A COMPARISON OF THE SOLVENT STABILITY OF THREE GRAM NEGATIVE ORGANISMS

Sunil Shrestha

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Department of Chemical and Biochemical Engineering
University College London
Torrington Place
London
Dedicated to my Family

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ABSTRACT

A growing interest lies in the use of biocatalysts in industry to carry out stereo- and regio-specific conversions. Many of these reactions involve poorly water-soluble reactants and/or products. This poses problems in attaining high substrate and product concentrations in the reactor, and two-liquid phase systems can be employed in such cases. The main disadvantage of this approach is destabilisation of the biocatalyst by the organic solvent.

Protection against solvent is afforded by the outer membrane in Gram negative cells. Naphthalene hydroxylation by Gram negative organisms was studied to compare solvent stability in an aqueous saturated buffer system, and a two-liquid phase system. The naphthalene dioxygenase gene, *nahA*, was isolated and inserted into plasmid pMMB66EH. The new plasmid construct was used to transform *E.coli* JM107 and *P.aeruginosa* strains PAC1R and PAC610. *P.putida* UV4 which expresses toluene dioxygenase activity from chromosomally encoded genes, was used to complete the array of microorganisms studied.

Exposure to solvent, both in two-liquid phase systems and in solvent saturated buffer, showed that differences in cell outer membrane composition affected the degree to which cells retained stability. Solvent hydrophobicity and presence of an aqueous:organic phase interface, were also important in determining the rate at which solvent damage occurred.

Loss of stability was shown to be a time dependent phenomenon, with sustained exposure leading to increased loss of stability, even with very hydrophobic solvents. PAC1R(pSS2) was unable to convert naphthalene at the same rate as PAC610(pSS2). This difference is thought to be due to differences in the extent of interactions between components of their outer membranes affecting the rate of uptake of the substrate. Partial permeabilisation by solvents increased the activity observed in PAC1R(pSS2).

Activity retention shown by UV4 was lower than that of JM107(pSS2) in hexane saturated buffer for the first 4 hours of biotransformation, but greater over the final 21 hour period. UV4 also retained a greater level of activity than JM107(pSS2) over the entire biotransformation in tetradecane saturated buffer. PAC1R(pSS2) activity appeared to be stimulated by the presence of saturating levels of hexane and tetradecane over the first 4 hours, but declined with continued exposure. This stimulated level of activity was still significantly lower than that of JM107(pSS2). Pentanol saturated buffer caused immediate and complete loss of activity in all cells.
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ABBREVIATIONS

Ap
Cb
DNA
EDTA
IPTG
kbp
Kn
LPS
MNNG
PAGE
SDS
STR
Superscript r
TBE
TE
Tris
UV
XGal

ampicillin
carbenicillin
deoxyribonucleic acid
ethylendiaminetetraacetic acid
isopropyl-β-D-thiogalactopyranoside
kilobase pairs
kanamycin
lipopolysaccharide
N-methyl-N’-nitro-N-nitrosoguanidine
polyacrylamide gel electrophoresis
sodium dodecyl sulphate
stirred tank reactor
indicates resistance
tris-borate-EDTA
tris-EDTA
tris(hydroxymethyl)aminomethane
ultraviolet
5-bromo-4-chloro-3-indolyl-β-D-galactoside

A.simplex: arthrobacter simplex
E.coli: Escherichia coli
S.aureus: Staphylococcus aureus
S.typhimurium: Salmonella typhimurium
P.putida: Pseudomonas putida
P.aeruginosa: Pseudomonas aeruginosa
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1. INTRODUCTION

1.1. BIOCATALYSTS

1.1.1. Biocatalysts in industry

Improved understanding of microbial biochemistry and genetics has increased the potential for the use of both whole cells and isolated enzymes as biological catalysts (biocatalysts) in industrial processes. In addition to their ability to produce a number of unique compounds, they may also be used to synthesize compounds traditionally produced by chemical means. Examples of products in which biocatalysts play a role in synthesis include, 1,2-chloropropionic acid (ICI), (S)-Naxopren ((S)-6-methoxy-α-methyl-2-naphthaleneacetic acid) (International Biosynthetics, BV-IBIS) [Pryce and Roberts, (1990)], polyphenylene (ICI), pinitol (ICI) [Ballard et al., (1983)] (Fig.1.1.), and 3-fluoroveratole [Johnston et al., (1987)].

1.1.2. Advantages of using biocatalysts

Regio- and stereospecificity:
Reactions catalysed by isolated enzymes and whole cells may confer strict regio- and stereospecificity [Ziffer et al., (1977); Gibson and Subramanian, (1984); Brand et al., (1992)] and so are potentially favourable for use in the pharmaceutical and agrochemical industries. Not only does this control the exact nature of the product, but in doing so, also reduces the amount of by-product formed.

Mild operating conditions:
Biocatalysts work optimally under relatively mild operating conditions of temperature, pressure and pH, reducing both the operating costs and the likelihood of unwanted side reactions occurring.
Fig 1.1. Synthesis of Polyphenylene and Pinitol from Benzene
Reduced reaction stages:
Compared to the chemical conversion processes, the biologically catalysed operations may be carried out more directly, often completing the conversion in a single step reaction [Johnston et al. (1987); Ballard et al. (1983)]. Loss of intermediates at each stage of the conversion process is thus reduced, as is the possibility of the occurrence of unwanted side reactions.

Ability to recognise a range of related compounds:
Biocatalysts are able to recognise and utilise a range of related compounds, often differing markedly in their structure, yet still maintain the same high degree of specificity [Ley et al. (1987); Johnston and Reganathan, (1987); Gibson et al. (1989)]. Gibson and colleagues (1990) identified 25 different compounds which could be recognised by the toluene dioxygenase of P. putida strain F39/D, and over 30 different compounds which the enzyme cloned into an E. coli strain, could utilise.

Whilst the biotransformation process can be carried out during the fermentation itself [Witholt et al. (1990); Favre-Bulle et al. (1991)], separation of the two stages may often be desirable. Where a possibility exists of the substrate or product affecting cellular growth [Favre-Bulle et al. (1991)], isolation of the two stages is beneficial to the overall process. This also allows for the growth and biotransformation phases to be optimised individually, without compromise, with cells harvested at the stage where they have optimal enzymic activity. Finally, if the biotransformation is carried out over a relatively short period, new cell growth is unlikely to occur, thus the necessity for sterile conditions during the biotransformation process is removed.

1.2. TWO-LIQUID PHASE SYSTEMS

1.2.1. Introduction to two-liquid phase systems

Many biological reactions of interest employ substrates which are only sparingly soluble in water. Yet an aqueous environment is essential for biocatalytic activity, in both isolated enzyme and
whole cell systems; dried *A. simplex* cells were observed to be unable to catalyse steroid conversion, but activity could be restored upon rehydration of the cells [Hocknull,(1989)]. For isolated enzymes, water molecules are required to maintain non-covalent interactions essential for activity. These include ionic bonds, van der Waals bonds, and hydrophobic interactions. The amount of water required appears to be dependent upon the nature of the enzyme. Klibanov (1989) proposed that only a single monolayer of water around the enzyme was required for activity, and that α-chymotrypsin could retain activity in various solvents due to the presence of 50 mole equivalents of water bound to only the charged groups of the protein [Zaks and Klibanov,(1988)]. Water may also be used as one of the active reagents in the reaction, for example during hydrolysis, or to dissolve any cofactors present.

1.2.2. Classification of two-liquid phase systems

Classification of two-liquid phase systems is important in order to understand the mechanisms of solvent distribution between the aqueous and organic phases. This allows the mass transfer of substrate and product in the reactor to be studied, factors that need to be taken into account when designing specific two-liquid phase biotransformation systems.

A classification system based upon concentration profiles for poorly water-soluble substrates dissolved in the organic solvent, has been proposed by Lilly and Woodley (1985). Three theoretical concentration profiles dependent upon solubility of the biocatalyst in the aqueous phase and the aqueous:organic phase ratio can be distinguished (Fig 1.2.).

**Profile A**
Where soluble biocatalysts, such as free enzymes, are used, the profile is independent of the phase ratio. The substrate concentration is greatest in the bulk organic phase and decreases at the film [Lewis and Whitman,(1924)] and phase interfaces, due to mass transfer resistance and partitioning between phases.
Fig. 1.2. Theoretical concentration profiles for transfer of substrate dissolved in organic phase in a two-liquid phase bioreactor (from Lilly and Woodley, 1985). Interface and film shown by a continuous line and a dashed line, respectively.
Profile B
When insoluble biocatalysts are used (immobilised enzymes, free or immobilised whole cells), a further set of partition coefficients and concentration gradients may be introduced by the biocatalyst and its support, in addition to those created at the liquid:liquid interface.

Profile C
If the phase ratio is altered such that the level of aqueous phase is decreased, so that it no longer forms a discrete phase between the organic phase and the insoluble biocatalyst, then in effect, only an aqueous film is retained around the biocatalyst. The concentration gradient produced by the aqueous phase becomes less significant, and the profile depicted by profile C is predicted to be observed.

Derived from the reactor concentration profiles, a classification system based upon the biocatalyst distribution and the phase ratio, can be distinguished (Figs 1.3. and 1.4.). The biocatalyst is assumed to be present in the aqueous phase either in a soluble (free enzyme), or insoluble form (free or immobilised whole cell, or immobilised enzyme). The aqueous phase can exist either as the continuous phase, a discrete discontinuous phase, or as a non-discrete phase.

Use of water-miscible organic solvents to increase the solubility of poorly water-miscible substrates, has been studied [Sonomoto et al.(1979;1984); Yokozeki et al.(1982)]. The main disadvantage of this system is that high levels of dissolved solvent in the aqueous phase can result in a loss of operational stability and reduced activity. When high concentrations of dissolved solvents are used, even immobilisation of the biocatalyst may not be able to prevent solvent disruption of the biocatalyst [Butler,(1979)]. Furthermore, addition of co-solvent is unable to alleviate the problems associated with substrate and/or product inhibition, and may exacerbate the situation by maintaining close proximity of these compounds with the biocatalyst.

The addition of a water-immiscible solvent, creating a second liquid phase, is an alternative method of carrying out...
biotransformations. The organic phase acts as a reservoir and/or sink into which the substrate and/or product dissolves. The aqueous layer retains the biocatalyst and any hydrophilic co-factors, acting as the site in which the reaction occurs. This system may enable biotransformation to occur without the deleterious effects associated with using a water-miscible solvent.

1.2.3. Advantages of two-liquid phase systems.

i) The addition of solvent to form a discrete phase can significantly increase the concentration of substrate present in the reaction vessel, thereby reducing the volume of the reactor necessary to form a given amount of product. The amount of dissolved substrate can reach high levels; 170g/L of cholesterol dissolved in carbon tetrachloride was reported to have been obtained by Buckland and co-workers (1975). Alternatively, the substrate may be used to form the organic phase [Brazier et al., (1990); Harbron et al., (1986)]. Similarly, the amount of product which can be formed, may also be increased as the reaction equilibrium is altered.

ii) Where the substrate is a solid at reaction temperatures, solubilisation by the solvent may make it more accessible to the biocatalyst [Ceen et al., (1987)].

iii) Hydrophobic products formed are readily partitioned into the organic layer, reducing the possibility of supersaturation in the aqueous phase, and any crystallisation of product upon the biocatalyst surface that may arise.

iv) Where high levels of substrate or product may lead to biocatalyst inhibition, partitioning away from the aqueous phase can limit these detrimental effects.

v) Finally, downstream processing may be greatly simplified. Isolation of the organic phase in which product and unreacted substrate accumulate, may ease the isolation and recovery of the biocatalyst.
Fig 1.3 Classification of two-liquid phase systems by the nature of the biocatalyst and the phase ratio. (from Lilly and Woodley, (1985))
Fig 1.4. Theoretical two-liquid phase profiles: biocatalyst solubility and phase ratio
(Adapted from Lilly and Woodley, (1985))
■ Insoluble biocatalyst;   ● Organic phase;  □ Aqueous phase
Also, recovery of product from the reaction medium may be simplified by virtue of the low boiling points of many organic solvents.

1.2.4. Disadvantages of two-liquid phase systems.

The main disadvantage of two-liquid phase biocatalytic systems results from the denaturing effect of the solvent upon the biocatalyst. This can occur either at the liquid:liquid interface, or within the aqueous phase itself, due to the presence of dissolved solvent. The actual mechanism(s) by which inhibition occurs is not clearly understood, and will be discussed later (section 1.3.).

1.2.5. Solvent selection criteria

The final selection of solvent to be employed must take into consideration a number of different factors [Lilly,(1982)]:

i) Reactant/product solubility in the solvent.

ii) Reactant/product partitioning between the two phases. The need to partition substrates and/or products exerting inhibitory effects upon the biocatalyst, has been mentioned. Complete partitioning of product into the organic layer is also desirable for downstream processing. However, high affinities of the substrate for the solvent phase may not necessarily be beneficial, if this hinders substrate transfer into the aqueous phase.

iii) Biocatalyst inhibition by organic solvent. The solvent itself can exert inhibitory effects upon the biocatalyst. However, these effects can be minimised by immobilisation of the biocatalyst to reduce denaturation at the liquid:liquid interface [Hocknull and Lilly,(1990)].

v) Solvent availability.

iv) Solvent safety.
The solvent to be used must be safe with respect to its toxicity and flammability. This must also be taken into consideration when deciding upon methods of disposal.

vi) Solvent cost.

1.3. EFFECTS OF SOLVENT ON BIOCATALYSTS

The actual mechanism(s) by which solvents affect biocatalysts is not clearly understood, with a wide range of effects observed [Lilly et al. (1987)]. The resultant effects are dependent upon a number of factors including the nature of the cell and solvent, the mode of presentation of solvent, the period of exposure, and also the agitation rate. Bar (1986), distinguishes between solvent effects resulting from the presence of a liquid:liquid interface (phase toxicity), and those caused by solvent dissolved in the aqueous solution (molecular toxicity) (Fig 1.5.). Only a few of the negative effects of solvents on the biocatalyst are illustrated, with a greater range of effects known to occur, both at the morphological and the physiological levels [Lilly, (1982); Lilly et al., (1987)].

**Loss of cell viability**

Loss of viability of cells exposed to a range of organic solvents has been reported in both *E. coli* and *Pseudomonas* [Jackson and DeMoss, (1965); Moses and Richardson, (1970); De Smet et al., (1983)].

**Loss of cell morphology**

Several changes in cellular morphology due to solvent damage have been observed by electron microscopy. Solvent treated cells showed cytoplasmic shrinkage [Jackson and De Moss, (1965)], chromosomal displacement [De Smet et al., (1978)] and membrane disruption [Favre-Bulle et al., (1991); DeSmet et al., (1978;1983)], with disruption occurring in both the cytoplasmic and outer membranes.
Loss of intracellular proteins
Intact cells were observed to lose intracellular proteins in a selective manner [DeSmet et al.,(1978)]. Malate dehydrogenase was released in toluene treated E.coli, but other cytoplasmic proteins, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase, were retained within the cell. This may reflect a possible peripheral location for malate dehydrogenase, or selective release by the peptidoglycan or outer membrane layers.

Loss of RNA
RNA loss was shown to be independent of the level of solvent present (1.5μl/ml to 50μl/ml), with up to 85% of the total cellular RNA being extracted into the medium. This may lead to impairment of enzyme synthesis required for maintenance and repair of the cell [Jackson and DeMoss,(1965)].

---

**SOLVENT**

**Molecular Toxicity**
- Enzyme inhibition
- Protein denaturation
- Membrane modification
  - membrane expansion
  - structure disorder
  - permeability change
  - etc.

**Phase Toxicity**
- Extraction of nutrients
- Disruption of cell wall
- extraction of outer cellular components
- Limited access to nutrients
- cell attraction to interface
- emulsion formation
- cell coating

Fig1.5. Toxic effects due to solvent
a) dissolved in the aqueous medium (molecular toxicity);
b) forming a distinct second phase (phase toxicity).
(from Bar, 1987)
Loss of lipopolysaccharide (LPS) and phospholipid
Exposure of cells to toluene was observed to result in removal of up to 41% of outer membrane LPS. In contrast, the outer membrane phospholipid remained relatively unaffected [De Smet et al., (1978)]. Despite this, the outer membrane appeared relatively intact, and the difference in the amount of the two lipid species lost may reflect the more exposed location of the LPS compared with the phospholipids.

Loss of selective permeability
The role of the outer membrane includes selectively limiting the flux of materials between the cell and its environment. Many solvents can disrupt this function. Cells treated with toluene were unable to concentrate C¹⁴-thiomethylgalactoside within the cell, and instantaneous loss of this compound was observed upon exposure to the solvent [Jackson and DeMoss, (1965)]. Similarly, increased susceptibility to hydrophobic antibiotics was observed following treatment of cells with polymyxin B nonapeptide, due to disorganisation of the outer membrane structure by the antibiotic [Vaara et al., (1984)].

