reslow \textit{et al}, 1987; Dordick, 1989). In such non-cofactor, non-electron transport chain linked systems, the log P profile obtained will be dependent upon the enzymes own susceptibility to solvent denaturation. This will vary depending upon the enzyme type, folding pattern and the solvents ability to distort the enzymes essential water (Khmelnitsky \textit{et al}, 1988; Zaks and Russell, 1988).

If the electron transport chain is found to be the weakest point ie. the most solvent sensitive and primary site of solvent attack,
then all enzyme reactions requiring its presence for activity will be dependent upon the log P at which the electron transport chain ceases to function. This was the case for A. simplex which lost activity below a log P of 9.8 (Hocknull and Lilly, 1987; section 4.3.1).

It is likely that sigmoidal log P correlations will be observed for biotransformations involving free Gram-positive bacteria, where the reactions are not linked to the electron transport chain.

When using whole cell biocatalysts for biotransformation it is important to select a group for example Gram-negative bacteria, which are likely to be resistant, and able to operate in a wide range of organic solvents (Harrop et al., 1989; Inoue and Horikoshi, 1989; Boeren et al., 1987; Harbron et al., 1986; Laane et al., 1985).

Nocardia rhodochrous, an actinomycete, was able to oxidise cholesterol to cholestenone in solvents of low log P such as dichloroethane (log P 1.3), (Duarte and Lilly, 1980). This one step conversion appeared not to be linked to the electron transport chain, when ethanol was used to regenerate NADH2.

Further breakdown of the cholestenone did not occur, however, complete breakdown was observed when cells were immobilised in calcium alginate or polyacrylamide gels (Duarte and Lilly, 1984).

The examples above, showing the various responses obtained using biocatalysts in the presence of organic solvents, strongly emphasise the need to take these variations into account when
selecting a suitable solvent on the basis of log P. As more data becomes available for the various biocatalyst types, a clearer picture as to the value of log P in predicting biocatalyst performance should emerge.

4.4 The role of the organic-aqueous interface in biocatalyst inactivation.

The organic-aqueous liquid-liquid interface has been observed to be important in bringing about biocatalyst inactivation for a variety of biocatalyst types (Inoue and Horikoshi, 1989; Hocknull, 1989, 1987; Brazier, 1989; Bar, 1987; Lilly et al, 1987; Rezessy-Szabó et al, 1987; Duarte and Lilly, 1984; Finn, 1966). The catalysts in the above mentioned examples were all thought to be caused by contact with the organic-aqueous interface as immobilisation or operation in the presence of sub-saturated levels of solvent in the buffer or growth medium, were in many cases found to prevent this inhibition.

4.4.1 Free cells.

P. putida when placed in the stirred tank, in the presence of organic solvents, produced linear product-time profiles for over a range of solvent log P values (figures 3.24, 3.25, 3.26). For solvents of log P > 4.0, product formation was at the same rate as the aqueous control. Similar levels of product were formed in shaken flasks (fig.3.29), indicating that the organic-aqueous
interface is not toxic for solvents above log P 4.0.

Solvents of log P < 4.0 were toxic to free cells, a log P < 3.5 producing little or no product. When cells were placed in shaken flasks in the presence of solvents of log P < 3.5, allowing only limited direct contact with the organic phase, no increase in product formation was observed, fig.3.29, indicating that dissolved levels of solvent alone are able to bring about biocatalyst inactivation, the organic-aqueous interface playing little part in inactivation for solvents of log P < 3.5.

The organic-aqueous interface was found to be important in bringing about inactivation by decanol (log P = 4.0) and di-iso-pentyl ether (log P = 3.9). When the amount of available interface present in the stirred tank was increased by increasing stirrer speed or phase ratio, (figures 3.20, 3.21, 3.23), for decanol, the product formation rate was observed to decrease. The product formation rate in buffer saturated with decanol, was identical to that for the aqueous control (fig.3.21), indicating that the toxic effects observed for decanol were due almost entirely to contact with the organic-aqueous interface. Di-iso-pentyl ether was observed to behave in a similar manner to decanol (fig.3.28, 3.29), producing product at 100% of the aqueous control in two-liquid phase shaken flasks. It can be seen from fig.3.28 that the presence of a second liquid phase in the shaken flask, even though the interface present was limited, was found to be toxic for decanol, di-iso-pentyl ether in such a system was able to form product as for the aqueous control.

It is important to note at this point that the behaviour of
*P. putida* was found to be variable in these two solvents, possibly due to slight variations in growth conditions from batch to batch.

When the level of available interface was increased for the non-toxic solvent dodecane (*log* P = 6.6), the product formation rate increased with rpm, reaching a maximum at 750 rpm and remaining at this level up to 1000 rpm, showing the presence of interface was not harmful for biotransformations in dodecane two-liquid phase systems.

The presence of organic-aqueous interface only appears to be detrimental for a few solvents in the log P range of ≈ 3.9 - 4.0. For solvents of log P below 3.9, the presence of interface appears not to be required to bring about biocatalyst inactivation, above a log P of 4.0 no inactivation was observed.

The exact mechanism by which the organic-aqueous interface brings about biocatalyst inactivation is not fully understood. For *A. simplex*, a Gram-positive bacterium, it is likely that the large concentration gradient brought about by direct contact with the interface allows for rapid diffusion of solvent into the cells, allowing the solvent to rapidly reach a toxic concentration. In buffer saturated with solvent this inactivation occurred at a reduced rate, but nevertheless inactivation did occur, as shown by the product-time curves, which indicated a reduction in productivity with time (Hocknull, 1989). The product-time profiles obtained for *P. putida*, both in the stirred tank and shaken flask, for non-toxic solvents, were linear over the reaction periods measured (up to 6 hours), indicating that
solvent could not reach a toxic level within the cell over this time period.

A second possible mechanism of inactivation by the organic interface could be the disruption of the cell membrane(s) by direct solvent contact. This disruption is likely to increase membrane permeability to solvent, allowing the concentration of solvent in the cell to rise more rapidly to a toxic level. Toluene has been shown to remove about 10% of the lipopolysaccharide (LPS) from the outer membrane of *E. coli* making the membrane more permeable (Smit *et al.*, 1975). LPS was shown in this study to act as a potent surfactant able to stabilise the potentially toxic organic-aqueous interface (section 3.7). It is likely that the inactivation of *P. putida* by decanol and di-iso-pentyl ether is due to disruption of the outer membrane, allowing the solvents to act upon the solvent sensitive inner membrane, which contains the electron transport chain.

The presence of the outer membrane is thought to be responsible for the resistance of *P. putida* to organic solvents of log P > 4.0 by preventing their entry, and by screening the more solvent sensitive inner membrane from direct contact with the organic-aqueous interface (see section 4.5). It is interesting to note that organisms which do not possess a structure equivalent to the Gram-negative outer membrane, as mentioned earlier in section 4.4, have in many cases been observed to be inactivated by the presence of the organic-aqueous interface.
4.4.2 Cells immobilised in calcium alginate beads.

Of these solvents tested, only decanol and di-iso-pentyl ether were able to bring about inactivation of *P. putida* due to contact with the organic-aqueous interface. Immobilisation of *P. putida* in calcium alginate beads protected the bacteria from the harmful effects of the interface for these two solvents. Immobilised cells with decanol present formed product at ≈ 81% of the rate for immobilised cells in buffer, whereas free cells formed product at only ≈ 26% of the rate of free cells in buffer (fig.3.33). Immobilised cells in di-iso-pentyl ether were observed to form product at the rate of immobilised cells in buffer.

Immobilisation conferred no advantage upon product formation rates for solvents above log P > 4.0, and was found to be ineffective for solvents of log P < 3.5 (see section 3.8.2). It would appear therefore, that shielding the cells from contacting the interface has no advantage for solvents which are found to be toxic in aqueous solution, log P < 3.5. An increase in biocatalyst activity was, however, observed for both decanol and di-iso-pentyl ether, indicating that the organic-aqueous interface was indeed responsible for a large proportion of the biocatalyst inactivation. Some of the inactivation brought about by decanol could not be removed by immobilisation, indicating that to some extent decanol was also toxic in aqueous solution. It is interesting to note that the product-time profile obtained with immobilised cells in the presence of decanol was linear over the 2.5 h period, indicating that biocatalyst activity and not
stability was affected (fig. 3.33). This will be discussed further in section 4.7.

Immobilisation of *P. putida* does not seem to confer any advantage upon biocatalyst activity or stability in the majority of organic solvents, and is therefore of little use. Another major drawback is the diffusional limitations brought about by immobilisation. Beads could not be operated at high cell loadings due to oxygen limitation, resulting in reduced product formation rates (figs. 3.31 and 3.32). Immobilisation may be of value in improving phase separation during downstream processing, however, due to oxygen transfer problems encountered for immobilised cells, this may prove to be impractical.

Immobilisation has been observed to be of great value in many systems where contact with the organic-aqueous interface has been shown to be toxic, for example, *A. simplex* when placed in a stirred tank and allowed to contact the organic-aqueous interface was found to produce full activity in only one solvent, di-iso-octyl phthalate, having a log P of 9.8 (Hocknull and Lilly, 1987). When immobilised, however, a high level of activity was observed in many solvents of log P 4 to 9.8, producing a sigmoidal-shaped curve (Hocknull, 1989), similar to that for free *P. putida* in the stirred tank (fig. 3.27). Immobilised *A. simplex* did not produce linear product-time profiles in any of the organic solvents tested, indicating that the diffusion of organic solvents into the beads, brought about a time dependent inactivation of the biocatalyst. This reduction was, however, small compared with the level of activity achieved in these
solvents by immobilisation.

*Lactobacillus delbrueckii*, a Gram-positive bacterium, has been observed to be inhibited in dodecanol-broth dispersions, however, entrapment in gels removed this inhibition (Bar, 1987).

Duarte and Lilly (1984), observed that the immobilisation of *Nocardia* allowed cholesterol to be broken down fully, free cells being able to perform only the first step to cholestenone.

From these few examples it would appear that immobilisation, preventing biocatalyst contact with the organic-aqueous interface, is a valuable technique for increasing the range of solvents within which the biocatalyst can operate. The results of this thesis show, however, that *P. putida* can perform just as well in a free form, and that immobilisation, with a few exceptions, is of no value. It is likely that this may be the case for other Gram-negative bacteria, due to the presence of the outer membrane (Harrop *et al*, 1989).
4.5 **The role of the Gram-negative outer membrane.**

The outer membrane of Gram-negative bacteria has been reported to confer increased resistance to many hydrophobic antibiotics and dyes (Leive, 1974; Gustafsson et al., 1973), including toxic organic solvents (Harrop et al., 1989). The outer membrane is thought to exclude the entry of these potentially toxic compounds due to the presence of lipopolysaccharide (LPS) on its outer surface.

The results of this thesis demonstrate that the disruption of this outer layer, by removing LPS, makes the cells sensitive to the toxic effects of organic solvents. The log P profile obtained, after removing a proportion of the LPS, was found to be similar to that obtained for free *A. simplex* in the two-liquid phase stirred tank (Hocknull and Lilly, 1987).

4.5.1 **The outer membrane as a permeability barrier to hydrophobic antibiotics and dyes.**

Most species of Gram-negative bacteria are relatively resistant to hydrophobic antibiotics, in spite of the fact that such organisms generally have sensitive internal sites for inhibition by these antibiotics (Hartmann et al., 1967; Staudenbauer, 1975). Resistance is thought to be due to the tight packing of the LPS molecules, which form a continuous layer, in the outer leaflet of the outer membrane which ensures impenetrability.
It is possible to measure outer membrane permeability by measuring the uptake of a hydrophobic dye. In this study the dye gentian violet was used to determine the effects of solvent exposure and chemical disruption of the outer membrane upon dye uptake. The method detailed in section 2.4.9 was adapted from Gustafsson et al (1973). Gustafsson observed that all cells, wildtype and envelope mutants, instantaneously took up 20% of the added dye which was found to be membrane associated, the wildtype strain did not take up any further dye over the 15 minute exposure.

*P. putida* in this study when exposed to the hydrophobic dye, took up ≈ 10% instantaneously. The untreated cells were found to take up ≈ 5 μg gentian violet / mg dry cell weight, see section 3.11.

In the secondary uptake of gentian violet for envelope mutants, observed by Gustafsson, dye was found in the ribosome fraction, indicating that the dye was taken up into the cytoplasm. Using a concentration of 10 μg / ml of gentian violet, the vast majority of dye uptake was completed within the first 10 minutes. In this study *P. putida* was exposed to gentian violet for a standard period of 30 minutes at 28°C in an orbital shaker, 150 rpm.

When *P. putida* was treated with EDTA, LPS was removed in increasing amounts as the concentration of EDTA was increased from 0 to 0.75 mM (fig. 3.39). No further release occurred with 1 mM. Exposure was carried out in 50 mM Tris buffer which alone was found to release about 10% of the total releasable LPS.

EDTA is thought to bring about the release of LPS from the outer membrane due to the chelation of the magnesium ions involved in the tight binding between the LPS molecules (for review see...
Nikaido and Vaara, 1985).

The removal of LPS from the outer membrane resulted in a higher level of gentian violet uptake (fig. 3.39). This increase in dye uptake mirrored the curve obtained for LPS release as the EDTA concentration rose, and suggests that the outer membrane is acting as a barrier to the entry of gentian violet, and that when LPS is removed, the outer membrane becomes more permeable to the dye.

When *P. putida* was grown repeatedly upon indole containing plates, the cells seemed to be more resistant to the removal of LPS by EDTA (fig. 3.46). LPS was not removed in Tris buffer, or at concentrations of EDTA below 0.6 mM. Above 0.7 mM, LPS was rapidly removed, reaching a maximum level of release, of the same magnitude as the non-selected strain, at 1 mM EDTA. The results obtained when the cells were exposed to gentian violet give a clear indication that the outer membrane permeability to the dye only increases as LPS is removed. Both the indole selected and non-selected cells released approximately the same amount of LPS, and upon release take up the same amount of dye.

It is not clear why the differences in EDTA sensitivity observed when cells were repeatedly grown on indole containing plates occurred. It is likely that growth in the presence of indole, which itself can be toxic at high concentrations or over long periods of exposure (Naylor, 1980), brought about a conformational change in the outer membrane LPS, giving them a greater affinity for binding $\text{Mg}^{2+}$, therefore making the release of LPS by EDTA more difficult. The adapted cells were not, however, found to reduce the level of gentian violet uptake for cells in
Tris buffer, being at the same level as for non-adapted cells = 5 µg / mg dw (figures 3.39 and 3.46).

The results obtained for the hydrophobic dye gentian violet do indeed show the outer membrane to be acting as a permeability barrier, its disruption resulting in an increased level of dye uptake.

It would be of interest to examine the uptake of a range of dyes, to determine the extent to which the outer membrane excludes molecules of various log P, in the absence of organic solvents. The levels of dye uptake obtained from such studies may be of use in predicting the type of response likely to occur in the presence of organic solvents, for a desired organism.

4.5.2 The role of the outer membrane in shielding against potentially toxic organic solvents.

The outer membrane has been reported to constitute an effective barrier to the entry of hydrophobic antibiotics and dyes (section 4.5.1). The work with gentian violet performed in this thesis also confirms this finding. It was thought likely, therefore, that the outer membrane of Gram-negative bacteria would also be likely to exclude the entry of potentially toxic organic solvents, and could explain the differences noted between the Gram-positive and Gram-negative log P profiles highlighted in this thesis.

In order to test out this hypothesis, the cells were chemically treated with EDTA to remove LPS from the outer membrane, before
being placed in the two-liquid phase stirred tank, and biocatalyst activity and stability measured over a range of solvent log P values.