Loss of substrate uptake
Non-competitive inhibition by solvents has been reported to affect sugar uptake mechanisms [Loureiro-Dias and Peinado, (1982); Leao and van Unden, (1982)]. Inhibition in these cases was attributed to alterations in the lipid environment disrupting the close lipid:protein interactions required to maintain the active conformation of the enzymes. The rate of loss of activity appeared to be correlated with the lipophilicity of the solvent used.

Loss of membrane associated functions
An intact membrane to generate an electron transport chain is essential for the formation of ATP and NADH. The NADH co-factor recycling step was shown to be the primary target of organic solvent disruption of steroid dehydrogenation in A. simplex, rather than the actual dehydrogenase enzyme itself [Hocknull and Lilly, (1988)]. Similarly, (Na⁺/K⁺)-ATPase activity was lost upon addition of alcohols, due to disruption of the ability to form a
membrane proton potential. As a result, respiration dependent lactose and proline transport functions were inactivated [Patel et al.,(1975)].

**Limited access to nutrients**

Aggregation of cells at the liquid:liquid interface and formation of emulsions may hinder the uptake of nutrients. In addition, coating of cells with an organic layer can lead to blockage of nutrient diffusion into the cell [Cho and Shuler,(1986)].

**1.4. PREDICTION OF SOLVENT STABILTY**

The wide array of effects produced by solvents upon biocatalysts makes the correlation of stability and activity with the nature of solvent more difficult. For example, Cremonesi and co-workers (1975) observed that \( \beta \)-hydroxysteroid dehydrogenase showed a significant decrease in activity in carbon tetrachloride, yet still remained stable.

Various methods to reduce the deleterious effects of solvent upon biocatalysts have been proposed. These include the addition of albumin and coenzyme to the reaction medium to increase the enzyme stability [Cremonesi et al.,(1975); Carrea et al.,(1979)], and controlling the extent of biotransformation, preventing complete conversion of substrate, where the substrate itself may have a stabilising effect upon the biocatalyst [Buckland et al.,(1975)]. Immobilisation techniques have been used by many workers and found to improve the stability of the biocatalyst [Hocknull and Lilly,(1990); Carrea et al.,(1979); Yamane et al.,(1979)]. However, problems may arise if the solvent interacts with the support medium itself, or if the support hinders the transfer of substrate to the active site. Not all immobilisation methods necessarily improve the activity or stability of the biocatalyst. Brink and Tramper (1985) observed that cells entrapped in calcium alginate were no more stable than cells used in the free form, depending upon the solvent used. Furthermore, immobilisation may not be able to protect the biocatalyst at high solvent concentrations [Carrea,(1984)].
Various workers have looked at the relationship between biocatalyst stability and solvent polarity. Brink and Tramper (1985) reported an association between solvent polarity (measured in terms of the Hildebrand coefficient ($\delta$)), and molecular weight of the solvent, with the biocatalytic activity of free and immobilized cells. Low activities were observed when solvents of high polarity ($\delta > 8$) and low molecular weight (less than 200) were employed. Similarly, Laane and co-workers (1985), looking at work carried out by various groups using a range of biocatalytic systems (free and immobilised cells and enzymes), in the presence of a variety of organic solvent:aqueous systems, suggested that polarity of the solvent could be more satisfactorily defined in terms of logP, where logP is represented by the equation:

$$\text{LOG } P = \frac{[\text{solvent}] \text{ in octanol}}{[\text{solvent}] \text{ in water}}$$

A correlation between the logP value of the solvent and the biocatalytic activity retained was found in which, solvents with logP values greater than 4 resulted in minimal damage to the biocatalyst, and those with LogP values less than 2 caused total loss of activity. This correlation has been used successfully by several workers. However, discrepancies can still occur; Hocknull and Lilly (1989) found that solvents of logP greater than 4 still caused inactivation of the immobilised biocatalyst, but that the rate of loss of activity increased with increasing water miscibility of the solvent.

Although the results of Laane's group may be used as a general guideline in the selection of solvents, it does not give us an understanding of the mechanism(s) involved in the disruption of biocatalysts. A closer inspection of cell:solvent interactions is required in order to elucidate this problem.
1.5. BACTERIAL CELL WALLS

Bacterial cells can be classified upon the structure of their cell walls, and the resultant ability to take up certain dyes. Gram positive and Gram negative cells share many features such as the peptidoglycan layer and the teichoic acids, although the proportions of these components found in the two classes of cells differ. Gram negative cells are further distinguished by the presence of a second membrane structure, different in composition to the cytoplasmic membrane, and overlying the peptidoglycan layer.

1.5.1. Gram positive cell walls

The Gram positive cell wall is morphologically distinct from its Gram negative equivalent. The major component of the cell wall is the peptidoglycan, contributing up to 50% of the total cell wall weight, with the remainder being made up of a variety of polymers.

The Peptidoglycan

The peptidoglycan is the main structural component of the cell wall providing rigidity and mechanical strength to the cell (Fig 1.6.). Isolated cell walls retain the original shape of the bacterial cell, whilst the remaining spheroplast either forms a sphere in an isotonic medium, or lyses in an hypertonic one. The peptidoglycan is made up of a series of glycan chains interlinked to form a complex three-dimensional matrix. Each glycan chain is made up of alternating units of N-acetylglucosamine and N-acetylmuramic acid linked by β-1,4-glycosidic bonds. A four amino acid chain of alternating L- and D-amino acids is attached to the carboxyl group of each muramic acid residue. The tetrapeptide is composed of L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine. Peptide bonds between the D-alanine and the meso-diaminopimelic acid of alternate strands provide the inter-strand linkages. Gram positive cells have a greater amount of such bonds than Gram negative cells, with up to 90% of the tetrapeptides being involved in cross-linking in Staphylococcus aureus.
Teichoic acids.
Teichoic acids are the main polymers associated with the peptidoglycan in Gram positive bacteria. These components are the major cell surface antigens of the Gram positive cells, and may also play a role in the movement of ions into the cells. Teichoic acids consist of glycerol or ribitol phosphate units joined by phosphodiester linkages, with up to 40 such units linked to form the polymer. Similar sugar-1-phosphate units are also found in some organisms which, although not strictly teichoic acids, are closely related in terms of their properties and functions (Fig. 1.7). The teichoic acids are joined to the peptidoglycan frame via a linkage unit which joins the teichoic acid chain to the 6' position of the muramic acid residue of the peptidoglycan.

Teichuronic acids.
Teichuronic acids, like the teichoic acids, form a linear polysaccharide bearing uronic acid residues. They are not phosphorylated, but probably bind to the peptidoglycan via a single phosphodiester bond.

1.5.2. Gram Negative cell wall

Transverse sections of cell walls from Gram positive and Gram negative organisms show several major differences in their structures. In Gram negative cells, the peptidoglycan layer is significantly smaller, but retains its role in maintaining the integrity of the cell. In addition, overlying the peptidoglycan layer is a second membrane structure composed of a lipid/protein bilayer, the outer membrane (Fig 1.8.). Associated with the outer membrane are a series of novel proteins, and the complex amphipathic LPS molecule. The periplasm occupies the space between the inner and outer membranes. This region contains a number of enzymes associated with transport, modification and degradation functions.

The periplasm
The periplasm defines the space between the outer and cytoplasmic membranes. Estimates of the volume occupied by the
periplasm range from 5% [Decad,(1976)] to 42% [Stock,(1977)]
dependent upon the growth phase of the cell [Decad,(1976)].
A large percentage of the total cellular protein content is located
in the periplasm, with the actual amount dependent upon growth
conditions and strain type [Oxender and Quay,(1975)]. A number
of different enzymic functions are found in this region, and these
may be classified into three groups:

i) Binding proteins;
this group of proteins mediates transport of solutes, including
sugars and amino acids, to cytoplasmic membrane permeases,
where they can then be transferred into the cytoplasm.

ii) Hydrolytic enzymes;
the hydrolytic enzymes catalyse the degradation of a number of
metabolisable compounds unable to traverse the cytoplasmic
membrane due to their size or charge. Partial degradation can
then allow these compounds to be translocated into the cytoplasm.

iii) Detoxifying enzymes;
cells are able to degrade or modify a number of antimicrobial
agents in the periplasm. Accumulation of these enzymes in this
region produces a concentrating effect, lowering the amount of
enzyme required to complete detoxification. Comparative enzymes
produced by Gram positive organism are secreted into the
medium, so that a greater amount of these enzymes are required
to bring about the same effect.

iv) Deoxyribonucleoside catabolising enzymes and nucleases;
a number of enzymes in the periplasm are involved in the
modification of deoxyribonucleotides and ribonucleotides.

The outer membrane
The outer membrane serves a variety of functions including:
forming a partial diffusion barrier to a number of hydrophobic
and large hydrophilic compounds. This is reflected in the greater
tolerance of Gram negative organisms to a number of hydrophobic
compounds compared with Gram positive cells [Nikaido and
Vaara,(1985)]; providing non-specific channels through which low
molecular weight hydrophilic molecules can pass; providing receptor sites for bacteriophage and bacteriocins; determining strain and species specificity of the cell; producing the main endotoxic effects associated with the cell.
The outer membrane is made up of three main components: the phospholipids; the proteins; and the lipopolysaccharides.

i) Phospholipid.
The main species of phospholipid identified in the Enterobactericeae is phosphatidylethanolamine, with lower levels of phosphatidylglycerol and cardiolipin also present. Freeze fracture studies indicate that the phospholipids are distributed asymmetrically in the outer membrane, with the LPS component found exclusively on the outer leaflet [Mühlradt and Golecki, (1975); Van Gool and Nanninga, (1971)]. It is thought that the outer leaflet may be composed entirely of LPS, with phospholipids forming the inner leaflet, or that small patches of phospholipid may exist together with LPS in this outer leaflet [Nikaido and Vaara, (1985)].

ii) Proteins
Lpo/OmpI
The most abundant protein isolated from the outer membrane of E.coli is Braun's lipoprotein [Braun, (1975)]. It can exist either in a free form, or covalently bound to the peptidoglycan [Inouye et al, (1972)]. This protein is thought to anchor the outer membrane to the peptidoglycan. Mutants defective in this lipoprotein dissociate their outer membrane when treated with EDTA and detergents, and form vesicles at their surface due to the reduced stability of the membrane [Suzuki et al, (1978)]. An analogous protein has been identified in P. aeruginosa. Protein I shows a number of similarities to Braun's lipoprotein, including similar mobility in SDS-polyacrylamide, and the presence of covalently bound glycerol and fatty acid residues [Mizuno and Kageyama, (1978)]. In addition, similarites in the amino acid composition and secondary structure, as well as stretches of sequence homology between the two proteins, have been reported [Cornelis et al, (1989)].
Fig 1.6. The N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA) residues of cell wall peptidoglycan. (Heavy arrow indicates inter-strand peptide linkage).
Fig 1.7 The structure of teichoic acid consisting of 1) glycerol phosphate, or 2) ribitol phosphate units. (adapted from Hammond et al. (1984))
OmpA/OprF
These analogous proteins play an important role in maintaining the structural stability of the *E. coli* and *P. aeruginosa* cells, respectively [Hancock *et al.* (1980)].

OprF deficient *Pseudomonads* produce a near spherical shape in contrast to the rod shape of the wild type cell. Furthermore, the cells are more prone to leakage of a number of periplasmic proteins and are more susceptible to osmotic shock [Gotoh *et al.* (1989)]. *E. coli* cells bearing a lipoprotein or an OmpA mutation appear morphologically normal, but where a double mutation involving both these proteins occurs (*lpo*/*ompA*), the cells take on a spherical form. This defect in *E. coli* can be corrected by expression of the oprF gene from *P. aeruginosa* [Woodruff and Hancock, (1989)] indicating the degree of similarity that exists between the OmpA and OprF proteins.

The porins
The porins make up the third major group of outer membrane proteins. These proteins are non-covalently associated with the peptidoglycan, and form non-specific channels for the movement of small hydrophilic molecules across the membrane.

A number of porin like proteins have been identified in *P. aeruginosa* although they appear to have a greater exclusion limit compared with the enterobacterial porins. *Pseudomonas* strains show a greater resistance to several hydrophobic and hydrophilic drugs than many of the Enterobactericeae. This greater resistance of *Pseudomonas* strains has been attributed to the inability of these compounds to traverse the porins. This may reflect either the highly selective nature of these proteins, or the lower percentage of these porins that are open at any given time in the pseudomonads [Angus *et al.* (1982); Yoshimura and Nikaido, (1982)].

Protein D1 appears to be a glucose-inducible porin with a preference for glucose and xylose [Hancock and Carey, (1980); Trias *et al.* (1989)]. Similarly, Protein D2 shows characteristics of a substrate-specific porin, selective for basic peptides or dipeptides, yet still allowing size dependent uptake of monosaccharides.
Fig 1.8. The Gram negative cell wall structure
(not drawn to scale)
(adapted from Hammond et al. (1984))
Other *Pseudomonas* major proteins

The function of Protein G is not yet clear, but it may be involved in uptake of fluoroquinolone or iron [Chamberland, (1989); Yates et al., (1989)].

Protein H1 is overexpressed when cells are grown in media deficient in Mg^{2+}, Ca^{2+}, Mn^{2+}, and Sr^{2+} [Bell and Hancock, (1989); Bell et al., (1991); Nicas and Hancock, (1983)]. These cells show increased resistance to antibiotics which are thought to act by chelating the divalent cations bound to the LPS. Protein H1 is thought to bind lipopolysaccharides in place of the cations normally present. The protein cannot be removed or substituted by the antibiotics, and so renders the cell resistant to them.

iii) The lipopolysaccharide (LPS)

LPS is a complex amphipathic macromolecule made up of three distinct regions joined by covalent linkages (Fig 1.9.). LipidA forms the innermost region of the LPS molecule integrating into the lipid bilayer. Joined to this is an oligosaccharide unit which can be separated into a low and a high molecular weight fraction [Fensom and Meadow, (1970); Koval and Meadow, (1977)]. The low molecular weight fraction consists of the core region which is linked directly to the lipidA. The high molecular weight fraction is made up of the O-antigenic saccharide units of the LPS, consisting of a number of unique repeating oligosaccharide units.

**LipidA**

LipidA forms the endotoxic centre of the LPS, and appears to be indispensable for cell survival, since no mutants deficient in this fraction have yet been identified. A strong interaction with the matrix protein O-8 in *E. coli* appears to be essential to maintain the overall lattice network of the outer membrane, which forms the basic framework from which the remaining outer membrane components can orientate themselves.

The lipidA component from many enterobacterial cells has been analysed and the structure elucidated [Rietschel et al., (1984)]. A glucosamine disaccharide unit forms the ‘backbone’ of the molecule, to which a number of fatty acid residues are attached. A high degree of homology exists between the lipidA of the
Pseudomonaceae and the Enterobactericeae, with antigenic cross reactivity observed between the lipidA components of *E. coli* and *P. aeruginosa* [Rivera and McGroarty,(1989)]. Kulshin and co-workers (1991) have characterised the lipidA fraction of two wild type and one rough mutant of *P. aeruginosa*, and shown them to share the same disaccharide ‘backbone’, but differ in the nature and the number of fatty acids present.

*Salmonella* species and *E. coli* lipidA display a highly ordered hexagonal lattice network of their fatty acyl components, which is postulated to influence the hydrophobic barrier property of the cells [Labischinski et al.(1985); Kato et al.(1990)].

**Core structure**

The core unit is a complex polysaccharide structure which is common to many members of the same species [Rowe and Meadow,(1983); Wilkinson and Galbraith,(1975)].

A 2-keto-3-deoxyoctonic acid (KDO) residue links lipidA to the core unit in both Enterobactericeae and Pseudomonaceae. The core unit of *P. aeruginosa* has been shown to contain a number of invariant sugars, although the molar ratio of these components and the order of linkage may differ between strains. Similar variations are known to exist amongst the enteric bacteria [Jansson et al.(1981)].

**O-antigenic region**

The O-antigenic region is made up of a series of repeating oligomer units whose length and composition is highly variable, thus forming the basis for immunological typing of cells. The O-antigenic polysaccharide unit can be resolved into an amino-sugar rich region and a neutral-sugar rich region [Yokota et al.(1987); Sawada et al.(1985)], with the shorter neutral-sugar region being antigenically related in *P. aeruginosa* of different serotypes.

Mutants defective in synthesis of the LPS are defined upon the extent to which the O-side chain and core are affected. Figure 1.10.1. illustrates the series of LPS mutants isolated from *S. typhimurium*. These are termed rough mutants, since the cells
Fig. 1.9 *Schematic diagram of the S-form lipopolysaccharide*
(Adapted from Labischinski *et al.*, (1985))
take on a grainy edged morphology in contrast to the smooth appearance shown by wild type cells. A similar series of mutants have been derived from *P. aeruginosa* strain PAC1 and PACIR, a spontaneous mutant of PAC1 but which shares its LPS structure [Chester and Meadow, (1975)] (Fig 1.10.2.).