4.5.2.1 The effect of outer membrane disruption upon biocatalyst activity and stability.

The outer membrane is thought to be effective in excluding hydrophobic molecules due to the tightly packed continuous layer of LPS in its outer leaflet (see fig. 4.2(A)). Chemical or genetical changes to the LPS are thought to cause the appearance of permeable phospholipid bilayer regions in the outer membrane (Nikaido and Vaara, 1985; Roantree et al, 1977), or discontinuities in the LPS (Hancock, 1984).

When E. coli was treated with EDTA, the cells were found to become sensitive to antibiotics, lysozyme and the complement system (Reynolds and Pruul, 1971; Tamaki and Matsuhashi, 1973; Nikaido and Vaara, 1985).

Leive (1965a) observed that treatment of E. coli with EDTA was only able to remove about 50% of the LPS. The removal of LPS by EDTA treatment was found not to affect internal metabolism and transport systems, which continued unimpaired (Leive, 1965b, 1968).

Treatment of P. putida with EDTA resulted in a reduction in activity in aqueous stirred tanks to \( \leq 80\% \) of the untreated control (fig.3.40). Product-time profiles were linear over the 2 h period of biotransformation, and biocatalyst stability would
appear to be unaffected by EDTA treatment. It is unclear, however, why a reduction in activity was observed. It is a possibility that limited cell lysis could have occurred, or the increased membrane permeability, coupled with the presence of EDTA, could have removed some of the Fe\textsuperscript{2+} ions required for full toluene dioxygenase activity (Geary et al., 1984; Gibson et al., 1982).

When cells exposed to EDTA of varying concentration were placed in stirred tanks containing the organic solvent octane (log P 4.5), the product-time profiles obtained were observed to be non-linear, the product formation rate falling off with time (fig.3.41). The results suggest a time dependent inactivation of the biocatalyst, appearing most prominent after 1 hour of biotransformation, presumably as octane reached a critical toxic level within the cell.

Activities after pretreatment with EDTA (0 - 0.8 mM) were calculated for the first hour of conversion, as the product formation over this first hour was approximately linear (fig.3.42). As the concentration of the EDTA used for pretreatment increased, the product formed in 1 h decreased in a linear fashion. Although all the releasable LPS was removed at 0.6 mM EDTA (fig.3.39). It is interesting to note that further reduction in product formation rate was observed after pretreatment with 0.65 - 0.8 mM EDTA. Although no further release of LPS is obtained above 0.6 mM EDTA, it is possible that higher concentrations could remove further Mg\textsuperscript{2+} ions from the outer membrane, making it more sensitive to the disrupting effects of the solvent.
Figure 3.43, showing the levels of product formed in octane two-liquid phase stirred tanks at various EDTA concentrations, as a percentage of the corresponding aqueous EDTA treated controls (fig.3.43), demonstrates clearly that the removal of LPS by EDTA treatment, making the membrane more permeable as shown by gentian violet uptake, makes the biocatalyst sensitive to the organic solvent octane, as shown by a reduction in activity and stability with time. Octane is shown not to be toxic to untreated cells (fig.3.41).

The reduction in productivity in the presence of octane, as a percentage of the corresponding aqueous EDTA treated control, was most pronounced at 0.8 mM EDTA, product formation being at 50 % of the control at 1 h and only 35 % after 2 h.

Further experiments performed using a wide range of organic solvents of various log P produced this same non-linear product-time profile (fig.3.44), adding further evidence to the idea that the outer membrane normally excludes these solvents, but when disrupted allows their entry, resulting in decreased biocatalyst activity and stability.

When the level of product formation was measured as a percentage of the aqueous control, against solvent log P, a very interesting profile was obtained (fig.3.45). The linear relationship observed between solvent log P and product formed, followed the same pattern as for the Gram-positive bacterium A. simplex (fig.4.1), which does not possess an outer membrane. This log P profile lends further evidence to the importance of the outer membrane in preventing biocatalyst inactivation by organic
solvents.
When the indole-selected strain was pretreated with EDTA, an increase in activity, above the level of the untreated control, was observed in the aqueous stirred tank (fig.3.47). Pretreatments with EDTA concentrations below the level required to remove LPS were also found to increase the productivity of the cells, above that of the untreated control. It appears that the removal of Mg$^{2+}$ from the outer membrane, although not at a high enough level to result in LPS release, was able to make the membrane more permeable to naphthalene. At EDTA concentrations where LPS was released, the observed productivities were found to be above the control level, and marginally higher than the pretreated cells where no LPS was removed. The product-time profiles were linear over the 3 hour period, with the exception of 1.0 mM EDTA, which after 90 minutes became non-linear, producing little more product. It is likely that cell lysis was responsible for this reduction in productivity, although cell viability studies would need to be performed to confirm this to be true.

The results suggest that selection in the presence of indole, although having no effect upon the uptake of the dye gentian violet, did result in a reduction in the rate of naphthalene entry into the cells. Disruption of the outer membrane by the removal of Mg$^{2+}$, or the release of LPS at higher EDTA concentrations, allowed naphthalene to enter at an increased rate, resulting in an ≈ 20% increase in productivity.

When indole-selected cells pretreated with 0.5 mM EDTA, (below the concentration required to release LPS), were placed
in stirred tanks containing organic solvents of various log P, an interesting observation was made (fig.3.49). The cells were not only resistant to the presence of organic solvents of log P 5.0 to 7.5, the rates of product formation were at a higher level than in the untreated control seen in the aqueous stirred tank. The product-time profiles were again linear over a 3.5 h period, indicating that biocatalyst stability was also unaffected.

It would appear that the removal of Mg$^{2+}$ from the outer membrane, without removing LPS, allows naphthalene to enter the cell at an increased rate, while excluding the entry of the potentially toxic organic solvents.

This observed resistance to organic solvents was not, however, present when LPS was removed (fig.3.50). The removal of ≈ 40 % of the releasable LPS resulted in solvents of Log P < 5.6 being totally toxic to the cells. Above a log P of 5.6, the level of product formation increased with increasing Log P, reaching ≈ 90 % of the untreated two-liquid phase control at a log P of 8.8. This profile again is of a similar nature to that observed for the Gram-positive A. simplex.

It is unclear as to why the indole-selected strain was found to be more sensitive to organic solvents once LPS was removed from the outer membrane. It is possible that the reduction in outer membrane permeability, brought about by repeated growth in the presence of indole, could alter the composition and / or fluidity of the inner membrane, which in some way reduced its solvent tolerance.
The removal of Mg$^{2+}$ is thought to be the reason for increased permeability resulting in a weakened LPS-LPS interaction, followed at higher EDTA concentrations by LPS release. The resistance to hydrophobic antibiotics and dyes observed for smooth strains of *Salmonella typhimurium*, was restored in rough mutants by the addition of cations such as Mg$^{2+}$ and Ca$^{2+}$ (Stan-Lotter *et al*, 1979). This finding supports the idea that divalent cations are required to stabilise the outer membrane LPS-LPS interactions, and that the addition of extra cations, in some cases can decrease membrane permeability, resulting in increased resistance to the entry of hydrophobic compounds.

There have been many studies using rough mutants which lack a large proportion of the polysaccharide moiety of the LPS in the outer membrane. These cells are found to be sensitive to hydrophobic antibiotics and dyes (Stan-Lotter *et al*, 1979; Roantree *et al*, 1977; Nikaido, 1976; Sanderson *et al*, 1974). Increased outer membrane permeability is thought to be due to the appearance of phospholipid bilayer domains (see fig.4.2(B)), which allow the passage of hydrophobic compounds more readily than the continuous LPS layer found in the wildtype organism (see fig.4.2(A)). Phospholipid domains are also thought to be formed after LPS has been released by EDTA treatment (see fig.4.2(D)), (Nikaido and Vaara, 1985). It has also been suggested that increased outer membrane permeability in rough mutants could be due to conformational changes in the Mg$^{2+}$ binding sites of the LPS, resulting in a weaker LPS-LPS interaction. This weaker interaction is thought to allow hydrophobic molecules to
penetrate through the LPS domains ( See fig.4.2(C), Nikaido and Vaara, 1985 ).

The results obtained using rough mutants also indicate the essential role of the outer membrane in shielding against potentially toxic molecules. Little information is available, however, as to how the various rough mutants would perform in the presence of organic solvents.

It would be of great interest to be able to isolate such mutants with the ability to hydroxylate naphthalene, and to characterise the role of the various components of the LPS in shielding against the entry of organic solvents.

In order to study the role of the outer membrane in shielding the inner solvent sensitive cytoplasmic membrane, an attempt was made to clone the *nah A* gene, coding for naphthalene dioxygenase, into a broad host range vector to allow the expression of the enzyme in a variety of these defined rough mutants, as well as in Gram-positive bacteria. The isolation and expression of *nah A*, however, proved to be more complex than first anticipated ( see section 4.10 ).
Fig. 4.2 Hypothetical structure of outer membrane in the wild type, deep rough mutants, and EDTA-treated wild-type cells of *E. coli* and *S. typhimurium*. (A) Wild-type strain in which the outer leaflet is almost entirely composed of LPS and proteins. (B) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the phospholipid bilayer domains. (C) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the LPS domains, which could have become more permeable due to alterations in LPS structure. (D) EDTA-treated wild-type cells. Phospholipid molecules are assumed to fill the void left by the selective removal of LPS by EDTA.

Taken from Nikaido, (1985).
4.5.3 The effect of solvent exposure upon membrane permeability.

In many studies the organic solvent toluene has been used to permeabilise *E. coli* to make the cell cytoplasm accessible to exogenous substrates, and to assay a variety of intracellular enzymes (de Smet *et al*, 1978). Toluene exposure has been observed to bring about the release of \( \approx 10\% \) of the outer membrane LPS, without removing phospholipids (Smit *et al*, 1975). It was of interest, therefore, to examine the extent to which solvent exposure increased membrane permeability to the hydrophobic dye gentian violet.

Cells were exposed to organic solvents of a variety of log P values, followed by measurement of the level of gentian violet uptake, as detailed in sections 2.4.8 and 2.4.9. Figure 3.36 shows the levels of dye uptake against log P for a range of solvents, excluding alcohols. Exposure to solvents of log P \( \geq 4 \), which were found to be non-toxic to cells during biotransformation (fig.3.27), resulted in no increase in dye uptake, being the same as for the untreated control. Exposure to solvents of log P \( < 4 \), which were found to be toxic to the cells during biotransformation, resulted in an increase in the level of dye uptake, being most pronounced below a log P of 3. Toluene (log P 2.5) exposure resulted in a level of dye uptake \( \approx 19 \) times the level of the untreated control, and \( \approx 7 \) times the maximum level of uptake achieved by the removal of LPS by EDTA treatment.
Exposure to toxic solvents of log P < 4 resulted in an increased membrane permeability to the hydrophobic dye. The reason for this increased dye uptake may be in part due to the removal of some of the outer membrane LPS by the solvent. However, when LPS was removed by EDTA treatment only a fraction of the level of dye uptake seen after solvent exposure was observed. LPS removal alone cannot account for these large increases in the level of dye uptake.

It is likely that the increased levels of dye uptake were due to increases in outer and cytoplasmic membrane fluidity, increased levels of fluidity being brought about by the partitioning of solvent into the membranes. Several studies have shown that organic solvents are capable of partitioning into membranes bringing about increases in fluidity (Baer et al., 1987; Vollherbst-Schneck et al., 1984; Nikaido et al., 1977; Ingram, 1976; Paterson et al., 1972). Other reports have shown that increased fluidity results in increased permeability and possible reduction in membrane bound enzyme activity (Meddings, 1989; Carruthers and Melchoir, 1986, Hoffmann et al., 1979). Although many studies on membrane fluidity have been performed, most studies have looked at only one or two solvents, and little information on the effect of solvent polarity upon membrane fluidity has been collected.

When cells were exposed to alcohols a linear relationship between dye uptake and log P was observed (fig.3.37). Only exposure to dodecanol was found not to increase dye uptake. When the alcohol and non-alcohol series were compared (fig.3.38), it was observed that alcohols were more effective than the non-alcohols at
Increasing the level of dye uptake. Although the reason for these differences is not fully understood, it is possible that the alcohols may be more effective at partitioning into the membranes to bring about increases in fluidity, resulting in an increased level of dye uptake. The alcohols were found to have an affinity for the dye gentian violet (see section 3.11). It is likely, therefore, that the presence of these alcohols in the membranes may result in higher levels of dye uptake, as the dye is also able to partition into the alcohol.

Only solvents found to be toxic to *P. putida* were able to bring about increased membrane permeabilities to gentian violet. This increase in permeability seems to correlate well with loss of biocatalytic activity (fig.3.27).

Growth in the presence of toxic solvents has been observed to alter membrane composition, regulating membrane fluidity to an optimal level. This growth dependent effect is discussed in section 4.9.

4.6 The effect of solvent exposure upon cell motility and viability.

Many groups of bacteria have been observed to be actively motile. This bacterial motility in many cases is generated by external organelles called flagella. In order for these flagella to bring about motility a proton motive force, (PMF), across the cytoplasmic membrane is required. This PMF allows the flagella to rotate generating rapid forward or backwards motion. The PMF generated
across the membrane is an energy requiring process, the energy being supplied via the electron transport chain. The presence of a proton gradient across the cytoplasmic membrane also relies upon the selective permeability to these ions (Macnab and Aizawa, 1984).

If organic solvents are able to dissipate this proton gradient by removing the membrane's selective permeability to protons, or by disrupting the electron transport chain which supplies the energy required to generate the proton gradient, motility would be expected to cease. The electron transport chain also requires the presence of proton gradients across the cytoplasmic membrane in order to generate ATP.

The motility of *P. putida* was therefore studied after exposure to a variety of organic solvents as another way of determining the effects solvent exposure may have upon membrane selective permeability.

As can be seen in fig.3.35, exposure to solvents of log P > 3.9 resulted in motility as for the aqueous control, indicating that these solvents could not bring about disruption of the proton gradient, probably because they were excluded from entering the cell by the outer membrane (see section 4.5.2). Cells exposed to solvents of log P < 3.3 were observed to be non-motile, indicating that the proton gradient had been disrupted. Exposure to hexane (log P 3.5) and phthalic acid diethyl ester (log P 3.3) resulted in some motility being observed. It is interesting to note that the log P at which cells are no longer able to hydroxylate naphthalene, a process which requires an active
electron transport chain, and the log P at which motility ceases are both around 3.9 to 3.3.

The results obtained would suggest that organic solvents of log P < 3.9 exert their toxic effects primarily upon the functioning of the electron transport chain, as was shown to be the case for *A. simplex* (Hocknull and Lilly, 1987). The possibility that motility ceased due to solvent interaction with the bacterial motor or inactivation of one of the electron transport chain components cannot be ruled out. It is more likely, however, that motility ceased due to loss of, or a reduction in membrane selective permeability to protons.

When cell viability was measured after exposure to organic solvents in phosphate buffer, as detailed in section 2.4.7, a profile identical to that obtained for cell motility was obtained, fig. 3.34. Cells were found to lose all viability when exposed to solvents of log P < 3.2 for 2 h. It would appear that the solvents attack upon the cytoplasmic membrane, resulting in the loss of the electron transport chain, prevented cell growth, as ATP is also required for cell division.

Exposure of *E. coli* to toluene has been shown to result in the release of 85% of the total RNA and up to 25% of the total cell proteins together with disaggregation of the ribosomes (Jackson and deMoss, 1965). The nuclear material has also been observed to be displaced to the cell periphery upon toluene treatment (Woldringh, 1973). It is possible that loss of cell viability could be due to inhibition of DNA or protein synthesis. Cells treated with toluene, although non-viable, were observed to
synthesize DNA if ATP was added, indicating that toluene destroys
the ATP generation system, usually the electron transport chain,
and not the DNA replication machinery (Moses and
Richardson, 1970).