LPS from smooth strains of *P. aeruginosa* have identified two distinct forms, a low molecular weight A band LPS, and a high molecular weight B band LPS. The two bands differ significantly in composition. The larger B band is thought to be the main antigenic structure exposed on the cell and is serotype specific, whilst the A band of *P. aeruginosa* was found to be antigenically related to a number of cells differing in their serotype. No amino-sugars have been detected in the A band LPS but a high rhamnose content was observed [Arsenault et al.,(1991)]. A further difference lies in the lipidA component of both LPS. LipidA antibody raised in *E. coli* was shown to cross-react with the lipidA of B band LPS of *P. aeruginosa*, but not with that from the A band. This may be a result of the low level of phosphate groups found in the A band LPS to which the antibodies bind.

1.6. NAPHTHALENE HYDROXYLATION

1.6.1. Naphthalene degradation

The complete degradation of naphthalene by many soil pseudomonads, is a multistep process involving the formation of the intermediate hydrocarbon, catechol. Catechol undergoes further degradation via a ring cleavage process to form the tricarboxylic intermediaries used in the production of ATP. Cleavage of catechol can either lead to the formation of cis,cis-muconic acid (*meta*-cleavage) (Fig 1.11), or 2-hydroxymuconic semialdehyde (*ortho*-cleavage). The final TCA products are dependent upon which cleavage pathway is used.

Naphthalene undergoes hydroxylation by both naphthalene dioxygenase and toluene dioxygenase via similar processes. Each enzyme is able to recognise a variety of compounds related to its own natural substrate, and catalyse the reaction in the same stereospecific manner.
Fig. 1.10.1 Core polysaccharides of rough mutants of *S. typhimurium* (Ra-Re)
P=phosphate; P.Etn=O-phosphorylethanolamine;
P.P.Etn=O-pyrophosphorylethanolamine
(adapted from Hammond et al., (1984))

Fig. 1.10.2 Partial structure of the lipopolysaccharide of *P. aruginosa* PAC1R
(from Rowe and Meadow (1983)).
1.6.2. Plasmid encoded genes involved in naphthalene degradation

Genes encoding the enzymes involved in naphthalene degradation are often found encoded upon plasmids. However, these genes may also be encoded by the chromosome [Weightman et al. (1984)], or distributed between the plasmid and chromosome such that part of the degradative process is encoded by genes on the chromosome and the remainder by those on the plasmid [Reineke (1980); Fennewald et al. (1979)].

A number of plasmids have been isolated from pseudomonads able to degrade naphthalene, the best studied being plasmid NAH7 isolated from *P. putida* strain PpG7 [Dunn and Gunsalus, (1973)]. Table 1.1. lists some of the plasmids found, not including those obtained by cloning or mutagenesis. Transposon Tn5 mapping of NAH7 has enabled the genes to be ordered into two distinct operons, *nah* and *sal*; *nah* (*nah A-nah F*), encodes the upper pathway enzymes catalysing breakdown of naphthalene to salicylate, and *sal* (*nah G-nah K*), the lower pathway enzymes encoding salicylate degradation via the meta cleavage pathway [Yen and Gunsalus, (1982); Yen et al. (1983)].

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Compound degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAM</td>
<td>camphor</td>
</tr>
<tr>
<td>OCT</td>
<td>octane, decane</td>
</tr>
<tr>
<td>SAL 1</td>
<td>salicylate</td>
</tr>
<tr>
<td>NAH</td>
<td>naphthalene</td>
</tr>
<tr>
<td>TOL</td>
<td>toluene</td>
</tr>
<tr>
<td>NIC</td>
<td>nicotin, nicotinamide</td>
</tr>
<tr>
<td>pEG</td>
<td>styrene</td>
</tr>
<tr>
<td>pWR1</td>
<td>3-chlorobenzoic acid</td>
</tr>
<tr>
<td>pAC25</td>
<td>3-chlorobenzoic acid</td>
</tr>
</tbody>
</table>

Table 1.1. Naturally occurring degradative plasmids
1.6.3. Naphthalene Dioxygenase

The first step in the degradation of naphthalene is its hydroxylation by the enzyme naphthalene dioxygenase. The enzyme catalyses the addition of two oxygen atoms from molecular oxygen at positions C1 and C2, in a stereospecific manner, to form (+)-cis-(1R,2S)-dihydroxy-1,2-dihydro-naphthalene [Jeffrey et al.,(1975); Jerina et al.,(1971)].

The isolated enzyme is unstable upon exposure to air [Catterall and Williams,(1971)], but can be stabilised by inclusion of ethanol, glycerol and dithiothreitol in the extraction buffer [Laborde,(1979); Ensley et al.,(1982)]. Subsequent isolation and purification of naphthalene dioxygenase [Ensley et al.,(1982)], indicated that the enzyme was in fact a multimeric protein made up of three subunits designated A, B and C. The subunits contain iron-sulphur chromophores at their active centre which coordinate the transfer of electrons from a suitable donor to the final acceptor molecule. Electrons are transferred sequentially from ferredoxin(nap) reductase (component A) to ferredoxin(nap) (component C), and finally to the terminal iron-sulphur protein (component B) (Fig 1.12.).

Component A: Ferredoxin(nap) Reductase.
Ferredoxin(nap) reductase is the primary unit of naphthalene dioxygenase, being a 2Fe-2S flavoprotein of molecular weight 36,000. In the presence of NADH, the enzyme binds the pyridine nucleotide transferring one or two electrons to a variety of electron acceptors including cytochrome C [Ensley et al.,(1982); Haigler and Gibson,(1990a)], ferricyanide and Nitrotetrazolium Blue [Haigler and Gibson,(1990a)]. NADPH can also act as an electron source, although the resultant activity was observed to be less than half that using NADH [Haigler and Gibson,(1990a)].

Component C: Ferredoxin(nap).
The intermediate unit of the dioxygenase enzyme is a protein of molecular weight 13,600 [Haigler and Gibson,(1990b)]. Ferredoxin(nap) bears an iron-sulphur chromophore at its redox centre arranged in a 2Fe-2S configuration. The presence of
Fig 1.11. Naphthalene degradation via the meta-cleavage pathway
Enzymes involved:
A, Naphthalene dioxygenase; B, cis-naphthalene dihydrodiol dehydrogenase; C, 1,2-dihydroxynaphthalene dioxygenase; D, 2-hydroxychromene-2-carboxylate isomerase; E, 2-hydroxybenzalpyruvate aldolase; F, salicylaldehyde dehydrogenase; G, salicylate hydroxylase; H, catechol-2,3-dioxygenase; I, hydroxymuconic semialdehyde dehydrogenase; J, 4-oxalocrotonate tautomerase; K, 4-oxalocrotonate decarboxylase; L, 2-oxopent-4-enoate hydratase; M, 2-oxo-4-hydroxypentanoate aldolase.
ferredoxin(nap) is essential for activity of naphthalene dioxygenase, and its function cannot be substituted by analogous ferredoxins from similar systems [Andrew et al. (1976); Subramanian et al. (1979); Gunsalus and Lipscomb, (1973)]. This suggests that a specific interaction between ferredoxin(nap) and the terminal component may be required for the transfer of electrons. However, the interaction with ferredoxin(nap) reductase appears to be less stringent, and spinach(nap) reductase is able to substitute its function, but at a reduced level of activity.

Component B: Iron-Sulphur Protein(nap)(ISP(nap)).
The terminal component of the naphthalene dioxygenase has a molecular weight of 158,000 which can be resolved into two polypeptide subunits of 55,000 and 20,000 [Ensley and Gibson, (1983)]. This is suggestive of an α2β2 quaternary structure. Both ferredoxin(nap) reductase and ferredoxin(nap) activities are required for ISP(nap) to accept electrons from NADH, and no activity is observed if only one or other of these functions is present.14C-naphthalene studies showed that ISP(nap) reversibly binds the substrate, most likely at the enzyme's active site.

1.6.4. Toluene dioxygenase

Comparisons of the toluene and naphthalene dioxygenases reveal a number of similarities. Both are tripartite systems utilising flavoprotein and iron-sulphur containing components and requiring NADH and molecular oxygen to effect hydroxylation [Haigler and Gibson, (1990a; 1990b); Subramanian et al., (1979; 1981; 1985). The individual subunits have been characterised and show similarities in both their gene order and molecular weights [Zylstra et al. (1988)]. However, naphthalene dioxygenase displays characteristics more reminiscent of benzoate dioxygenase. These include: its ability to reduce cytochrome c. in the absence of components B and C; recognition of both NADH and NADPH as electron sources; and stimulation of activity by FAD and FMN [Yamaguchi and Fugisawa, (1978)].
Fig 1.12. Direction of electron transfer through naphthalene dioxygenase
Comparison of the nucleotide sequences of naphthalene dioxygenase and benzene dioxygenase from *P. putida* strains identified regions of DNA showing more than 60% homology [Kurkela et al., (1988)]. However, the amino acid homology was less stringent, suggesting that both enzymes had emerged from a common ancestral gene, but that the gene products had diverged over time.

1.6.5. Regulation of the *nah* and *sal* operons

Enzymes involved in naphthalene degradation were found to be active at low levels in the absence of any exogenously added factors [Barnsley, (1974); Yen and Gunsalus, (1982); Schell, (1985)]. However, when salicylate or its analogue, 2-aminobenzoate, was added to *Pseudomonas* strains, NCIB 9816, ATCC 17483 and PpG277 harbouring the Nah plasmids, induction of a number of *nah* encoded enzymes produced a 20fold increase in activity [Barnsley, (1974)].

Salicylate is able to induce all the enzymes encoded by the *nah* and *sal* operons, with an intact *nahR* gene essential for this coordinated induction to occur [Schell, (1983); Schell and Wender, (1986); Grund and Gunsalus, (1983); Yen and Gunsalus, (1982)]. *nahR* cells produce a pleiotropic effect resulting in a *nah-*/*sal-* phenotype, which can be corrected by in trans complementation using a second, intact *nahR* allele [Schell, (1985); Grund and Gunsalus, (1985)].

The *nahR* protein is expressed at very low levels (less than 10fold lower than the uninduced levels of naphthalene degrading proteins), irrespective of the presence or absence of inducer [Schell, (1985)]. This suggests that the *nahR* gene is constitutively expressed and that the level of expression is not affected by salicylate. Furthermore, the interaction of salicylate with the *nahR* protein does not affect the binding affinity of the protein, nor the location at which it binds the DNA [Schell and Poser, (1989)]. However, the level of detected mRNA was shown to increase 30fold in the presence of salicylate, indicating that the *nahR* protein acts at the level of transcription as an activator of transcription rather than a transcription regulator. It is possible
that salicylate alter the conformation of the nahR protein such that an active state is attained which allows full initiation of transcription to occur.

1.7. PREVIOUS WORK

*P. putida* strain development was carried out in order to isolate an organism able to accumulate benzene *cis*-glycol [Ballard *et al.* (1983)]. The glycol is an important intermediate in the formation of polyphenylene, and derivatives of benzene *cis*-glycol have enormous potential in the synthesis of a wide range of organic products. Organisms were originally isolated from soil samples heavily contaminated with waste hydrocarbons, and a *P. putida* strain able to utilise benzene as a carbon source was further studied. MNNG-treatment was used to develop a strain blocked in the benzene dihydrodiol dehydrogenase encoding gene. This strain, UV4, was shown to accumulate benzene *cis*-glycol, as well as a number of glycols from a variety of benzene derivatives, such as fluorobenzene, toluene and naphthalene. The genes of this degradative pathway are encoded upon the chromosome and expressed constitutively, with addition of inducers shown to have no effect on the level of dioxygenase activity observed [Hack,(1992)]. However, pH and DOT were both found to adversely affect the enzyme activity [Hack,(1992); Harrop,(1990)]. Similarly, high glucose concentrations appeared to repress the enzyme activity, but if the level of glucose was reduced for a period prior to harvesting, the activity was found to be unaffected.

Solvent exposure studies of two different systems, looking at steroid dehydrogenation by *A. simplex* (a Gram positive organism) [Hocknull,(1989)], and naphthalene hydroxylation by *P. putida* UV4 (a Gram negative organism) [Harrop,(1990)], showed differences in the retained activity which may be due to shielding effects produced by the outer membrane of the Gram negative organism [Harrop *et al.*,(1992)]. Although not directly comparable, these studies do give an indication of the solvent protection afforded by the outer membrane. Fig 1.13. shows the logP:activity retention profile produced by the two organisms [Harrop *et al.*, (1992)], demonstrating the differences in activity retention
between the two upon exposure to a range of solvents. \textit{P.putida} strain UV4 is able to retain all its activity in solvents with log P > 4, as predicted by Laane and co-workers (1985). In contrast, \textit{A.simplex} is affected by all solvents up to a log P of 9.8, the actual amount of activity retained being dependent upon the solvent hydrophobicity. Solvent interfacial effects also showed differences between the two organisms. Immobilisation of \textit{A.simplex} to reduce exposure to the interface, resulted in significant improvements in its solvent stability. The resultant logP:activity retention profile was comparable to that produced by UV4. Immobilisation of UV4 however, only improved stability in the presence of two solvents, decanol (log P=4) and di-iso-pentyl ether (log P=3.9), but solvents with log P < 3.5 inactivated the enzyme regardless of the immobilisation process [Harrop,(1992)]. The involvement of the outer membrane in protecting UV4 against solvent was shown by pretreatment of cells with EDTA; the damage caused to the outer membrane resulted in an increased sensitivity of the cells to solvents and the new logP:activity profile was comparable to that produced by \textit{A.simplex}

1.8. AIM OF RESEARCH PROGRAMME

The denaturing of biocatalysts by solvents forms the main barrier to the effective implementation of two-liquid phase technology. Solvent exposure studies using \textit{P.putida} UV4 and \textit{A.simplex} show that the two cell types differ in the extent of damage sustained. The difference in the structure of the cell wall may account for the greater stability of the \textit{P.putida} UV4 strain compared with \textit{A.simplex}, with the outer membrane thought to play a significant role in protecting the cell against solvent. However, amongst the Gram negative cells, the differences in the outer membrane structure observed may be expected to give rise to different permeability profiles towards hydrophobic compounds. This is suggested by the lower permeability of many \textit{Pseudomonads} to a range of hydrophobic compounds.
Fig 1.13 Log P: Product formation profile for *A. simplex* and *P. putida* UV4
From Harrop et al. (1992)

Triangles = *P. putida*; Circles = *A. simplex*; open symbols = free cells; filled symbols = immobilised cells.
Although comparison of the effect of solvents upon dioxygenase activity of *P. putida* UV4 and the steroid Δ¹-dehydrogenation activity of *A. simplex*, may reflect differences in the susceptibility of the two cells to solvents, a more direct comparison in which different organisms are subject to the same reaction parameters would give a better reflection of the solvent tolerances of these different organisms.

Using hydroxylation of naphthalene as a model system, the aim of this project was to compare the effect of a range of solvents upon the activity of three different cells able to carry out this reaction, maintaining the same reaction conditions between them. The solvent tolerances of three Gram negative organisms, *P. putida* UV4, *P. aeruginosa* PAC1R, and an *E. coli* JM107 strain were compared.

In order to do this, the naphthalene dioxygenase producing gene, *nahA*, was expressed in a broad host range plasmid, in both *P. aeruginosa* PAC1R and *E. coli* JM107. The toluene dioxygenase of UV4 was able to utilise naphthalene to produce naphthalene dihydrodiol. The toxic effects of the solvents upon the biocatalyst were looked at both dissolved in the aqueous medium, and at the liquid:liquid interface, in order to identify the extent of damage caused by each.

Results obtained from the comparison of activities produced by the three biocatalytic systems may be used to elucidate possible mechanisms by which solvents affect whole cells. There also lies the possibility of tailoring biocatalysts to given reactions in order to maximise bioconversions in solvent/aqueous two-liquid phase systems through the use of rDNA technology.
2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Organisms

*Pseudomonas putida* UV4 was developed from a *Pseudomonas* strain isolated from soil samples with a high hydrocarbon content, and able to grow in benzene saturated aqueous solution. MNNG treatments produced a *P. putida* isolate able to constitutively express the toluene degrading genes, and which accumulated toluene-cis-glycol, the first product of toluene degradation catalysed by toluene dioxygenase.

*Pseudomonas aeruginosa* strains PAC1R and PAC610 are derivatives of strain PAC1 [Kelly and Clarke,(1962)]: PAC1R synthesizes the complete lipopolysaccharide structure; strain PAC610 is isogenic to PAC1R, but is unable to generate the antigenic O-side chain, whilst retaining the core polysaccharide and lipid A components [Meadow et al.(1978)].

*Escherichia coli* strains used are derivatives of *E.coli* strain B or K12 developed for laboratory use and have the following markers:

i) *strain JM107* supE 44, endA1, hsdR17, gyrA96, relA1, thi Δ (lac-pro AB)
F[traD, proAB + lacIq lacZ ΔM15], [Yanisch-Perron et al.(1985)]

ii) *strain HB101* supE 44, hsdS2 (r-m-) rec A13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1 [Rodriguez et al.(1977)].

Plasmids

Table 2.1. Obtained plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRK2013</td>
<td>Km^r</td>
<td>Rodriguez et al.(1977)</td>
</tr>
<tr>
<td>pBGS18-</td>
<td>Km^r</td>
<td>Spratt et al.(1986)</td>
</tr>
<tr>
<td>pMB66EH</td>
<td>Ap^r</td>
<td>Furste et al.(1985)</td>
</tr>
</tbody>
</table>
Table 2.2. Constructed plasmids (this work).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Markers</th>
<th>Gene insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQR113</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>nahA-B</td>
</tr>
<tr>
<td>pQR155</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>nahA</td>
</tr>
<tr>
<td>pQR156</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS1</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS2</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS2.1</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS3</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS3.1</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS4</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
<tr>
<td>pSS4.1</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt; (Cbf&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>nahA</td>
</tr>
</tbody>
</table>

2.1.2. Reagents

All reagents were obtained from BDH except for the following:

- Agarose: Sigma
- Bactoagar: Gibco BRL
- Indole: Aldrich
- IPTG: Pharmacia
- Lysozyme: Sigma
- Naphthalene: Aldrich
- Nutrient Broth: Difco
- Restriction endonucleases: Anglia Biotechnology Ltd.
- X-gal: Northumbrian Biotech. Ltd.
- Sodium hydroxide: Fisons
- RNase: Anglia Biotechnology Ltd.
- Phenol: Fisons
- Chloroform: Fisons
- Trizma acid/base: Sigma
- EDTA: Fisons
- Bromophenol blue: Aldrich
- T4 DNA ligase+buffer: Northumbrian Biotech. Ltd.
- Dithiothreitol: Sigma
Solvents
Solvents used are listed together with their logP values.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>LogP</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>-0.76</td>
<td>Fisons</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-0.24</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Dichloroethane</td>
<td>1.3</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Pentanol</td>
<td>1.33</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.5</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Hexane</td>
<td>3.5</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>7.8</td>
<td>Aldrich</td>
</tr>
</tbody>
</table>

Tris.Cl
DNA treatments were carried out using Tris.Cl buffer. 1M stock solution was made and diluted as required.
For 1L of 1M stock:
Tris.HCl  106.4g
Trizma base  39.4g

Phenol-chloroform
Phenol was prepared by dissolving 500g of phenol in the following:
Distilled water  200ml
Tris.Cl (1M, pH7.4)  30ml
NaOH (2M)  15ml
EDTA (0.2M)  60ml
The mixture was allowed to equilibrate prior to addition of an equal volume of chloroform.

Lysis buffer
Lysis buffer was used in plasmid DNA preparations.
50mM Glucose
25mM Tris.Cl (pH8)
10mM EDTA (pH8)
**Tris-EDTA (TE) buffer**
10mM Tris.Cl (pH8)
1mM EDTA

**Tris-Borate-EDTA (TBE) buffer**
DNA separation was carried out in agarose gels using TBE buffer as a running medium.
90mM Tris.Cl
90mM Boric acid
2.5mM EDTA

**5X Loading buffer**
Loading buffer was used to stop DNA reactions where samples were to be run in agarose gels. Ficoll was added to increase the density of the DNA to enable it to be run in an agarose gel. Buffer was made up in TBE.
25%w/v Ficoll
0.1M EDTA
0.025%w/v bromophenol blue

**T4-DNA ligase buffer**
200mM Tris.Cl (pH7.6)
50mM MgCl₂
50mM dithiothreitol

**Phosphate buffer**
Biotransformations were carried out in 50mM potassium phosphate buffer, pH7, made up by mixing equal volumes of 50mM solutions of K₂H₂PO₄ and KH₂PO₄, the final pH being adjusted with 3M KOH.

**2.1.3. Growth Media**

**Nutrient broth**
_E. coli_ were grown in Nutrient broth (Oxoid No2) made up at 2.5% w/v in distilled water, and autoclaved at 15lb/in² for 15mins. Antibiotics were added as required.
For plate cultures, purified agar was added at 2% w/v, prior to sterilisation.

**M9 minimal medium agar for E. coli JM107**

*E. coli* JM107 were also maintained on M9 minimal medium plates [Maniatis *et al.*(1982)].

(Per litre).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>6 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>1 g</td>
</tr>
<tr>
<td>Na$_3$C$_6$H$_5$O$_7$.2H$_2$O</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
</tbody>
</table>

The medium was sterilised by autoclaving at 15lb/in$^2$ for 15mins prior to addition of the following:

1M MgSO$_4$ (2mls)
1M CaCl$_2$ (0.1ml)
1M Glucose (10mls)
1%w/v Thiamine (1ml)

**M9 minimal medium agar for Pseudomonads**

Pseudomonads were grown in nutrient broth for biotransformations, and plated either on nutrient agar plates, or M9 minimal medium plates, made up in the following manner.

(Per litre).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>6 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>1 g</td>
</tr>
<tr>
<td>Na$_3$C$_6$H$_5$O$_7$.2H$_2$O</td>
<td>5 g</td>
</tr>
<tr>
<td>Trace elements</td>
<td>5 ml</td>
</tr>
<tr>
<td>Citrate (sodium salt)</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
</tbody>
</table>

After autoclaving, the following were added:

1M MgSO$_4$ (2ml),
1M CaCl$_2$ (0.1ml)
Pseudomonas defined salts medium [Evans et al. (1970)]

Growth of pseudomonads was carried out in shake flasks using defined salts medium supplemented with a carbon source of either sodium succinate or sodium salicylate at 0.5% w/v (per litre).

- NaH$_2$PO$_4$.2H$_2$O (2M) 5 ml
- NH$_4$Cl (4M) 20.5 ml
- KCl (2M) 5 ml
- Na$_2$SO$_4$.10H$_2$O 2 ml
- Citric Acid (1M) 2 ml
- MgCl$_2$ (1M) 1 ml
- CaCl$_2$ (0.02M) 1 ml
- Na$_2$MoO$_4$ (0.01M) 0.01 ml
- Na$_2$HPO$_4$.12H$_2$O (0.2M) 15 ml
- Trace elements 5 ml

The pH was adjusted to 7.0 with 3M KOH prior to sterilisation.

Trace elements solution [Evans et al. (1970)]

(per litre)
- HCl (conc) 1.0 ml
- ZnO 0.41 g
- FeCl$_3$.6H$_2$O 5.4 g
- MnCl$_2$.4H$_2$O 2.0 g
- CuCl$_2$.2H$_2$O 0.17 g
- CoCl$_2$.6H$_2$O 0.47 g
- H$_3$BO$_4$ 0.06 g

Antibiotics

Antibiotics were added to the growth media after sterilisation as required. These were added to both plates and broths, at the following concentrations:

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>PAC1R/610</th>
<th>E. coli strains</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>NA</td>
<td>100μg/ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>500μg/ml</td>
<td>NA</td>
<td>Sigma</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>NA</td>
<td>50μg/ml</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

NA = not added
2.2. EQUIPMENT

75ml Stirred Tank Reactors (STR)
Biotransformations were carried out in 75ml glass stirred tank reactors at a working volume of 60ml (Fig2.1.). The temperature was maintained at 28°C using a heated water bath, and mixing effected by a constant torque motor at 750rpm. Aeration was provided by 6 in-line flow pipes powered by an air pump at 1-1.3v/v/m (60-80cc air/min).

High Pressure liquid Chromatography
HPLC was used to quantify levels of naphthalene dihydrodiol. An LDC/Milton Roy CM4000 multiple delivery pump was used in line with an LDC/Milton Roy SpectroMonitor 3000 detector at 254nm. A Techsil 10CN 25cmx4.6mm column (HPLC Technology) was operated as a normal phase system with a mobile phase of 98% 1,2-Dichloroethane/2% Methanol, both of HPLC grade. The mobile phase was degassed in a sonicated water bath prior to use, and run at 1ml/min. Samples were loaded using a Promis II autosampler, and peaks calculated using an LDC/Milton Roy CI-10B integrator.

Sample preparation for HPLC
200μl samples were removed from the stirred tank reactor and added to 5ml of dichloroethane in a 10ml screw-capped glass vial. The contents were thoroughly mixed to stop further reaction. If the samples were not to be analysed immediately, they were stored at 4°C until required, without any subsequent degradation of the product [Harrop,(1990)]. 1ml of the sample was placed into 2ml glass HPLC vials and analysed on the HPLC.

Nuclear magnetic resonance identification of naphthalene dihydrodiol.
Naphthalene dihydrodiol was crystallised from a prepared stock solution dissolved in phosphate buffer [Harrop,(1990)]. 1 volume of the dihydrodiol was vortexed with two volumes of dichloroethane, in a 25ml, universal bottle, and the two phases allowed to
Fig 2.1. 75ml stirred tank reactor

(sizes shown in mm)
The organic phase was identified as containing the dihydrodiol by HPLC analysis. The organic phase was removed, and a further two volumes of dichloroethane added to the aqueous phase. The organic phases from the two extraction procedures were bulked. The naphthalene dihydrodiol was crystallised by rotary evaporation at 45°C, and further dried in a cool vacuum oven for 6 hours. Crystallised dihydrodiol was analysed by NMR verifying the nature of the product. Deuterated chloroform and deuterated water were used to identify the positions of the hydrogen, carbon and oxygen groups. Analyses were carried out by the Department of Chemistry, University College London.

**Naphthalene dihydrodiol standard curve**

Naphthalene dihydrodiol was dissolved in dichloroethane to a stock concentration of 10mg/ml. Serial dilutions were made, and samples analysed by HPLC (Fig 2.2.). Naphthalene dihydrodiol was observed at a retention time of 4.2mins and naphthalene at 3.0mins.

**2.3. METHODS**

**2.3.1. DNA manipulations**

**Small scale plasmid isolation**

Small scale plasmid preparation was carried out by alkaline lysis. 1.5mls of cell culture was used from a 5ml overnight broth. Cells were spun out in a 1.5ml microcentrifuge tube at 13000 rpm for 5mins in a microfuge. Supernatant was discarded and the cell lysed in 100μls of lysis buffer. 200μls of 0.2N NaOH/1% (w/v) SDS was added, the contents mixed gently, and then left on ice for 5 mins. 150μls of an ice cold solution of 4M potassium acetate, pH6, was added and the mixture vortexed prior to incubating on ice for 20mins. The precipitate formed was removed by centrifugation for 10mins and the supernatant transferred to a fresh tube. An equal volume of buffered phenol/chloroform was added and mixed thoroughly. After centrifuging for 2mins, the upper aqueous layer was transferred to a new tube. 2 volumes of
Fig 2.2. Naphthalene dihydrodiol standard curve.
Ethanol were added to the DNA, and the tube stored at -20°C for 20 mins. Precipitated DNA was collected by centrifugation for 10 mins, and the supernatant discarded. The resultant pellet was rinsed in 70% ethanol and dried at 5°C. DNA was resuspended in 50 μl of TE buffer, pH 8, and RNase added to a final concentration of 20 μg/ml and allowed to stand at room temperature for 30 mins.

**Large scale preparation of plasmid DNA**

Large scale preparations of plasmid DNA were carried out using a modification of the alkaline lysis procedure. 400 ml cultures were grown overnight and harvested by centrifugation in a Sorvall RC2-B at 7000 rpm, 4°C for 10 mins. 20 ml of lysis buffer was added and the cells vortexed until thoroughly resuspended. The cells were incubated at room temperature for 5 mins. 50 ml of 0.2 N NaOH/1% (w/v) SDS was added and the mixture inverted gently several times prior to storage on ice for 5 mins. 40 ml of ice cold 4M potassium acetate/pH 6 was added and the mixture shaken vigorously. The resultant solution was held on ice for 20 mins – 1 hour and then spun in a Sorvall RC2-B at 4°C, 7000 rpm for 30 mins. The supernatant was transferred to a fresh tube and 50 mls of 50% polyethylene glycol 6000 was added. The mixture was placed on ice for 1 hour. Plasmid DNA was precipitated by centrifugation for 30 mins at 10,000 rpm, 4°C, and the pellet recovered and resuspended in 5 ml of TE buffer, pH 8. DNA was extracted twice in phenol/chloroform. 1.1 g CsCl was added per 1 ml of DNA solution. 200 μl of a 10 mg/ml solution of ethidium bromide was also added to visualise the plasmid DNA after centrifugation. The solution was loaded into Beckman quickseal polyallomer tubes, and spun in a Beckman L-7 ultracentrifuge at 20°C, 50,000 rpm for 20 hours. During the final 30 mins, the rotor speed was reduced to 40,000 rpm to relax the gradient. The plasmid DNA was isolated by removing the top of the tube and a bent hypodermic needle used to extract the plasmid band. 4 ml of TE buffer was added to the DNA solution to prevent precipitation of the CsCl. The ethidium bromide was removed by extracting with an equal volume of CsCl-saturated isopropanol several times. The aqueous phase containing DNA was ethanol precipitated and resuspended into 1 ml of TE buffer.
**Agarose gel electrophoresis of DNA**
High melting point agarose gels were made up at 0.8%w/v, using 0.5% TBE buffer. DNA samples were mixed with 5X loading buffer and run at 100-150mV. The gels were stained in an ethidium bromide solution (1µg/ml) for 10mins, and the bands visualised under UV irradiation using a 309nm UV transilluminator.

**Restriction enzyme digests**
All restriction enzyme digests were carried out in the manufacturer's buffers and the volume of restriction enzymes was maintained below 10% of the total volume. Reactions were carried out at 37°C for 2hours and terminated by the addition of loading buffer.

**DNA ligation**
Precut vector and insert DNA were mixed in a sterile microfuge tube in a 1:3 molar ratio. The DNA was phenol-chloroformed and ethanol precipitated to remove residual restriction enzyme and the pellet resuspended in 50µl TE buffer. 5µl of 10X bacteriophage T4 DNA ligase buffer, 1µl T4 DNA ligase and 1µl of 5mM ATP were added to the DNA and the mixture incubated overnight at 4°C. 2µls were removed prior to incubation and run on an agarose gel beside a 2µl aliquot removed after incubation to check the ligation.

**Preparation of competent E.coli cells**
Fresh competent cells were prepared prior to each transformation.
Cells were grown up overnight in a 5ml nutrient broth with 20mM MgSO₄ added. 0.5ml of overnight culture was used to inoculate a fresh 10ml nutrient broth containing 20mM MgSO₄, and the cells grown at 37°C with shaking for 2-3hours to an OD600 of 0.6-0.7. Cells were harvested in an MSE benchtop centrifuge at 4,500rpm for 10mins. The supernatant was removed and cells resuspended into 2mls of ice cold,100mM CaCl₂. The cells were transferred into two1.5ml microfuge tubes and held on ice for 30mins. Cells were reharvested and resuspended into 400µls CaCl₂ solution prior to immediate transformation.
Transformation of *E.coli* cells

5-50ng of DNA in a 20-50μl volume was added to 200μls of competent cells and gently mixed. Cells were incubated on ice for 45mins then immediately heat shocked by placing in a 37°C water-bath for 5mins. Cells were returned to ice for 1-2mins prior to addition to 5ml nutrient broths and grown with shaking at 37°C for 2hours. 100μls of cells were plated out onto nutrient agar plates with the appropriate antibiotic supplement, and grown overnight.

Conjugal transfer of DNA

DNA was transferred from *E.coli* cells to the *Pseudomonas* strains by conjugal transfer using the helper plasmid, pRK2013. *E.coli* strain HB101 harbouring plasmid pRK2013, was initially transformed with the plasmid to be transferred. Cells carrying both plasmids were selected for by resistance to kanamycin and ampicillin, and also for their ability to convert indole to indigo. Donor and recipient cells were grown up overnight in 5ml nutrient broths supplemented with antibiotics as required. Cells were harvested in an MSE benchtop centrifuge at 4000rpm for 10mins and supernatant removed. Donor cells were diluted in 100μls of fresh nutrient broth and recipient cells into 50μls. The cells were mixed together gently in a microfuge tube and spotted onto a nutrient agar plate. The plates were incubated upright overnight. Conjugated cells were lifted off using 1ml of 1xM9 salts solution, and pelleted in a microcentrifuge for 2mins. Cells were washed twice in 1xM9 salts solution and resuspended into 1ml of the salts solution. Cells were diluted x10⁻² and x10⁻³, and 100μls of each plated onto minimal media supplemented with carbenicillin. Plates were incubated for 3-4 days before colonies were observed. Colonies were isolated onto fresh minimal medium plates supplemented with carbenicillin, IPTG and indole, to check for dioxygenase activity.