It seems more likely, therefore, that the loss of cell viability
in solvents of log P < 4 is due to solvent action upon the
cytoplasmic membrane, resulting in an inability to generate ATP.

4.7 Proposed mechanism of biocatalyst inactivation
by organic solvents.

As mentioned earlier (section 4.2), the hydroxylation of
naphthalene requires the presence of a co-substrate in order to
regenerate NADH. In this study glucose was used to provide this
reducing power. Glucose is utilised by the Entner-Doudoroff
pathway, the Embden-Meyerhof pathway being completely absent from
Pseudomonas (Entner and Doudoroff, 1952). Energetically, the
operation of the Entner-Doudoroff pathway is only half as
efficient as the Embden-Meyerhof rout, for the net yield of ATP
per mole of glucose is 1 mole instead of 2, because only 1 mole of
triose phosphate is produced and oxidised. This gives 2 moles of
ATP but the net yield is only one because of the need to
phosphorylate glucose.

The pathway does however work well, allowing the generation of
high levels of NADH and ATP, when linked to the tricarboxylic acid
cycle and electron transport chain.

If the electron transport chain were inactivated, for example with
KCN, then NADH would accumulate with the production of no ATP. This depletion of ATP would result in the breakdown of active transport systems which rely upon ATP for transport of molecules into the cell. Glucose is one such molecule which relies upon active transport to enter the cell, being phosphorylated to gluconate-6-phosphate, a process requiring ATP (Midgley and Dawes, 1973; Whiting et al., 1976). It can be seen, therefore, that if the electron transport chain is inactivated, little glucose uptake will be able to occur. If glucose is present as the only carbon source, then production of NADH will also cease as glucose will be prevented from entering the Entner-Doudoroff pathway due to the breakdown of the active transport system for glucose uptake. The effective uptake of glucose is therefore seen to rely upon the presence of an active electron transport chain. The hydroxylation of naphthalene by *P. putida* requires the presence of NADH, which in this study was generated using glucose.

When no carbon source was provided, little product was formed (section 4.2), indicating the strict requirement for a NADH regenerating carbon source. When the electron transport chain was inactivated with the addition of KCN (fig.3.8), no product formation was observed, this could, however, have been due to inactivation of the dioxygenase enzyme itself by KCN.

It is likely that the toxic effects observed for solvents of log P below 4.0 are due to the inactivation of the electron transport chain by the removal of the cytoplasmic membranes selective permeability to ions, in particular protons. As mentioned above the inactivation of the electron transport chain results in a very
limited uptake of glucose into the cell. Since glucose is required
to regenerate the $NADH_2$ utilised during the hydroxylation of
naphthalene, the bioconversion stops.

It is interesting to note that those solvents which brought about
a loss of cell motility, also resulted in the loss of cell
viability (fig.3.34). This loss of cell viability is likely to
be a result of an inactivated electron transport chain, as the
process of bacterial cell growth and division requires ATP. The
addition of ATP to non-viable toluene treated cells resulted in
the observation of extended DNA synthesis, indicating that the DNA
replication machinery was unharmed by exposure to toluene
(Moses and Richardson, 1970). These results show that it is the
depletion of ATP that inhibits DNA replication. This ATP depletion
is again likely to be due to the inactivation of the electron
transport chain by toluene.

The results obtained for growth in the presence of organic
solvents (fig.3.51), indicate that solvents of log $P < 3.2$ are
detrimental to cell growth on glucose, and below a log $P$ of 2.9,
no growth was observed. This observation lends further evidence to
the proposal that the toxic solvents are acting upon the
cyttoplasmic membrane, resulting in the loss of its selectively
permeable nature, without which little ATP can generated to fuel
the further uptake of glucose and cell division, in this case
resulting in no growth.

The results do not conclusively show that it is the electron
transport chain that is inactivated; growth inhibition may be due
to the destruction of some other essential component of the cell replication machinery.

It would be of great interest to measure the effects of solvent exposure upon the cellular ATP levels. A method of measuring changes in cellular ATP levels has been developed by Antonietti and Ferrini (1986). It is likely that no significant change in ATP level would be detected for solvents of log P > 4.0, as these solvents do not appear to be toxic to the cells, allowing high levels of biotransformation to occur. These solvents were found to be non-toxic due to their exclusion from the cell by the outer membrane (section 4.5.2). If the hypothesis that organic solvents of log P < 4 bring about the inactivation of naphthalene hydroxylation by disrupting the selective permeability of the cytoplasmic membrane, and consequently the electron transport chain and co-factor regeneration, then the ATP pool would be expected to be small.

The increased resistance of *P. putida*, compared to the Gram-positive *A. simplex*, to organic solvents in the log P range 3.7 to 9.8 (fig.4.1), is thought to be due to the presence of the outer membrane. The outer membrane has been shown to exclude these solvents from the solvent sensitive cytoplasmic membrane by preventing their entry into the cell. The disruption of the outer membrane by removing LPS was observed to increase cell susceptibility to these organic solvents (section 4.5.2.1), a log P profile similar to that for the Gram-positive *A. simplex* being obtained.

Solvents of log P < 4 were found to be toxic to *P. putida*. These
solvents must therefore be able to penetrate the outer membrane in order to bring about inactivation of the electron transport chain. When cells were exposed to solvents and their permeability to gentian violet measured, solvents of log $P < 4$ were shown to bring about increased membrane permeability to the dye, being most pronounced below a log $P$ of 3 (fig. 3.36). The non-toxic solvents of log $P > 4$ were not able to bring about an increase in membrane permeability. It appears that the toxic solvents are able to bring about their toxic effects upon the cytoplasmic membrane, by first breaking down the outer membrane permeability barrier. It is important to note, that where this outer membrane disruption is greatest, in solvents of log $P < 3$, no biocatalyst activity was observed.

The mechanism of outer membrane disruption is not fully understood. It has been observed that toluene can remove LPS from the outer membrane even in low concentrations (Smit et al, 1975), and it is likely that in the stirred tank, in the presence of a large amount of organic-aqueous interface, that even more LPS may be removed. It has been shown in this study, that the removal of LPS did increase permeability to the hydrophobic dye gentian violet (section 4.5.1). It is likely that the removal of LPS from the outer membrane is the first site of action for the toxic solvents. These solvents may interact with the magnesium binding sites of the LPS, bringing about conformational changes which weaken the LPS-LPS interactions, leading to LPS release. The displaced LPS is thought to be replaced with phospholipids from the inner leaflet of the outer membrane (Nikaido and Vaara,
forming phospholipid bilayer regions in the outer membrane. These phospholipid bilayer regions are thought to allow organic solvents to partition rapidly through them, allowing access to the inner membrane. Once the solvents gain access into the outer membrane, it is likely that they will dissolve in the phospholipid region of the outer membrane and in the phospholipid bilayer of the inner cytoplasmic membrane, resulting in increased membrane fluidity. This increased membrane fluidity is thought to bring about increased membrane permeability to hydrophobic molecules, and explains why cell exposure to these toxic solvents results in a much higher level of hydrophobic dye uptake than observed for the removal of LPS alone (section 4.5.3).

The increased membrane fluidity is likely to be responsible for the loss of the cytoplasmic membrane selective permeability, resulting in the loss of the electron transport chain and cell motility, both requiring the presence of proton gradients across the membrane to function. The loss of electron transport, as mentioned earlier, is likely to result in the depletion of the ATP pool, this ATP depletion resulting in loss of cell viability, motility, glucose uptake and utilisation, and loss of biocatalytic activity with regard to the hydroxylation of naphthalene.

The solvent log P at which the enzyme toluene dioxygenase is inactivated cannot be determined from these studies. The addition of NADH to the stirred tank during biotransformation may shed some light on the situation. When ethanol was used to regenerate NADH, a co-substrate not requiring active transport into the cell, in the presence of toluene, no hydroxylation activity was observed
when toluene (log \( P \) 2.5) was present as a second phase (Lilly et al, 1987; Brazier, 1989). The regeneration of NADH in this system relies upon the enzyme alcohol dehydrogenase. If the enzyme alcohol dehydrogenase is more sensitive to the action of toluene than toluene dioxygenase, then it is possible that the toluene dioxygenase enzyme may still be in an active form but unable to hydroxylate toluene due to a lack of NADH. The enzyme ethanol dehydrogenase is known to require the presence of zinc ions as a co-enzyme for activity (Stryer, 1981). The enzyme may therefore be susceptible to inactivation due to loss of the coenzyme upon solvent treatment. Toluene dioxygenase itself requires the presence of \( \text{Fe}^{2+} \) ions (Gibson et al, 1892), and if the loss of membrane selective permeability allows these ions to diffuse out of the cell, enzyme activity may be reduced.

The study of the free enzyme, although difficult due to its inactivation in air, may be able to determine the log \( P \) at which the enzyme is denatured and no longer active.

The organic-aqueous interface was not found to be important in bringing about biocatalyst inactivation, with the exception of decanol (log \( P \) = 4) and di-iso-pentyl ether (log \( P \) = 3.9). Solvents above a log \( P \) of 4 were not toxic in any way, and those of log \( P \) < 3.9 exerted their toxic effects even in solvent saturated buffers alone (fig.3.29). The two solvents mentioned above were found to inactivate the biocatalyst by contacting the organic-aqueous interface, shielding in calcium alginate beads restoring activity (section 4.4.2).

The response in the stirred tank was found to be variable between
batches for these two solvents. It is likely that slight variations in growth conditions are responsible for this variation (see section 4.9).

It is clear that these two solvents require direct contact with the cells in order to bring about their toxic effects. Exposure to these solvents, followed by the measurement of dye uptake was observed to produce only a slight increase in dye uptake within the bounds of experimental error. This may be the reason why the cells have to contact the organic solvent directly, generating a large diffusion gradient, to be inactivated by them.

The product-time profiles for both decanol and di-iso-pentyl ether were linear over several hours (fig. 3.25, 3.26), although the rate of product formation was reduced. The toxic effects of these solvents in the two-liquid phase stirred tank was seen to be an instantaneous effect, after which no further inactivation occurred. It is unclear why this should be the case. If solvent is able to gain access at a limited rate, a reduction in product formation rate would be expected with time and a linear product-time profile would not be observed.

It is possible that the two solvents were only just able to reach the required membrane concentration to cause limited membrane disruption, and that further exposure to the solvents could not bring about any further increase in the level of solvent within the membrane.

The initial reduction in product formation rate, indicates that the level of solvent required to bring about this effect enters the cells over a short time period, less than 20 minutes.
The reduction in product formation rate is unlikely to be due to a proportion of the cells becoming non-viable, as exposure to these solvents over 2 h was observed not to be detrimental to them (fig. 3.34, 3.35), although it is important to note that the viability results of fig. 3.34 were obtained without mixing of the organic and aqueous phases. To measure the level of cell viability in a two-liquid phase stirred tank may prove to be difficult, as many viable cells may be removed to the organic-aqueous interface, giving an artificially low value for cell viability.

Cells when in aqueous buffer or non-toxic solvent were observed to be able to bring about the hydroxylation of naphthalene for about 8 hours. It is likely that loss of activity resulted from breakdown of the toluene dioxygenase. No further synthesis of the enzyme could occur in the stirred tank due to the presence of glucose.

If enzyme production could be brought about during the biotransformation, then the biocatalyst life may be greatly extended.

4.8 Adhesion of cells to the organic-aqueous interface.

As can be seen in fig. 3.30, and described in section 3.7, P. putida is found to adhere to the organic-aqueous interface upon mixing, in many cases forming stable emulsions, see plate 4. The non-toxic solvents of log P > 4 were found to remove about 20% of the cells from the aqueous phase. It is interesting to note that the solvents observed to be toxic of log P < 4 were found to
remove a much larger proportion of the cells from the aqueous phase forming stable emulsions, 60% being removed by hexanol (log P 1.8). The increased adherence of cells to the organic-aqueous interface may be a function of cell surface hydrophobicity, as suggested by Rosenberg *et al.* (1982). Many workers have used cell adhesion to organic solvents as a measure of cell surface hydrophobicity (Mozes and Rouxhet, 1987; Dillon *et al.*, 1986; Rosenberg *et al.*, 1982). When the results of Rosenberg *et al.* (1982), are plotted as the percentage cells removed from the aqueous phase against solvent log P, a similar profile to that obtained in this study was obtained (fig.4.3).

It is likely that the removal of LPS from the outer membrane, known to be brought about by some of the toxic solvents of log P < 4 (Smit *et al.*, 1975), also contributed to the stabilisation of the emulsion. When LPS extracted from cells with EDTA was mixed alone, in the absence of cells, with hexanol and a range of other solvents, emulsions were formed which were found to be stable for several weeks, showing LPS to be a very effective surfactant (see section 3.7). The results shown in fig.3.30 may also indicate that LPS is being removed by solvents of low log P, although this remains to be proven for *P. putida*.

*P. putida* was also observed to produce a surfactant, found to be a rhamnolipid, during biotransformations in phosphate and Tris buffers, together with an observed increase in emulsion stability with time in the stirred tank (section 3.7). It is unclear, however, if this surfactant was released LPS or a separate rhamnose containing lipid. The production of surfactant is likely
4.3 Adherence of *Proteus mirabilis* to hydrocarbons versus solvent log P. Xylene (log P 3.1), Octane (log P 4.5) and Hexadecane (log P 8.8).

Data taken from Rosenberg, V (1982).
to bring about the stabilisation of organic-aqueous emulsions, creating problems for downstream processing. Venkata and Karanth (1989) also observed the production of a rhamnolipid in pseudomonads. They stated that the rhamnolipid was produced under nitrogen limiting conditions, and that the presence of high levels of phosphate could reduce the levels of production. *P. putida* throughout this study was operated in the absence of a nitrogen source, which according to Venkata may result in elevated levels of surfactant production. It would be interesting to compare the levels of surfactant formed in both Tris and phosphate buffers in order to examine the role of phosphate in reducing surfactant production. Operation in phosphate buffer may have a selective advantage over Tris buffer, when considering phase separation during downstream processing.
4.8.1 Implications for downstream processing.

One of the potential advantages of operating a two-liquid phase system is the ease with which the water insoluble product, dissolved in the organic phase, can be separated from the biocatalyst contained within the aqueous phase.

This separation in the case of *P. putida*, however, is likely to be far from straightforward. The rhamnolipid surfactant produced by *P. putida* as well as some LPS released from the outer membrane in some of the more toxic solvents of low log P both act to stabilise the organic-aqueous emulsions formed during mixing, which were in many cases found to be stable for many hours or even days.

The separation of the organic and aqueous phases during downstream processing for *P. putida* does not appear to be straightforward, increasing the cost of product and biocatalyst recovery.

Solvents of a log P > 4 were however observed to result in a much smaller amount of stable emulsion, possibly due to the cells reduced affinity for more hydrophobic solvents, or the inability of these solvents to remove LPS from the outer membrane.

The production of rhamnolipid surfactant may be reduced by operating in the presence of phosphate, and possibly a nitrogen source (Venkata and Karanth, 1989).

The production of stable emulsions in certain solvents is obviously a factor which needs to be taken into consideration when choosing an organic solvent for biotransformation. It may also be possible to select for a mutant lacking the ability to produce surfactant,
and which may be less susceptible to removal of LPS from the outer membrane by organic solvents of low log P. The inability to produce surfactant may have detrimental effects upon the biocatalysts resistance to organic solvents, these would obviously need to be determined.