Cells producing active naphthalene or toluene dioxygenase are able to convert tryptophan or indole to indigo. These cells produce a blue-black colouration upon growth, due to formation of indigo.
Enzyme activity is greatest when cells are incubated at 30°C, but colouration was still observed at 37°C. Naphthalene and toluene dioxygenase are able to hydroxylate indole to indole-2,3-dihydrodiol, which undergoes two non-enzymatic steps, eliminating 3 moles of water, resulting in the formation of indigo (Fig 2.3.).

2.3.2. Naphthalene dioxygenase activity measurements

Enzyme activity measurement by spectrophotometry
The catalytic step of indole conversion to indigo involves the hydroxylation of indole to indole dihydrodiol. Spontaneous elimination of one water molecule produces the coloured compound, indoxyl. A slower air oxidation step, converts indoxyl to indigo (Fig 2.3.). Therefore, the rate of formation of indoxyl, measured at OD$_{400}$, can be used as a measure of enzyme activity. Reactions were carried out in a total volume of 1 ml using plastic, 1 ml cuvettes (Sarstedt) and monitored in a CECIL CE272, temperature controlled spectrophotometer. Cells were resuspended in 950 μl phosphate buffer containing 25 μl glucose (final concentration, 25 mM) to an OD$_{600}$ of 0.25-0.3. The volume was made up to 990 μl with phosphate buffer, and the reaction initiated by addition of 10 μl indole dissolved in ethanol (final concentration =1 mM). The reaction was carried out at 30°C and monitored at OD$_{400}$.

Enzyme activity measurement by HPLC
Accumulation of naphthalene dihydrodiol was measured by HPLC. 200 μl samples were diluted into 5 ml of dichloroethane, of which 1 ml samples were analysed. A mobile phase of 98% 1,2-dichloroethane/2% methanol was run. Naphthalene dihydrodiol peaks were observed at 4.2 minutes and integrated by an in-line integrator. The concentrations were calculated from the standard curve derived earlier.
Fig 2.3. Conversion of tryptophan and indole to indigo
2.3.3. Fermentations

**Fermentation of E. coli**
An active colony, identified by its ability to convert indole to indigo, was grown up overnight in 5ml of nutrient broth. This was used to seed a 2L shake flask containing 500ml of nutrient broth. Cells were grown in an orbital shaker at 250rpm, 30°C and growth monitored at OD\textsubscript{600}. Cells were induced at early-log phase (OD\textsubscript{600}=0.22-0.3) with IPTG (final concentration, 1mM) and growth continued for a further 4 hours (late-log phase/ OD\textsubscript{600}=2.0). Cells were harvested at 4°C in a SORVALL high speed centrifuge, at 8000rpm for 10mins. Cells were then washed in 50ml of phosphate buffer and reharvested.

**Fermentation of P. putida UV4**

*P. putida* cells were grown in either Pseudomonads defined salts medium supplemented with sodium salicylate or sodium succinate at 0.5%w/v or nutrient broth. An active colony from a plate growth was grown up overnight in 5ml of nutrient broth. The culture was used to seed a 500ml volume of broth in a 2L shake flask, and cells grown at 30°C, 250rpm. Growth was monitored by spectrophotometry at 600nm using distilled water as a blank, until late-log phase was reached (OD\textsubscript{600}=2.5-2.7). Cells were harvested by centrifugation at room temperature in a SORVALL high speed centrifuge at 8000rpm for 10mins. The cell pellet was washed in 50ml phosphate buffer and reharvested.

**Fermentation of P. aeruginosa PAC1R and PAC610.**
Fermentations were carried out in nutrient broth as for *E. coli* JM107. Cells were induced at early-log phase (OD\textsubscript{600}=0.4-0.5) and growth continued for a further four hours. The cells were harvested as for *E. coli* JM107.

**Dry cell weight determination**
Dry cell weight was calculated as a percentage of the wet cell weight. Cells were grown and prepared as described. 20ml cell suspension was harvested in preweighed 10ml test tubes which had been predried at 90°C for 48 to 72 hours, and buffer removed.
using a pasteur pipette. Wet weight was noted, and cells dried to constant weight at 90°C over 24 hours.

2.3.4 Solvent exposure studies

Cell exposure to naphthalene dissolved in ethanol (Fig 2.t*)

Cells were prepared as described and resuspended into phosphate buffer to give a final concentration in a total volume of 5ml. Biotransformations in STRs were carried out using a naphthalene solution made up in ethanol to a stock concentration of 500mM. To monitor the effect of ethanol upon the cells, preincubation was carried out in phosphate buffer in the presence of ethanol at the same level to which the cells would be exposed during biotransformation. Cells were stirred at 200rpm, 28°C, 1-1.33v/v/min, for two hours. Controls, in which cells were pretreated in phosphate buffer alone, were used for each different level of ethanol exposure. During the biotransformations, naphthalene solution was added to a final concentration of between 4.2mM and 50mM. A final control, in which cells were not challenged to ethanol, either during pretreatment or during biotransformation, was also carried out.

Biotransformations of UV4 using 4.2mM and 8.4mM naphthalene in ethanol concentrations were not subject to preincubation in ethanol, since it had been shown that preexposure at these levels had no detrimental effect on the cells.

Solvent saturation of phosphate buffer

Solvent was added to phosphate buffer in a 1L Duran bottle, until a second layer of approximately 2cm was formed. The vessel was sealed and the mixture stirred for 48hrs to ensure complete saturation of the buffer. The upper organic layer was retained as a solvent reservoir.

Cell exposure to solvent saturated buffer

Cells were resuspended in phosphate buffer to a final concentration in a 1ml volume of buffer. Cell suspensions were then added to 50ml of solvent saturated buffer and incubated with stirring at 200rpm and 28°C in STRs. At specific intervals, the cells...
were removed and harvested on a benchtop centrifuge at 4800rpm for 10mins. Cells were washed twice in an equal volume of phosphate buffer, and finally resuspended into 5ml of buffer. Biotransformations were carried out in STRs as described.

Cell exposure in a hexane:buffer two-liquid phase system
Cells were resuspended in phosphate buffer to the desired concentration in a volume of 1ml. Cell suspension was then added to a 30ml hexane/29ml buffer mix in STRs, and mixing carried out at 750rpm/28°C. Cells were removed at 2 and 4hour intervals and washed twice in equal volumes of phosphate buffer. Controls were carried out using cells mixed in phosphate buffer alone. Biotransformations were performed as described.
Fig 2.4. Schematic diagram illustrating biotransformation in naphthalene/ethanol.

V1', V2', and V3' are the control samples to V1, V2, and V3, respectively.
3. RESULTS

3.1. GENETIC MANIPULATIONS

3.1.1. Restriction enzyme map of pQR113

In order to express naphthalene dioxygenase in various *E. coli* and *Pseudomonas* strains, it was decided to utilise the *nahA* gene from the NAH plasmid of *P. putida* PpG7. A 6.9kbp EcoRI-HindIII fragment containing the *nahA*, *nahB* and part of the *nahC* genes had previously been incorporated into plasmid pKK223.3 to form the plasmid pQR113 (made by K.Seehra).

In order to isolate the *nahA* gene, a restriction enzyme map of pQR113 was constructed to identify restriction enzyme sites peripheral to, and not contained within, the *nahA* gene (Fig 3.1).

3.1.2. Construction of plasmids pSS1, pQR155 and pQR156

EcoR1 (at site 11440) and BamH1 (at site 6504) were chosen to construct plasmid pSS1. Ligation of this 4.9kbp fragment with plasmid pBGS18- was carried out to form the new construct pSS1 (Fig 3.2.1.). Induction and expression of *nahA* in *E. coli* JM107 transformed with pSS1 enabled the synthesis of active naphthalene dioxygenase, as shown by its ability to convert indole to indigo.

It was thought that expression could be improved by removing DNA regions upstream of the *nahA* gene in order to bring the promoter into closer proximity with the transcription start site. Initial attempts using exonucleaseIII were unsuccessful. Therefore, it was decided to carry out partial cleavage of the 6.9kbp *nah* fragment using restriction enzyme Sau3A. These fragments inserted into the BamH1 site of pBGS18-, and the resultant constructs used to transform *E. coli* JM107.

Two constructs, pQR155 and pQR156 (made by J.Ward), were identified as having lost DNA regions upstream of the start site, and able to express the *nahA* gene (Fig 3.2.2.).
Fig 3.1. Restriction map of pQR113: shaded region contains DNA isolated from the NAH plasmid; single line is plasmid pKK223.3 cleaved with EcoRI/HindIII.
3.1.3. Construction of plasmids pSS2, pSS3 and pSS4

In order to be able to express *nahA* in both *E.coli* and *Pseudomonas* strains, a broad host range vector able to replicate in both these species was chosen. The pMMB family of plasmids based upon plasmid RSF1010 are known to be able to replicate stably in both *E.coli* and *Pseudomonas* hosts. Plasmids pMMB66EH and pMMB66HE were chosen for this reason. Plasmids pMMB66EH and pMMB66HE differ only in the orientation of their multiple cloning sites with respect to the Ptac promoter.

The isolated *nah* fragments from plasmids pSS1, pQR155 and pQR156, were cleaved at their EcoR1-HindIII sites and inserted into the equivalent sites in plasmid pMMB66EH, to produce plasmids pSS2, pSS3, pSS4 (Fig 3.3.). Insertion of these fragments into plasmid pMMB66HE produced plasmids pSS2.1, pSS3.1, and pSS4.1.

*E.coli* JM107 cells were transformed with these plasmids, and expression of the *nahA* gene shown to enable the conversion of indole to indigo, and naphthalene to its diol form (see section 3.2.4., Fig 3.6.)

Transfer of plasmid pSS2 into the *Pseudomonas* strains PAC1R and PAC610, was carried out by conjugation using the helper plasmid pRK2013 in *E.coli* strain HB101. *P.aeruginosa* exconjugants able to express the naphthalene dioxygenase gene, were identified by their ability to convert indole to indigo upon induction (see section 3.2.7, Fig 3.7.) and naphthalene to the diol (see section 3.2.8, Fig 3.8.).

Plasmids pSS2.1, pSS3.1, and pSS4.1 showed no naphthalene dioxygenase activity.

3.2. GROWTHS AND ACTIVITIES

3.2.1. UV4 growth in complete and minimal media

*P. putida* was grown in shake flasks in either nutrient broth or in pseudomonads defined salts medium, supplemented with either succinate or salicylate as carbon source, at 0.5% w/v.
Fig 3.2.1. Construction of plasmid pSS1.
Plac=lac promoter; Kn=kanamycin resistance; kbp=kilobase pairs; =nahA
DNA: Restriction enzyme sites: H=HindIII; S=SphI; A=AccI; He=HinCII; X=XhoI;
B=BamHI; K=KpnI; Sa=SaiI; E=EcoRI; lacPOZ'=lac operon.
Fig 3.2.2. Construction of plasmids pQR155 and pQR156
Cleavage of pQR113 with Sau3A and ligation to pBGS18 cut with BamH1 to form pQR155 (from fragment 1) and pQR156 (from fragment 2): Plac=Iac promoter; Kn=kanamycin resistance; kbp=kilobase pairs;  =naliA DNA
Restriction enzyme sites: H=HindIII; S=SphI; A=AccI; Hc=HinCII; X=XhoI; B=BamH1; K=KpnI; S=SalI; E=EcoRI; lacPOZ'=lac operon.
Fig 3.3. Construction of plasmids pSS2, pSS3, and pSS4.
pSS2 constructed from pSS1; pSS3 constructed from pQR155; pSS4
constructed from pQR156: n gene fragment; Ap=ampicillin
resistance; Kn=kanamycin resistance; lacIq=lac repressor; Ptac=tac
promoter.
Cells were unable to grow when salicylate was used as a carbon source.
The rates of growth of UV4 in both minimal medium+succinate and nutrient broth were similar, reaching stationary phase after approximately 9 hours (Fig 3.4.).
Dry weight of cells at late-log phase were calculated at 25% of wet cell weight.

3.2.2. UV4 dioxygenase activity comparisons

Cells were harvested at mid-log, late-log and stationary phases of growth, and the activities compared spectrophotometrically.

i) Defined salts+succinate
The level of naphthalene dioxygenase activity produced after harvesting, was considered to be equal, at all stages of growth, since only a 5% variation in activity was observed between the highest and lowest values obtained (Table 3.1.).

ii) Nutrient broth
Highest activity was obtained at late-log phase of growth, producing a rate of 0.0286 abs.units/min, after values were normalised for cell concentration. A 16% difference in the dioxygenase activity was observed between the highest and lowest rates. This was thought to have occurred as a result of variations arising during aliquoting of samples, and not of differences in the level of transcription in the cells.

Table 3.1 UV4 growth and activity in various media
Activity measured after normalisation for cell concentration (as measured at OD₆₀₀). Rate of conversion of indole measured at OD₄₀₀.

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Time of harvesting</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(OD₆₀₀)</td>
<td>(abs.units/min)</td>
</tr>
<tr>
<td>Nutrient broth:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mid-log</td>
<td>7 hrs (1.28)</td>
<td>0.0240</td>
</tr>
<tr>
<td>late-log</td>
<td>9 hrs (2.36)</td>
<td>0.0286</td>
</tr>
<tr>
<td>stationary phase</td>
<td>12 hrs (2.68)</td>
<td>0.0263</td>
</tr>
</tbody>
</table>
Defined salts medium+succinate:
mid-log 7hrs (1.39) 0.0272
late-log 9hrs (2.40) 0.0258
stationary phase 12hrs (2.49) 0.0263

The similarities in the levels of dioxygenase activity between cells grown in minimal medium and nutrient broth (Fig 3.5.) suggest that both media enable cells to grow and to express nahA at similar levels. No advantage appears to be gained from cultivating the cells in one or other of the media. It was therefore decided to continue growth in nutrient broth and to harvest cells at late-log phase of growth. This would ensure that all cells were subject to the same growth conditions with respect to media, temperature and rate of mixing.

3.2.3. JM107 growth in nutrient broth

*E.coli* JM107 cells were grown in shake flasks using nutrient broth. Cells were induced at early-log phase and growth continued for a further 4 hours. Stationary phase was reached after 9 hours. Dry cell weight at the end of growth (late-log phase), was calculated at 20% of wet cell.

3.2.4. Comparison of enzymic activities from *E.coli* JM107 harbouring plasmids pSS2, pSS3, and pSS4

All three DNA constructs were tested for their levels of activity, in stirred tank reactors (Fig3.6.). The greatest activities were observed over the first 5 hours of biotransformation, with specific activities ranging from $6 \times 10^{-3}$ g/g/hr to $8 \times 10^{-2}$ g/g/hr (table 3.2).
Fig. 3.4. *P. putida* UV4 growth in various media:
Cells grown in 100ml volume in 500ml shake flasks, seeded with a 1% v/v overnight growth culture. Growth carried out at 30°C, 250rpm, in nutrient broth or defined salts medium supplemented with succinate or salicylate (sodium salts) at 0.5% w/v.
* succinate; ○ salicylate; ◦ nutrient broth
Abs units

Fig 3.5. *P. putida* UV4 indole conversion to indoxyl; comparative activities
Phase of growth: ■NB-late log; ♦ MIN+succ-mid log
Indole conversion measured at OD_{400} in 1ml cuvettes, using phosphate buffer (pH7/50mM), 20mM glucose, and 1mM indole dissolved in ethanol.
Comparison of the dioxygenase activities from cells expressing the three different plasmids indicated that the activity produced by cells expressing plasmid pSS2 was 10-fold greater than that produced by cells bearing plasmid pSS4, and 13.3-fold greater than cells expressing plasmid pSS3. The differences in the activities may reflect differences in the DNA regions upstream of the transcription start site, acquired during the initial construction of these plasmids. Loss of the activity of the ferredoxin(nap) reductase subunit and the loss of the nahA promoter region in plasmids pSS3 and pSS4, have both been proposed as possible explanations for these differences in activity. A detailed discussion is carried out later (see section 4.1.2.).

The naphthalene dioxygenase activity was observed to decrease with prolonged biotransformation, with the largest decrease seen in cells expressing plasmid pSS2. Cells harbouring plasmids pSS3 and pSS4 showed almost negligible decreases in specific activity over the entire reaction period.

The loss of activity in JM107(pSS2) may be a result of product inhibition occurring when naphthalene dihydrodiol concentration approached 4-5 g/L. Depletion of glucose required for cofactor recycling may also have influenced the decrease in activity observed. Similar activity profiles were observed with P. putida UV4 [Harrop, (1990)]. JM107(pSS2) was chosen to carry out all further biotransformations because of the higher level of activity produced.