4.9 Cell growth in the presence of toxic organic solvents.

When *P. putida* and *A. simplex* were grown in the presence of organic solvents, as described in section 2.4.3.8, a major difference in cell growth verses solvent log P profile was observed. *A. simplex* was shown not to grow in solvents of log P < 5.1, full growth as for the control only occurring at a log P of 7.5. *P. putida* was found to grow at the rate of the aqueous control in all solvents tested with a log P > 3.2. Some growth was observed in solvents with a log P of 3.1 to 2.9, below which no growth occurred. This marked difference between *A. simplex* and *P. putida* is likely to be a Gram-positive - Gram-negative difference, due to the presence of the outer membrane. This Gram-positive - Gram-negative difference in tolerance to organic solvents has also been demonstrated by Rezessy-Szabó *et al.* (1987) and Inoue and Horikoshi (1989). Both groups report that Gram-negative organisms are able to grow in a wider range of organic solvents, and at lower solvent log P, than Gram-positive bacteria. The yeast *Saccharomyces uvarum* was also observed to behave in a similar manner to the Gram-positive bacteria, for growth in organic solvents. Inoue and Horikoshi (1989), obtained a novel *P. putida* isolate which was
found to grow in the presence of solvent dispersions down to a log P of 2.4. These cells were observed to be actively motile, indicating that the selective permeability of the cytoplasmic membrane was still intact, and that the electron transport chain was able to function. The isolate was shown to be able to grow in toluene, (log P 2.5), 50% (v/v) and was observed to be actively motile. The isolate was observed to be unable to grow upon toluene as a nutrient, unlike other strains of *P. putida*. It was not clear from the report of Inoue and Horikoshi (1989), whether the strain was unable to utilise toluene due to its total exclusion from entering the cell by the outer membrane, or due to the lack of the enzymes necessary for its breakdown. If the isolate does not contain the necessary enzymes, it would be of great interest to transfer a plasmid coding for the constitutive production of these enzymes into the bacterium. If the entry of toluene is excluded by the outer membrane, then growth will not be seen to occur on toluene as a sole carbon source. Although such isolates may be observed to be tolerant to a wide range of low log P solvents, they may be of little use for the biotransformation of many hydrophobic compounds due to their exclusion from the cell. The isolate of *Incur* may only be able to effectively act upon compounds with a log P < 2.4.

It is interesting to note that *P. putida* used here in this study was able to grow, with active motility, down to a solvent log P of 2.9 (fig.3.52; section 3.13), whereas cells grown in aqueous media were found to be totally inactivated by solvents of log P below 3.2, no motility being observed (fig.3.35). These results
suggest that the cells have been able to adapt to the presence of organic solvents, making them more resistant to them.

The adaption of cells to organic solvents has been studied in great detail (Hoek and Taraschi, 1988; Park et al., 1988; Baer et al., 1987; Tsuchiya et al., 1987; Vollherbst-Schneck et al., 1984; Ingram, 1976). Growth of Saccharomyces cerevisiae in the presence of tri-butyl phosphate resulted in a reduction in cell surface hydrophobicity (Park et al., 1988). A reduction in cell hydrophobicity is likely to reduce the level of cell adherence to the organic-aqueous interface, making phase separation more straightforward. This has been observed in many cases, adherence to organic solvents being used as a measure of cell hydrophobicity (Rosenberg et al., 1980).

The presence of butanol during growth has been observed to alter the lipid composition and membrane fluidity of Clostridium acetobutylicum (Baer et al., 1987; Vollherbst-Schneck et al., 1984). Butanol was found to bring about an increase in the levels of saturated acyl chains, and a reduction in the levels of unsaturated acyl chains in the cytoplasmic membrane, this alteration is thought to be in response to the 20 to 30% increase in membrane fluidity brought about by butanol in the membrane. The observed shift toward a higher saturated chain to unsaturated chain ratio is thought to occur to reduce the membrane fluidity to an acceptable level for cell function. Such homeoviscous adaption has been described for E. coli and other bacteria in response to increases in membrane fluidity brought about by increased temperature (Sinensky, 1974; Russell, 1984). The level of
phospholipid fatty acid saturation was also increased when *Streptococcus mutans* was grown in the presence of lidocain, resulting in a reduction in membrane fluidity, as determined by electron spin resonance spectroscopy (Tsuchiya *et al.*, 1987). Hoek and Taraschi (1988) observed that ethanol stimulated phospholipase C in isolated cell systems, and proposes that this solvent interaction may act as a trigger in inducing long-term adaptive responses at the cellular level.

*E. coli* has also been observed to radically alter its fatty acid composition in the presence of alcohols (Ingram, 1976). The alterations in membrane composition are thought to compensate for the direct interaction of alcohols with the membrane. It was proposed that these adaptive responses were able to increase tolerance to alcohols.

It is clear that growth in the presence of organic solvents is able to bring about adaptive responses which result in reduced membrane fluidity. This reduced membrane fluidity may also reduce the susceptibility of the electron transport chain to inactivation by solvent. Certain solvents may not be able to dissolve into the adapted membrane to a sufficiently high enough level to cause the membrane to become leaky to ions and other co-factors.

It is clear that the method of biocatalyst growth may contribute to its ability to tolerate a wider range of organic solvents. It was found to be extremely difficult to test this hypothesis out using *P. putida* UV4, as slight changes in growth conditions resulted in the production of no toluene dioxygenase. This strain produced no enzyme in the presence of high levels of glucose. Glucose was
normally fed continuously into the system to prevent a large build up of glucose. When the growth rate was reduced, or the lag period increased by the addition of organic solvent to the growth vessel, glucose was allowed to build up to levels inhibitory for enzyme production.

This problem could be solved by either using a carbon source which is unlikely to bring about catabolite repression, or to control the glucose feed rate to the cell growth rate, this would, however, necessitate on-line optical density measurements.

The strain also had another major drawback, in that no enzyme production could be obtained above 32°C. This meant that active cells could not be grown at elevated temperature to increase the level of saturated fatty acids in the membranes.

Growth rate and nutrient limitation have also been observed to alter the ratio of high to low molecular weight LPS within the outer membrane, as well as producing chemically different LPS forms (Dodds et al, 1987). At low growth rates under glucose limitation E. coli, grown in a chemostat, produced high molecular weight LPS identical to that previously characterised from cells grown in batch culture. At a high growth rate the ratio of high molecular weight LPS to low molecular weight LPS produced greatly decreased. High growth rates were also observed to produce small amounts of chemically different LPS. The LPS formed during rapid growth was found to have a slightly higher molecular weight than LPS obtained from rough mutants. This form of LPS was also observed during growth under mg⁺⁺ limiting conditions. Changes in temperature have also been observed to bring about this partial smooth-rough
transition (Cadieux et al., 1983; Kawaoka et al., 1983; McConnell and Wright, 1979). The work of Wright and Gilbert (1987), showed that the growth rate in a chemostat affected the overall hydrophobicity of the cell envelope and thereby the optimal value of $\log P$ for compounds to traverse it. Wright also suggests that alterations in phospholipid ratios within the cell membranes may also influence the permeation of hydrophobic compounds through them.

It is clear from the results discussed above that growth conditions are likely to influence the way the biocatalyst interacts with its environment and would appear to be a valuable area for further study in the context of resistance to organic solvents.

4.10 Strategies for strain development.

As mentioned earlier, $P. putida$ is not the ideal strain to study the effects of growth conditions upon solvent tolerance, due to catabolite repression, and cannot be grown above 32°C for the production of active cells. The level of specific enzyme production could not be controlled, preventing studies with varying levels of enzyme present. The enzyme toluene dioxygenase is also known to have a requirement for the addition of extra $Fe^{2+}$ ions when purified (Gibson et al., 1982). It is possible that the increased cell permeability brought about by solvent action could result in the leakage of these ions out of the cell, resulting in a reduced enzyme activity. The enzyme naphthalene dioxygenase has been observed not to require these extra $Fe^{2+}$ ions and may possibly be
more stable in such solvents.