Table 3.2. Specific activities from JM107(pSS2), (pSS3) and (pSS4)

<table>
<thead>
<tr>
<th>SPECIFIC ACTIVITY (g/g/h)*</th>
<th>JM107 (pSS2)</th>
<th>JM107 (pSS3)</th>
<th>JM107 (pSS4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 hours</td>
<td>8x10⁻²</td>
<td>6x10⁻³</td>
<td>8x10⁻³</td>
</tr>
</tbody>
</table>

*sp activity measured as (g/L product formed)(g/L drycell weight)(h⁻¹)
Fig 3.6. Comparative activities of JM107 expressing plasmids (pSS2), (pSS3), and (pSS4)

Cells biotransformed in 60ml phosphate buffer (50mM/pH7) at 750rpm, 28°C, air sparged at 1-1.33v/v/min, g/g=gdiole/cells.
Line from 5hrs to 6.5hrs shows theoretical product profile.
Active plasmid: □ pSS2; ■ pSS3; ♦ pSS4;
3.2.5. Effect of cell concentration on dioxygenase activity of JM107(pSS2)

In order to maximise substrate conversion during biotransformations, various cell concentrations were tested. Cell loadings of between 0.33g/L and 1g/L (dcw) were used. The dioxygenase function is dependent upon glucose for cofactor recycling and aeration for the provision of oxygen molecules. A higher cell loading may be expected to limit the amount of cofactor and oxygen available to the cells, thus affecting the final activity.

Specific activity was observed to decrease by almost 25% when cell loadings were raised from 0.33g/L to 0.67g/L or 1.0g/L (table 3.3).

However, this drop in specific activity was not considered significant enough to offset the 2.3fold increase in total activity achieved, and it was decided to carry out all subsequent biotransformations at 1g/L.

<table>
<thead>
<tr>
<th>Cell concentration (g(dcw))/L</th>
<th>0.33</th>
<th>0.67</th>
<th>1.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity (g/L/h)</td>
<td>3.55X10^-3</td>
<td>5.36X10^-3</td>
<td>8.09X10^-3</td>
</tr>
<tr>
<td>Specific activity (g/g/h)</td>
<td>10.65X10^-3</td>
<td>8.04X10^-3</td>
<td>8.09X10^-3</td>
</tr>
<tr>
<td>% Specific activity decrease*</td>
<td>0</td>
<td>24.5</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Table 3.3 Effect of cell concentration on specific activity in JM107(pSS2)
* Specific activity decrease expressed as a percentage of specific activity obtained using 0.33g/L cell loading to carry out biotransformation.
3.2.6. PAC1R(pSS2) and PAC610(pSS2) growth in nutrient broth

Strains PAC1R and PAC610 were grown in shake flasks using nutrient broth as a growth medium. Cells were induced at early-log phase (OD\text{600} 0.4-0.5) and growth continued for a further 4 hours. The cells showed very similar growth profiles, reaching stationary phase after 11 hours. Dry cell weight was calculated at 15% of wet cell weight.

3.2.7. PAC1R(pSS2) and PAC 610(pSS2) conversion of indole

Hydroxylation of indole by PAC1R(pSS2) and PAC610(pSS2) showed similar rates of conversion over the reaction period, as measured by spectrophotometry (Fig 3.7.). This suggests that the level of expression of naphthalene dioxygenase may be similar in both cells, such that the resulting activities are approximately equal.

3.2.8. PAC1R(pSS2) and PAC610(pSS2) conversion of naphthalene

Biotransformations carried out in stirred tank reactors showed a significant difference in activity between strains PAC1R and PAC610, both expressing plasmid pSS2 (Fig 3.8.). Near linear reaction profiles were observed over the two hour period of biotransformation, with PAC1R accumulating less than 4 times the amount of naphthalene dihydrodiol produced by strain PAC610 (8.46X10^{-4}g/L: 3.5X10^{-3}g/L).

It is thought that differences in the outer membrane structure of PAC610 and PAC1R may give rise to the different activities observed. The outer membrane acts as a barrier to the movement of hydrophobic compounds into the cell. The intact outer membrane structure of PAC1R may be forming a very effective barrier to the movement of hydrophobic molecules, significantly retarding the flux of naphthalene. Because of its inability to synthesise the O-antigenic polysaccharide chains, the degree of interaction between LPS molecules required to mediate the exclusion of hydrophobic molecules, may be significantly reduced. Therefore, strain PAC610 may be unable to produce as effective
an hydrophobic barrier as strain PAC1R, and so allows a greater movement of substrate into the cell.
These results contrast with the approximately equal rates of conversion of indole by the two strains. The reduced size and higher solubility of indole in water, may allow a greater rate of diffusion of the molecule into PAC1R compared with naphthalene. Also, the ethanol used to dissolve the indole, may act to partially permeabilise the outer membrane of PAC1R, allowing an increased flux of indole into the cell. All results are discussed further in section 4.1.5.

**Cell storage stability**
Both *E.coli* strain JM107(pSS2) and *P.aeruginosa* PAC1R(pSS2) could be stored overnight at 4°C as cell pellets, with little loss of activity. Both cells were observed to retain 93%-95% of their activity.
In contrast, *P.putida* UV4 rapidly lost activity at low temperatures. A 20.9% decrease in activity is reported upon storage at 4°C (A.Collins. pers comm.).

**3.3. USE OF NAPHTHALENE DISSOLVED IN ETHANOL**

**3.3.1. Explanation for use of ethanol**

Due to the low solubility of naphthalene in water, naphthalene crystals were often observed to be lost during biotransformation through foaming produced upon stirring and aeration. Also, naphthalene crystals were found remaining at the end of the biotransformation period, indicating that much of the substrate had not been utilised by the cells. Samples containing high and random naphthalene levels were often detected, producing irregular naphthalene concentration profiles throughout the reaction making it difficult to quantitate substrate utilisation (results not shown).
In order to try and resolve these problems, it was decided to increase the solubility of naphthalene in the buffer using ethanol as a solvent. Also, ethanol could be used to recycle NADH through
Fig 3.7. Conversion of indole by PAC1R (pSS2) and PAC610 (pSS2)

- ■ PAC610(pSS2); ○ PAC1R(pSS2).

Indole conversion carried out in 1ml vol using phosphate buffer (50mM/pH7)/20mM glucose/1mM indole dissolved in ethanol. Cells added to an OD$_{600} = 0.2$. Reaction followed at OD$_{400}$.
Fig 3.8. Conversion of naphthalene by PAC1R(PSS2) and PAC610(PSS2) in STRs
Biotransformations carried out in 60ml phosphate buffer, at 750rpm, 28°C, air sparged at 1-1.33 v/v/min, g/g=gdiol/gcells.
■PAC1R(pSS2); ♦PAC610(pSS2)
the activity of the cell's alcohol dehydrogenase system, thus alleviating the need to provide glucose as the source of co-factor recycling. The maximum concentration of naphthalene that could be successfully dissolved in ethanol was limited to 500mM. Addition of low levels of naphthalene solution to phosphate buffer still resulted in some crystallisation of the naphthalene. However, the amount of crystallised naphthalene was low, and the amount lost during foaming was negligible.

3.3.2. UV4

UV4 cells were able to utilise the naphthalene even when provided at a concentration of 4mM. Furthermore, the amount of ethanol at this level was sufficient to maintain the enzymic activity over the three hour biotransformation period. However, for extended biotransformations, the low level of ethanol may prove to be inhibitory to the enzymic activity, with depletion of ethanol leading to a loss in the ability to recycle NADH.

The maximum activity observed during the initial biotransformations using 12.5mM-50mM naphthalene approached 8.7X10^-3 g/g/h (Fig 3.9.). This was achieved by both cells presented with 25mM naphthalene which had not been preexposed to ethanol, and cells presented with 12.5mM naphthalene, irrespective of pre-treatment.

Any damaging effects on cells due to the ethanol was tested for by preexposing cells to ethanol for a 2hour period, and comparing the resultant activity against a control group of cells that had been exposed to phosphate buffer over the same period. Cells presented with naphthalene at a 50mM concentration showed reduced levels of biocatalytic activity; cells preexposed to ethanol at 10%v/v were totally inactive, and the control group (preexposed to phosphate buffer/biotransformed in 50mM naphthalene), produced the lowest level of activity of all the samples at 5.47X10^-3 g/g/h. This represents a 38% lower activity than the maximum level observed. The activity level of cells presented with 25mM naphthalene solution was dependent upon whether cells had been pretreated with ethanol or not, with pretreatment
in the presence of 5%v/v ethanol resulting in almost an 17% lower activity than its control group.

When naphthalene was added at a concentration of 12.5 mM, no inhibitory effects were observed, even after preexposure to 2.5%v/v ethanol. Decreasing the amount of naphthalene in ethanol to 8.4 mM and 4.2 mM, did not result in any significant change in the level of activity (Fig 3.10.).

It was of interest to note that the level of product formed, using naphthalene dissolved in ethanol, was greater than that when crystalline naphthalene and glucose were used. This may reflect an increased availability of substrate to the cell, and/or the possible partial permeabilisation of the cells by ethanol, allowing a more rapid uptake of the substrate.

3.3.3. JM107(pSS2)

Reactions were performed at 8.4 mM and 4.2 mM naphthalene, in order to minimise the amount of ethanol to which the cells were exposed. Exposure of JM107 cells to ethanol caused a rapid loss of activity, even when naphthalene was added at only 4.2 mM. Cells preexposed to ethanol, at 1.67%v/v and 0.83%v/v, showed no activity during biotransformation (Fig 3.11.). Cells that had not undergone preexposure to ethanol showed a low level of activity (8.4 mM producing 0.047X10^-2 g/g/h; 4.2 mM producing 0.194X10^-2 g/g/h). Increasing the naphthalene concentration (from 4.2 mM to 8.4 mM) resulted in an over four fold decrease in the level of product formed. In both cases, the activities observed were significantly lower than that of the control sample in which cells were not challenged with ethanol at any stage.
Fig. 3.9. UV4 activity in STR using naphthalene dissolved in ethanol (12.5-50 mM): PE, cells pre-exposed to ethanol; UE, cells not pre-exposed to ethanol; □ 12.5 mM (UE); ■ 12.5 mM (PE); ○ 25 mM (UE); ● 25 mM (PE); △ 50 mM (UE); ▲ 50 mM (PE); ◊ control

Biotransformations carried out in 60 ml volume at 750 rpm/28°C/air sparged at 1-1.3 v/v/min. Naphthalene added as a solution dissolved in ethanol to a final concentration of 500 mM, g/g=gdiol/gcells.

<table>
<thead>
<tr>
<th>naphthalene concentration (mM)</th>
<th>ethanol content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4.2 mM: 0.83%)</td>
<td></td>
</tr>
<tr>
<td>(8.4 mM: 1.67%)</td>
<td></td>
</tr>
<tr>
<td>(12.5 mM: 2.5%)</td>
<td></td>
</tr>
<tr>
<td>(25 mM: 5%)</td>
<td></td>
</tr>
<tr>
<td>(50 mM: 10%)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.10. UV4 activity in STR using naphthalene dissolved in ethanol (12.5-4.2mM); cells were not pre-exposed to ethanol □ 12.5mM; ● 4.2mM; ▲ 8.4mM; ◆ Control (naphthalene xtals/glucose) Biotransformations carried out in 60ml volume at 750rpm/28oC/air sparged at 1-1.33v/v/min. Naphthalene added from a stock solution made up in ethanol to a final concentration of 500mM, g/g=gdioI/gcells.

<table>
<thead>
<tr>
<th>Naphthalene concentration (mM)</th>
<th>Ethanol content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4.2mM:0.83%)</td>
<td>(8.4mM:1.67%); (12.5mM:2.5%); (25mM:5%); (50mM:10%)</td>
</tr>
</tbody>
</table>
Fig 3.11. JM107(pSS2) activity in STR using naphthalene dissolved in ethanol (4.2mM-8.4mM)

Biotransformations carried out in a volume of 60ml at 750rpm, 28°C, air sparged at 1-1.33v/v/min, g/g=gdiol/gcells.

UE= cells not pre-exposed to ethanol; PE= cells pre-exposed to ethanol
Control= cells incubated in phosphate buffer; 4.2mM (UE); 4.2mM (PE);
8.4mM (UE); 8.4mM (PE); Control

<table>
<thead>
<tr>
<th>Naphthalene concentration (mM):ethanol content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4.2 mM: 0.83%); (8.4 mM: 1.67%); (12.5 mM: 2.5%);</td>
</tr>
<tr>
<td>(25 mM: 5%); (50 mM: 10%)</td>
</tr>
</tbody>
</table>
3.4. SOLVENT EXPOSURE STUDIES

3.4.1. Solvent choice.

Solvents were chosen on the basis of their hydrophobicity measured in terms of logP. Correlation of biocatalyst activity retention to solvent logP [Laane et al. (1985)], identified three groups of solvents based upon their logP values: solvents with logP > 4 produced no loss of activity; solvents of logP between 2 and 4 caused limited damage to the biocatalyst; and solvents of logP < 2 resulted in complete loss of biocatalytic activity. The solvents were chosen to fall into each of these categories and have been shown to produce the predicted effects upon P. putida UV4, in two liquid-phase systems [Harrop et al. (1992)]. Therefore, it was of interest to compare the effects of the solvents on this wider range of cell systems.

3.4.2. Exposure to solvent saturated buffer

**JM107**

i) Pentanol (LogP=1.33)

Exposure to pentanol saturated phosphate buffer resulted in complete loss of cellular activity (Fig 3.12.). Centrifugation was unable to completely pellet all the cellular matter, with a cloudy supernatant formed, which is thought to include cellular lipid components partitioned into the solvent.

ii) Hexane (Log=3.5)

Two-liquid phase biotransformations using solvents in the range logP=3.5 to logP=4, indicated that these solvents were all capable of causing limited cellular damage upon UV4 [Harrop, (1990)]. It was observed that immobilisation of UV4, to reduce exposure to the liquid:liquid interface, was unable to prevent loss of activity when decanol (logP=4) was used as the second phase. Hexane, logP=3.5, would therefore be predicted to cause some loss of activity to the cells tested. Loss of activity was observed as a two stage process with an initial rapid loss over the first 30 minutes to two hours leading to
Fig 3.12. JM107(pSS2) activity after exposure to pentanol saturated buffer.
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer, at 750rpm, 28°C, air sparged at 1-1.33 v/v/min.
g/g=gdiole/gcells
- unexposed;  30 min exp;  60min exp;  120min exp;
240min exp;  21hrs exp
Fig 3.12.1. JM107(pSS2) % activity after exposure to pentanol saturated buffer
% activity as % of untreated control.

No activity was observed in any of the samples tested.
Fig 3.13. **JM107 (pSS2) activity after exposure to hexane saturated buffer.**

Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33v/v/min.

\[ \text{g/g = diol/gcells} \]

- Unexposed; • 30min; ■ 60min; • 120 min; ▲ 240min; □ 21hr
Fig 3.13.1. JM107 (pSS2) % activity after exposure to hexane
saturated buffer.
%activity as % of untreated control. Dashed line indicates
theoretical decline in activity.
Fig 3.14. JM107 (pSS2) activity after exposure to tetradecane saturated buffer.
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33v/v/min, g/g=gdiol/gcells.
- unexposed; * 0.5hr exp; 1hr exp; 2hr exp; 4hr exp; 21hrs exp.
Fig 3.14.1. JM107 (pSS2) % activity after exposure to tetradecane saturated buffer. % activity as % of untreated control. Dashed line indicates theoretical decline in activity.
an approximately 10% decrease in activity, followed by a steadier decline up to 21 hours (Fig 3.13, 3.13.1.). Dissolved hexane produced a decrease in activity after 30 mins exposure. This level of activity was maintained by cells exposed for up to 2 hours in solvent saturated buffer. Further decrease in activity was observed with increased exposure time. Cells exposed for 4 hours lost approximately 25% of their activity, and cells exposed for 21 hours show an almost 78% drop in activity.

iii) Tetradecane (LogP=7.8)
Tetradecane is poorly-miscible in water, with a logP value of 7.8. Activity was expected to be unaffected by exposure to the saturated buffer. However, loss of activity was still observed, although to a much lesser extent. Cells lost between 10% and 15% of their activity when treated with solvent saturated buffer for between 30 mins and four hours. Prolonged exposure for up to 21 hours produced a 68% decrease in overall activity (3.14, 3.14.1.).

UV4
i) Pentanol (LogP=1.33)
Action of pentanol on UV4 was comparable to that observed when JM107(pSS2) was exposed to the solvent. No activity was observed in any of the samples tested (3.15, 3.15.1.). Cellular matter could not be completely pelleted, producing a cloudy supernatant, and cells were observed to have lost their pigmentation. This may suggest that disruption of the outer membrane had occurred allowing the release of periplasmic contents, including cell pigment [Garrard,(1972)].

ii) Hexane (LogP=3.5).
The pattern of solvent damage upon cell activity observed for previous cell:solvent combinations is evident in UV4 exposed to hexane saturated buffer (3.16.). A rapid initial decline in the level of activity over the first 2 hours of exposure produced almost 26% drop in activity. Further decline in activity occurred at a slower rate, with 57.47% activity retained after 4 hours and 45% after 21 hours (3.16.1.).
iii) Tetradecane ($\text{LogP}=7.8$)
Biotransformations previously carried out in tetradecane: phosphate buffer two-liquid phase systems, showed no loss of activity in toluene dioxygenase expressed by UV4 [Harrop,(1990)]. Therefore, it would be expected that exposure to the lower concentrations of tetradecane found dissolved in phosphate buffer, would not have any effect on the activity of the biocatalyst. Loss of activity was still observed, leading to a 5% decrease in activity in cells exposed for up to four hours in saturated buffer. Cells retained almost 87% of their activity after 21 hours (3.17, 3.17.1.). Therefore it seems that, although UV4 is still subject to disruption by tetradecane saturated buffer, the extent to which the solvent damages cells is significantly lower than JM107 treated in the same manner.

**PAC1R**
The high degree of interaction of lipopolysaccharides in the outer membrane of PAC1R, which forms a barrier to the diffusion of hydrophobic compounds, was earlier postulated to account for the retardation of movement of substrate into the cell, producing the low levels of activity observed (see section 3.2.3.). Exposure to solvent was therefore predicted to show a greater degree of solvent stability compared with both UV4 and JM107(pSS2).

i) Pentanol ($\text{LogP}=1.33$)
Dissolved levels of pentanol caused complete loss of activity in PAC1R (3.18, 3.18.1.). Cellular matter could not be completely pelleted, and outer membrane disruption was thought to have resulted in the loss of pigmentation [Garrard,(1972)].

ii) Hexane ($\text{LogP}=3.5$)
Exposure to hexane saturated buffer resulted in a continued increase in the %activity retained in cells exposed for up to 4 hours (3.19). This was observed as an initial increase to almost 107% activity of the untreated control, in cells exposed over the first hour. %activity rose to 112% activity after the second hour. This was followed by a more rapid increase to approximately 157% activity after four hours of exposure (3.19.1.).
Fig 3.15. UV4 activity after exposure to pentanol saturated buffer
No activity observed with exposure to pentanol saturated buffer
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33 v/v/min, g/g=gdioI/gcells.
- Control; ◇ 0.5hr exp; ★ 1hr exp; ♦ 2hr exp; ✯ 4hr exp
Fig 3.15.1. UV4 % activity after exposure to pentanol saturated buffer
%activity as % of untreated control.
No activity observed.
Fig 3.16. UV4 activity after exposure to hexane saturated buffer

Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28oC, air sparged at 1-1.33v/v/min, g/g=gdiol/gcells.

- control; 0.5hr exp; 1hr exp; 2hr exp; 4hr exp; 21hr exp
Fig 3.16.1. UV4% activity after exposure to hexane saturated buffer.
%activity as % of untreated control. Dashed line indicates theoretical decline in activity.
Fig 3.17. UV activity after exposure to tetradecane saturated buffer
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33 v/v/min, g/g=diole/gcells.
- control  - 0.5hrs exp;  - 1hr exp;  - 2hr exp;  - 4hr exp;  - 21hr exp
Fig 3.17.1. UV4 % activity after exposure to tetradecane saturated buffer. % activity as % of untreated control. Dashed line indicates theoretical decline in activity.
The increases in activity are thought to result from a limited permeabilisation of the outer membrane, allowing an increase in the flux of substrate. Prolonged exposure resulted in a decline in the activity level to approximately 85% of the control. This is thought to result from cumulative solvent damage to cellular functions required for cell maintenance, occurring either within the periplasm or the cytoplasmic membrane.

iii) Tetradecane (LogP=7.8)
Exposure to tetradecane saturated buffer resulted in a continued increase in the %activity in cells exposed for up to 4 hours (3.20, 3.20.1.). An initial increase in %activity was shown by cells exposed for up to 1 hour, resulting in almost 200% of the %activity produced by the untreated control. Increased exposure time (up to 4 hours) resulted in a smaller rise in the %activity observed. Long term exposure (21 hours) showed that %activity retained by the cells had significantly decreased, and that a final activity level of 101.13% of the control sample was retained. Permeabilisation of the cells by the solvent, is again thought to be responsible for the increase in activity, with long term exposure causing damage to functions required for cell maintenance.
Fig 3.18. **PAC1R activity after exposure to pentanol saturated buffer.**

Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28oC, air sparged at 1-1.33v/v/min. g/g=gdiol/gcells.

- Control;  
- 0.5hr exp;  
- 1hr exp.
Fig 3.18.1. PAC1R(pSS2) % activity after exposure to pentanol saturated buffer
% activity as % of untreated control.
No activity observed in any samples tested.
Fig 3.19. PAC1R activity after exposure to hexane saturated buffer.
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33v/v/min, g/g=gdioI/gcells.

□ Control; ● 0.5hr exp; ◆ 1hr exp; ○ 2hr exp; ◊ 4hr exp; × 21hr exp
Fig 3.19.1. PAC1R (pSS2) % activity after exposure to hexane saturated buffer.

% activity as % of untreated control. Dashed line indicates theoretical decline in activity.
Fig 3.20. PAC1R (pSS2) activity after exposure to tetradecane saturated buffer.
Cells preexposed to 50ml hexane saturated buffer at 150rpm, washed and biotransformation carried out in 60ml phosphate buffer at 750rpm, 28°C, air sparged at 1-1.33 v/v/min, g/g=gdiol/gcells.
.control; ♦ 0.5hr exp; □ 1hr exp; ■ 2hr exp; ◆ 4hr exp; ♠ 21hr exp.
Fig 3.20.1. PAC1R (pSS2) % activity retained after exposure to tetracane saturated solvent.

% activity as % of untreated control. Dashed line indicates theoretical decline in activity.
3.4.3. Hexane:buffer two-liquid phase system

Two-liquid phase systems allow direct contact of cells with solvent at liquid:liquid interface. Cell:solvent interaction at the interface is thought to bring about a series of disruptive effects distinct from those produced by interaction with dissolved solvent [Bar,(1986)]. Protection to the interface by immobilisation has been shown to increase the stability of the biocatalyst [Hocknull and Lilly,(1990)].

Hexane was used to form the organic phase, with former studies using UV4 exposed to solvent:buffer two-liquid phase systems having shown that up to 60% of activity was lost by cells treated in this manner [Harrop,(1992)].

All cell samples tested showed a complete loss of activity after 2 hours of exposure in the two-liquid phase system (Figs 3.21, 3.22., 3.23.). The amount of cell pellet recovered during harvesting was observed to have decreased after the exposure period, suggesting that cells may have been lost by lysis, or been retained at the liquid:liquid interface.
Fig 3.21. JM107(pSS2) activity after exposure to a hexane: buffer 2
-liquid phase system
Cells preexposed to hexane(30ml):phosphate buffer(30ml) buffer
at 750rpm/28oC/air sparged at 1-1.33v/v/min.
Biotransformations carried out in 60ml phosphate buffer, at
750rpm, 28oC, air sparged at 1-1.33v/v/min, g/g=gdiol/gcells.
□ control; ■ 2hr exp; ◆ 4hr exp.
Fig 3.22. UV4 activity after exposure to a hexane: buffer 2-liquid phase system.
Cells preexposed to hexane(30ml):phosphate buffer(30ml) buffer at 750rpm/28oC/air sparged at 1-1.33v/v/min. Biotransformations carried out in 60ml phosphate buffer, at 750rpm, 28oC, air sparged at 1-1.33v/v/min, g/g=gdial/gcells.
= control; □ 2hr exp; • 4hr exp.
Fig 3.23. PAC1R (pSS2) activity after exposure to a hexane: buffer 2-liquid phase system.
Cells preexposed to hexane (30ml):phosphate buffer (30ml) buffer at 750rpm/28oC/air sparged at 1-1.33v/v/min. Biotransformations carried out in 60ml phosphate buffer, at 750rpm, 28oC, air sparged at 1-1.33v/v/min, g/g=gdiol/gcells.
▪ control; □ 2hr exp; * 4hr exp.
4. GENERAL DISCUSSION

4.1. DIOXYGENASE ACTIVITY LEVELS

4.1.1. Variations in UV4 dioxygenase activity

Initial studies were carried out to look out the effects of the growth medium on the dioxygenase activity produced by UV4. Since nutrient broth is a complex medium, it was decided to see if it contained a component(s) which was detrimental to the production of the active dioxygenase enzyme. It was also possible that since the medium was unbuffered, changes in pH may have reduced the level and/or rate of growth.

Cell growth and activity in nutrient broth was compared with those of cells grown in defined salts medium buffered to pH7 and supplemented with sodium salicylate or sodium succinate at 0.5% w/v. Previous studies in which UV4 growth had been carried out in this medium have shown that it is capable of sustaining its growth and enabling the production of dioxygenase from the cell.

Cells grown in non-repressing media, minimal medium+salicylate or succinate, were used as controls. Succinate supplemented medium enabled growth of UV4 to occur at a rate comparable to that observed when cells were grown in nutrient broth.

No difference was observed, between the levels of activity obtained by cells grown in nutrient broth and those grown in minimal medium+succinate. This may suggest that nutrient broth had no detrimental effects upon the growth of UV4 or dioxygenase activity produced by the cell. Any changes in pH that may have occurred during fermentation were insufficient to produce a significant change in the level of dioxygenase synthesised.

The level of activity was shown to be independent of the phase of growth, suggesting that there was no change in the rate of the enzyme produced during the growth period. All further fermentations were therefore carried out in nutrient broth since it was able to support growth of all the cells, allowing standardisation of growth conditions.
Highest levels of activity were produced during the initial bio-transformations, and were observed to decline to approximately \(5 \times 10^{-3}\) in later reactions during later fermentations. Subculturing of active cells onto nutrient agar/indole plates produced some inactive colonies, unable to convert the indole to indigo. The actual cause(s) of this decrease in activity is unknown, but it is postulated that mutations during fermentations causing reversion of the mutated form of the cell may have occurred. This may have resulted in either reactivation of the toluene dihydrodiol dehydrogenase gene, or the loss of the ability to constitutively express the degradative genes.

4.1.2. JM107 dioxygenase activity variation with plasmids pSS2, pSS3, and pSS4.

The level of naphthalene dioxygenase activity produced by *E.coli* JM107 in STRs, was dependent upon the plasmid used to mediate the expression of the *nahA* gene (plasmid pSS2, pSS3, or pSS4). The level of dioxygenase activity produced by transcription from plasmid pSS2 was 10-13.3 fold greater than that achieved from pSS3 and pSS4.

Isolation of the *nahA* gene fragment from plasmid pQR113 used in the construction of plasmids pSS3 and pSS4, resulted in the truncation of 0.8kbp and 1.8kbp of DNA from the initial coding region of the gene fragments, respectively. The genes encoding the subunits of *nahA* are arranged so that the first subunit synthesised is the ferridoxin\(_{(nap)}\) reductase [Yen and Gunsalus, (1982)], and excision of these DNA regions may have resulted in loss of this subunit of naphthalene dioxygenase. There is no absolute requirement for this subunit and it has been shown that its function can be substituted by analogous proteins [Haigler and Gibson, (1990)]. It is possible therefore that an *E.coli* reductase protein may act as a substitute, enabling naphthalene dioxygenase to function in JM107, at a rate lower than if ferredoxin\(_{(nap)}\) reductase had been present.

Alternatively, the *nahA* promoter region may have been removed during construction of plasmids pSS3 and pSS4, but retained in pSS2. Sequencing of the promoter region of the *nah* operon has
identified regions of homology to the *E.coli* -10 and -35 consensus sequences [Yen and Serdar,(1988); Schell,(1986)]. Transcription in pSS2, therefore, may be occurring from both the Ptac promoter present in the vector, and the nahA promoter, enhancing the level of transcription achieved.

DNA sequencing of the 5' region of the nahA fragment may reveal the actual reason for the differences in activity observed.

Plasmid pSS2 was stable in its *E.coli* host, enabling dioxygenase activity to occur at between $0.4 \times 10^{-2}$ to $0.8 \times 10^{-2}$ g/g/h, with an observed maximum of $1.1 \times 10^{-2}$ g/g/h.

4.1.3. Dioxygenase activity inhibition of JM107(pSS2)

At levels of product approaching $4.5 \times 10^{-2}$ g/L the rate of formation of naphthalene dihydrodiol was observed to decline (Figs 3.4., 3.19.). This may be due to product inhibition and/or depletion of glucose required for co-factor recycling. Increasing amounts of glucose supplied may alleviate the problem of glucose depletion, whilst, biotransformation in a two-liquid phase system may overcome inhibition due to build up of product.

4.1.4. Dioxygenase activities of UV4 and JM107(pSS2) using naphthalene in ethanol

Naphthalene was presented to the cells dissolved in ethanol, from a 500mM stock solution. Cells preexposed to ethanol at the same level as that present during biotransformation, were run alongside cells that had been pretreated in phosphate buffer, to determine the influence of ethanol on the cells. The activities obtained were compared with the activities produced by cells that had not been challenged with ethanol either during pretreatment, or during biotransformation.

**UV4**

Naphthalene dissolved in ethanol was degraded by the dioxygenase produced by UV4, except when cells had been preexposed to 10%v/v ethanol. The activities obtained when naphthalene in ethanol was used, were consistently higher than if naphthalene
was presented to cells in the absence of ethanol, with levels approaching $1.8 \times 10^{-2} \text{g/g/h}$ when 12.5 mM naphthalene/ethanol was added.

The level of activity was dependent upon: i) the concentration of naphthalene/ethanol used, with optimal concentrations of 12.5 mM or less; ii) whether cells had been preexposed to ethanol (for samples preexposed to 5%-10% v/v ethanol, only). Cells presented with naphthalene at 12.5 mM, or less, were unaffected by preexposure to ethanol.

**JM107(pSS2)**

Presentation of naphthalene in ethanol to JM107(pSS2) resulted in a decrease in the level naphthalene dioxygenase observed. No dioxygenase activity was observed in cells which had been preexposed to ethanol, and the level of activity declined with increased concentrations of naphthalene/ethanol supplied to the unexposed cell samples.

The control sample (no ethanol challenge) retained dioxygenase activity at a significantly greater level than cells utilising the naphthalene when ethanol was present.

The differences in the activities observed in UV4 and JM107(pSS2), may reflect either differences in the structure of the cells, particularly the outer membrane, or differences in the way in which the cells take up the substrate.

Addition of dissolved naphthalene to the reaction mix resulted in the formation of very fine particles of naphthalene, as it came out of solution. The resulting greater surface area of substrate presented to the cells, may allow more efficient uptake of the naphthalene in UV4, resulting in the increased activity observed. Hsieh and Wang (1980) identified an optimum size of naphthalene particles of 0.4-0.8 mm, which produced maximal activity from their *Pseudomonas* strain. Deviations from this optimum size resulted in a reduction of the levels of activity, suggests that specific cell:substrate interactions may be required for the uptake of substrate. For *E. coli* JM107(pSS2), increasing the substrate surface area may have resulted in the reduced activities observed. However, if cell:substrate interactions were the only contributory factor in the increased activity observed in UV4, an equivalent
activity would be expected when cells were presented to naphthalene at either 4.2mM or 8.4mM, since the surface area of naphthalene would be expected to be similar in both these samples. But the decline in activity observed associated with an increase in the substrate level, and the absence of activity in cells preexposed to ethanol, suggests that the presence of the ethanol may have a destabilising effect upon the cells.

The LPS of the outer membrane exists in a highly organised state, with strong ionic interactions between neighbouring units [Labischinski et al., (1985); Nikaido and Vaara, (1985); Kato, (1985; 1990)]. This degree of organisation extends to the lipid A region of the molecule, producing a highly ordered hexagonal lattice network of fatty acyl chains, which is thought to contribute to the hydrophobic barrier of the outer membrane [Kato, (1985)].

Disruption of this regular structure may be expected to increase the permeability of the membrane. Earlier studies in which unsaturated fatty acid chains were substituted for the saturated units, thus creating a greater degree of disorder, resulted in the cells being more permeable to hydrophobic compounds [Rosen and Hackette, (1972)]. Similarly, intercalation of ethanol into the lipid bilayer of the outer membrane may be expected to disrupt this ordered state, enabling increased flux of naphthalene into the cell, and producing the greater activity observed in UV4. This assumes that the rate limiting step, in this case is the diffusion of substrate into the cell.