An attempt was made to clone the *nah A* gene from the plasmid NAH 7 into a broad host range vector under the control of a *tac* promoter. It was hoped that the enzyme could be placed in a number of Gram-negative and Gram-positive bacteria, including rough mutants which have outer membrane defects, and expressed to a desired level, under the control of the *tac* promoter, in these organisms. The ability to move the enzyme around from strain to strain is likely to yield more evidence for the role of the various cell wall components in shielding against toxic solvents. It would also be possible to place the enzyme into strains shown to have high tolerance levels to organic solvents, eg. the novel isolate of Inoue and Horikoshi (1989). This would answer the question posed earlier, can the substrates toluene or naphthalene gain access into such cells in order to be broken down?

The enzyme would no longer be under catabolite repression, and could be expressed at any stage during the cell growth under the control of the *tac* promoter. The *tac* promoter can be turned on to express the enzyme by the addition of IPTG and possibly lactose. The level of enzyme per cell could also be controlled to a desired level. Such a vector would be able to express the enzyme regardless of growth conditions, and would be ideal for the study of the effects of growth conditions upon the hydroxylation of naphthalene in the presence of organic solvents.

The dihydrodiol formed would not be able to be broken down further due to the lack of the other enzymes of the pathway.

The isolation of the *nah A* gene from the *nah B* gene coding for the
second enzyme in the breakdown of naphthalene was not as straightforward as expected, there being no convenient restriction sites in \textit{nah}\textit{B} that were not also in \textit{nah}\textit{A}. The presence of the native NAH 7 promoter in front of the \textit{nah}\textit{A} gene also prevented the full expression of the enzyme from the \textit{tac} promoter. The native NAH 7 promoter has been observed to require the presence of the \textit{nah}\textit{R} gene product for full expression. This problem is likely to be solved in either one of two ways, either the native promoter is removed and the distance between the \textit{tac} promoter and the \textit{nah}\textit{A} gene optimised, or the \textit{nah}\textit{R} gene could also be cloned into the vector.

It would also be of great interest to be able to express the enzyme in yeasts and filamentous fungi.

The expression of the enzyme naphthalene dioxygenase in many different organisms in the presence of organic solvents is likely to yield a great deal of information upon the way in which these organisms interact with organic solvents, and should indicate which organisms are best suited for use in the presence of organic solvents.

The isolation and cloning of the \textit{nah}\textit{A} gene is still in progress in collaboration with Dr. John Ward, Department of Biochemistry, University College London.
5. CONCLUSIONS.

1. *P. putida* is able to perform the hydroxylation of naphthalene in the presence of a large number of organic solvents without loss of activity or stability.

2. The solvent log P is a very good indicator of predicted biocatalyst behaviour in the two-liquid phase stirred tank, with solvents of log P > 4 allowing full activity, and little or no activity below a log P of 3.5.

3. The organic-aqueous interface plays little role in biocatalyst inactivation, the outer membrane preventing solvent penetration to the inner solvent sensitive cytoplasmic membrane.

4. Solvents of log P < 3.5 are found to be toxic when present in the aqueous phase alone, immobilisation not preventing cell inactivation.

5. Biocatalyst inactivation by organic solvents of log P < 4 is brought about by the inactivation of the electron transport chain, resulting in a loss of NADH regeneration using glucose.

6. The electron transport chain and active motility are destroyed due to solvent action upon the cytoplasmic membrane, resulting in a loss of membrane selective permeability.
7. The presence of the outer membrane is thought to be responsible for the ability to tolerate organic solvents of $\log P > 4$, and its disruption results in increased sensitivity to these solvents.

8. The outer membrane is likely to be the reason for the observed Gram-positive - Gram-negative differences in the stirred tank reactions. Gram-positive organisms lacking this membrane being more sensitive to the toxic effects of these solvents.

9. Exposure to organic solvents of $\log P < 3$ results in the partial breakdown of the outer membrane permeability barrier, as shown by the greatly increased level of dye uptake.

10. Where the reaction being performed is not electron transport chain linked, and is not performed by a membrane bound enzyme, it is likely that Gram-positive organisms will give sigmoidal activity - $\log P$ profiles similar to that obtained for Gram-negative organisms.

11. It is unlikely that a Gram-positive bacterium will be observed to produce a sigmoidal $\log P$ profile if the reaction is electron transport chain linked.

12. The $\log P$ concept although a good indicator of activity when applied to a single system, breaks down when applied to predict the behaviour of biocatalysts in general. If biocatalyst form and reaction type are taken into account along side $\log P$, then a
better prediction of biocatalyst behaviour in the presence of organic solvents may be achieved.

13. *P. putida* was shown to produce surfactants during the hydroxylation of naphthalene which brought about stabilisation of the organic-aqueous emulsions. These emulsions are likely to create problems for downstream processing. Selection for a non-surfactant producing mutant may help to reduce these effects.

14. Cells adhered more strongly to the organic-aqueous interface for solvents of log P < 3. This was thought to be due to a greater affinity of the cells for these solvents and possibly due to the removal of LPS from the outer membrane, LPS itself proving to be a very effective surfactant.

15. The cloning of the *nah A* gene into various broad host range vectors, followed by expression in various cell types, is likely to yield much information as to the role of the various cell wall components in resisting solvent damage. This information should be of use when selecting the most suitable biocatalyst for a particular bioconversion.

16. The method of biocatalyst growth is likely to affect its performance in organic solvents. This is an area where much research needs to be carried out, as it is likely that product yields may be greatly improved.
6. **FUTURE WORK**

1. The relationship between solvent log P and cytoplasmic membrane fluidity should be determined using electron spin resonance. The effects of membrane fluidity should also be correlated to dye uptake and biocatalyst activity.

2. The uptake of a range of hydrophobic dyes of various log P should be examined to determine the log P at which the outer membrane becomes impermeable to these hydrophobic compounds. The log P at which the membrane becomes permeable should also be determined. These studies may give an indication of the extent to which protection from the organic solvents occurs at the expense of limiting substrate uptake rate.

3. The *nah A* gene should be cloned into a broad host-range vector to allow the enzyme to be expressed in both Gram-positive and Gram-negative bacteria, including mutants with known cell wall deficiencies, e.g. rough mutants with deficient LPS. Study of the hydroxylation of naphthalene in the presence of organic solvents of various log P should lead to a better understanding of the role of the various cell wall components in solvent tolerance.

4. The effects of varying growth conditions upon membrane permeability to dyes and fluidity, as well as upon biocatalyst activity in the presence of organic solvents, should be studied.
using a nah A containing bacterium. Such studies with the existing strain would prove to be difficult due to catabolite repression.

5. Further studies using the EDTA treated P. putida should be performed to study the role of interface in biocatalyst inactivation of a Gram-negative bacterium with a disrupted outer membrane. The product-time profiles in the two-liquid phase stirred tank were observed to be non-linear, the product formation rate falling off with time, indicating that inactivation occurred as the solvent reached a critical concentration in the cytoplasmic membrane, this being time dependent. It is likely that if the EDTA treated cells are immobilised or operated in solvent saturated buffers, that the biocatalyst stability will be improved, the solvent taking longer to reach the critical level in the membrane.

6. Little information is available as to the interaction of organic solvents with certain biocatalyst types, e.g. yeasts, filamentous fungi, animal and plant cells. There is obviously a need to characterise these interactions to form a clearer picture of the way organic solvents interact with the biocatalyst, and to allow for the selection of the biocatalyst type and form best suited for the reaction.

7. Most of the studies using whole cells to carry out biotransformations in the presence of organic solvents have involved reactions which are either directly or indirectly linked to the electron transport chain, for obvious reasons, as these
reactions cannot be easily performed using free enzymes. It would be of great interest to study a number of reactions which are not electron transport chain linked especially in Gram-positive and Gram-negative bacteria.
## APPENDIX I

**LDC/MILTON ROY CL-10B INTEGRATOR METHOD**

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<tr>
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All other values are set at default values.

This program also allows data to be stored on disc.
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