However, interactions between the solvent and intracellular proteins would be expected to lead to loss of their activity. This can occur either through direct interaction between proteins and solvent, or indirectly by disruption of the protein environment required to maintain enzyme functions [Costerton et al., (1974)]. Where proteins are involved in cell maintenance functions, for example energy generation and sugar transport, the disruption of the protein-lipid interaction required for activity could lead to cell destabilisation. Also, disruption of the intact cytoplasmic membrane may result in loss of the ability to conduct the electron transfer required for NAD/NADH recycling [Hocknull and Lilly, (1988)]. Loss of activity would be cumulative, as shown by decreased activity in preexposed cells, and lead to eventual cell
death. Exposure of cells to high levels of ethanol (5%-10% v/v for UV4; all levels for JM107) may allow a greater rate of flux of the solvent into the cell, causing a rapid loss of activity.

The greater ethanol tolerance shown by UV4 is not entirely unexpected, since Pseudomonads, in general, are more restrictive to the movement of hydrophobic molecules into the cells. But most of this work has been performed using the pathogen, P. aeruginosa. However, studies of antibiotic susceptibility by a number of non-fermentative, Gram negative, soil bacteria, showed that several Pseudomonas species had an inherently high resistance to hydrophobic antibiotics [Fass and Barnisham, (1980)]. This suggests that P. putida may also have a greater tolerance to many hydrophobic compounds.

However, the low tolerance of JM107(pSS2) to ethanol, even when present at less than 1% v/v, suggests that movement of ethanol may also be occurring through channels other than diffusion through the lipid bilayer. Several β-lactams and quinolones have been shown to enter both E. coli and Pseudomonas cells through specific porins (as well as by diffusion through the membrane) [Chapman and Georgopapadakou, (1988); Michea-Hazehpour et al., (1991); Hooper et al., (1989); McCaffrey et al., (1992)], and that the rate of movement is dependent upon their hydrophobicity [Nikaido and Rosenberg, (1983)]. In Pseudomonas, it is postulated that a smaller fraction of these porins are open at any given time, and/or the exclusion limit is greater compared with those found in enterobacteria [Angus et al., (1982); Yoshimura and Nikaido, (1982)]. The small size and hydrophilic nature of ethanol may allow its rapid influx via such channels, in JM107, but to a much lesser extent in UV4.

In order to look at the influence of naphthalene particle size upon the rate of reaction, the cells could be presented with naphthalene ground down to predetermined sizes, as measured by fine sieving, in single aqueous phase STR systems. If particle size were a contributory factor in the activity level observed, activity would be expected to increase until an optimum size had been reached. This would separate the effect of particle size from that of ethanol. The effect of ethanol on the cells may be determined by altering either the naphthalene concentration or the ethanol concentration,
during biotransformation. Large naphthalene particles would be used whilst the concentration of ethanol would be increased to determine the effect of the solvent upon the cells.

4.1.5. Dioxygenase activity comparisons of PAC1R(pSS2) and PAC610(pSS2).

Naphthalene dioxygenase could be expressed from pSS2 in both *Pseudomonas aeruginosa* strains, as observed by conversion of indole to indigo, and naphthalene to its diol. Both cells retained this property throughout several subculturings, indicating that the plasmid remained stable in these strains. Both PAC1R(pSS2) and PAC610(pSS2) carried out the conversion of indole to indoxyxl at the same rate, over the 25min reaction period, suggesting that both cells were likely to be able to express the *nahA* gene to similar levels. However, during biotransformations in STRs, using naphthalene as the substrate, the dioxygenase activity produced by PAC610(pSS2) was observed to be more than 4 times higher than that of PAC1R(pSS2). Earlier, it had been proposed that transcription of *nahA* in both strains was likely to occur at a similar rate, in order to explain similar rates in conversion of indole. If this were the case, then the different rates of activity produced in biotransformation of naphthalene may reflect differences in their ability to take up the new substrate. It is proposed that diffusion of the large hydrophobic naphthalene molecules through the outer membrane bilayer of PAC1R may be the rate limiting factor in this reaction. The highly structured nature of the LPS, producing a network of LPS molecules with a very low degree of fluidity, may severely hinder the movement of the substrate. The organisation of the lipopolysaccharides is partly maintained by ionic bridges formed within and between the charged saccharide units of neighbouring molecules [Nikaido and Vaara,(1985)]. Limited disruption of the outer membrane structure by solvent would therefore be expected to allow a greater movement of substrate into the cell, producing an increase in activity. The presence of ethanol was shown to result in such an increase during the conversion of indole to a rate equivalent to
that produced by PAC610(pSS2). Similarly, PAC1R(pSS2) exposed to dissolved levels of tetradecane and hexane also resulted in a greater activity than cells not treated to these solvents, suggesting that partial disruption of the outer membrane had indeed enabled cells to take up the substrate at a greater rate.

The composition of the outer saccharide chain of a _P. aeruginosa_ PAO1 serotype 05 and 06 were observed to consist of a number of negatively charged sugar residues, each able to form ionic interactions with similarly charged sugars on neighbouring LPS molecules [Lam et al.(1982)]. Absence of outer saccharide chains in PAC610, may therefore be expected to produce a less effective hydrophobic barrier to the diffusion of naphthalene into the cell, resulting in the increased dioxygenase activity observed. In addition, rough mutants defective in their LPS appear to produce fewer outer membrane proteins, and have a significantly larger phospholipid content, possibly extending into the outer leaflet of the outer membrane, which normally has little or no phospholipid, forming extensive phospholipid bilayer domains [Smit et al.(1975)]. These changes may increase the overall fluidity of the outer membrane in a number of ways:

i) LPS-protein interactions contribute to the stability of the membrane by maintaining the overall structured network of the LPS [Nikaido and Vaara,(1985)];

ii) phospholipid-phospholipid arrays are much more fluid than the equivalent LPS-LPS conformations, with LPS lateral diffusion rates calculated at 10,000X lower than the movement of phospholipids [Mulhradt et al.,(1974)];

iii) hydrophobic molecules preferentially diffuse through phospholipid bilayers (Nikaido et al.,(1977)).

During the conversion of indole, partial permeabilisation of the outer membrane of PAC1R by ethanol used to dissolve the substrate, is thought to have enabled the increased uptake of indole by PAC1R, in a manner similar to that postulated for UV4 (see 4.1.4.). Increasing the time of reaction and/or the level of
ethanol used, would be expected to produce a decrease in the activity as the solvent destabilises the cell.

4.2. SOLVENT EXPOSURE STUDIES

4.2.1. Factors influencing cell disruption

The actual mechanism(s) by which solvents destabilise cells is unclear, with a variety of effects being observed. These range from disruption of co-factor recycling [Hocknull and Lilly, (1988)] to extensive release of intracellular components [DeSmet et al. (1978)].

The action of the solvents studied here show some similarities in their effects upon cell activity, despite the marked differences in the hydrophobicities of these solvents. *E. coli* JM107(pSS2) and *P. putida* UV4 showed the most similarity in their patterns of loss of activity, with initial exposure producing a small but rapid decline in activity in UV4 and JM107(pSS2) treated for up to 4 hours in hexane and tetradecane saturated buffers. This was followed by a slower rate of loss of activity in cells exposed for a greater period of time. Similar patterns of activity loss were observed in *A. simplex* exposed to a variety of solvents [Hocknull, (1989)], and this may represent a general pattern of activity loss amongst whole cell biocatalysts. *P. aeruginosa* PAC1R(pSS2) displayed a unique pattern of activity retention, which will be discussed later.

The changes in the naphthalene dioxygenase activities were observed to be dependent upon four main factors:

i) solvent presentation;

ii) solvent hydrophobicity;

iii) time of exposure;

iv) cell structure.

**Solvent presentation**
The severity of the action of solvents appears to be affected by the way in which the solvent is presented to the cell. Disruption due to dissolved levels of solvent, and that produced by exposure to the liquid:liquid interface, show marked differences [Bar,
The extent of solvent disruption in two liquid phase systems can be limited by immobilisation of the biocatalyst, thereby effectively reducing interfacial contact [Hocknull and Lilly, (1990)].

Cells exposed to dissolved levels of hexane retained activity, even after 21 hours of treatment (Figs. 3.13.1, 3.16.1, and 3.19.1). However, when an hexane interface was introduced in two-liquid phase systems, complete inactivation of all cell samples was observed (Figs 3.21., 3.22., and 3.23.).

A loss in the amount of cells recovered after exposure in the two-liquid phase system was noted, suggesting that cell lysis may have occurred, or cells been retained in the liquid:liquid interface formed during the harvesting process. Therefore, not all of the cells may have been recovered after the washing process, producing a lower activity than might have been possible. Sample measurements carried out during biocatalysis in the two liquid phase systems may give a truer representation of the loss of activity effected by interaction at the liquid:liquid interface.

**Solvent hydrophobicity**

Laane et al (1985) and Brink and Tramper (1985) both demonstrated the relationship between solvent water miscibility and activity retention of the biocatalyst. Although the parameters used to calculate the hydrophobic values of solvents by the two groups differed, both studies indicated that the higher the degree of water miscibility, the greater the loss in activity observed. This predicted pattern of cell inactivation was seen for the solvents used in this study. The most hydrophilic solvent used in the saturated buffer exposure studies, pentanol (logP=1.33) caused total loss of activity in all cells, after just 30 minutes of exposure in the saturated buffer. Cells treated in hexane and tetradecane saturated buffers retained activity, although the extent of activity loss and the rate of loss of activity appeared to be dependent upon the solvent hydrophobicity; cells exposed to hexane saturated buffer showed a greater final decrease in activity compared with cells exposed to tetradecane saturated buffer (Fig 4.1. and Fig 4.2.).
Solvent hydrophobicity may be associated with the solvent concentration present in the buffer, with more hydrophilic solvents expected to dissolve to a greater final concentration. This would result in cells being challenged with a higher level of solvent which may diffuse through the membrane, causing a greater rate of cell inactivation.

The loss of pigmentation in UV4 and PAC1R(pSS2), and the formation of a cloudy emulsion upon harvesting by all cells after exposure to pentanol saturated buffer, indicates that outer membrane disruption is likely to have occurred, solubilising lipids and releasing the periplasmic contents; similar losses of cytochrome C were observed when the outer membranes of cells were disrupted [Garrad,(1972)]. For this to occur, severe damage to the outer membrane must be effected. Electron microscopy studies of *P.oleovorans* exposed to 10% to 80%v/v solvents showed loss of lipids from both the cytoplasmic membrane and the outer membrane, with the formation of protein free vesicles thought to be composed of extracted lipopolysaccharides and phospholipids [Witholt et al.(1990)]. In order to explain these observations, Witholt proposed two mechanisms, in which cells coming into contact with solvent molecules partitioned their lipids around a core of solvent, forming the vesicles seen. Most damage was observed to occur in the cytoplasmic membrane, suggesting that internalisation of the solvent enabled disruption of the membrane.

**Time of exposure**

Studies into the action of solvent damage on biocatalysts have looked at reactions over a limited time span. Whilst this gives an indication of the activity retention over that specific reaction period, long term effects of solvent disruption on the biocatalyst are not considered. Microbial biocatalysts are likely to be subject to non-specific, cumulative solvent damage, affecting a number of different cellular functions. Therefore, increasing exposure to solvents is likely to produce a continuous loss of activity. The decrease in activity was observed as a two stage process in UV4 and JM107; an initial rapid loss of activity over the first four
hours, followed by a slower rate of decrease over the remaining period of exposure.

**Cell structure**

The role of the outer membrane structure in protecting cells against solvent disruption is seen by the greater levels of activity retained by *P. putida* UV4 compared with *A. simplex* exposed to a range of solvents [Harrop et al. (1992)]. Amongst the Gram-negative organisms, compositional variations in the outer membrane are known to result in differences in the ability to take up a number of hydrophobic compounds. *P. aeruginosa* displays a high tolerance to many hydrophobic antibiotics, this property being associated with the greater exclusion limits of its porins to these compounds, or the greater number of porins found in the closed state. However, uptake of many hydrophobic antibiotics, such as quinolones, has been shown to occur either through the porins, or by diffusion through the lipid bilayer [Chapman and Georgopapadakou, (1988); Michea-Hazehpour et al. (1991); Hooper et al. (1989); McCaffrey et al. (1992)].

The comparative activity retentions of the cells (Figs 4.1, 4.2), shows differences in the solvent tolerances between these organisms. UV4 and JM107 show similarities in their patterns of activity retention in the solvents tested, but differ in the rate of activity loss (after the initial 4 hour period of exposure) and the final activity retained at the end of the 21 hour exposure period. JM107 appears to be more susceptible to the effects of solvents than UV4; exposure of UV4 in hexane saturated buffer resulted in an almost 55% final loss of activity (Fig 3.16.1.), whilst activity of JM107(pSS2) declined to almost 78% of the control sample (Fig 3.13.1.). Similarly, exposure to tetradecane saturated buffer produced a decrease of approximately 13.4% for UV4 (Fig 3.17.1.) compared with a 67.76% decrease for JM107(pSS2) (Fig 3.14.1.), showing a greater long term solvent tolerance by UV4 compared with JM107, in both hexane and tetradecane, when solvents were presented in a saturated buffer.

JM107(pSS2) and UV4 cells exposed to between 0.87% and 10% v/v ethanol again resulted in very different activity retentions. The presence of ethanol appears to severely disrupt
the activity of JM107(pSS2), as shown by the inability of cells which had been preexposed to ethanol, to convert naphthalene to the diol, and the decrease in activity with the increase in naphthalene solution, in cells that had not been pretreated this way. In contrast, preexposure to 10% ethanol was required to bring about a total loss of activity in UV4. These cells were able to tolerate preexposure up to 2.5%v/v ethanol without any loss of activity.

In all cases, the level of activity was significantly greater than if cells had not undergone any ethanol exposure, either during pretreatment, or during biotransformation. This may be due to the greater surface area of the substrate presented to the cell, the partial permeabilisation of the cell wall allowing a greater rate of uptake of the substrate, or a combination of these effects.

The greater rate of activity loss observed in the E.coli strain was suggested earlier to be due to the greater accessibility of the solvent into the cell. This may have occurred both through movement via the porin channels, and diffusion through the outer membrane.

Significant increases in activity were observed for P.aeruginosa PAC1R exposed for up to 4 hours in both hexane and tetradecane saturated buffers (Figs 3.19.1., 3.20.1.). This is thought to be due to disruption of the highly ordered LPS array of the outer membrane by solvent, enabling an increased rate of diffusion of substrate into the cell. Cells treated for 4 hours in tetradecane saturated buffer showed over 251% increase in activity compared with the control sample. An equivalent exposure time in hexane saturated buffer only produced an increase in the activity level of just under 157% of the control. The lower rate of increase of activity produced upon exposure to hexane saturated buffer, compared with the tetradecane saturated buffer, may reflect the greater damaging potential of this solvent. Hexane is thought to effect a greater disorganisation of the outer membrane, allowing internalisation of the solvent to occur more readily, where it may destabilise cellular functions.

Prolonged exposure (up to 21 hours) results in a decrease in activity, as continued solvent damage to cellular functions destabilises the cell.
Fig 4.1. Activity retention after exposure to hexane saturated buffer
Cumulative graph from Figs 3.11.1, 3.14.1, and 3.17.1. % activity as %
of untreated control. Dashed line indicates theoretical decline in
activity. Cells preexposed to 50ml hexane saturated buffer
at 150rpm. Biotransformation carried out in single aqueous phase
60ml phosphate buffer at 750rpm, 28oC, air sparged at
1.1.33\text{v/v/min}.

- UV4; \# JM107(pSS2); \Delta PAC1R(pSS2)
Fig 4.2. Activity retention after exposure to tetradecane saturated buffer
Cumulative graph from Figs 3.12.1,3.15.1, and 3.18.1. %activity as % of untreated control. Dashed line indicates theoretical decline in activity. Cells preexposed to 50ml hexane saturated buffer at 150rpm. Biotransformation carried out in single aqueous phase 60ml phosphate buffer at 750rpm, 28oC, air sparged at 1-1.33v/v/min.
● UV4; ■ JM107(pSS2); △ PAC1R(pSS2)
It is postulated that the structure of the outer membrane of PAC1R is thought to produce a very effective hydrophobic barrier, as observed by the lower rate of naphthalene conversion, compared with strain PAC610(pSS2) (see section 4.1.5.).

Tetradecane and hexane would be expected to be similarly hindered in their ability to traverse this lipid bilayer. However, some diffusion of these solvents is still likely to occur, with integration into the lipid bilayer, resulting in the disorganisation of this highly organised structure. Because the membrane appears to be more resistant to hydrophobic compounds compared with the outer membranes of both *E.coli* JM107 and *P.putida* UV4, it is predicted that only limited movement through the membrane would occur. This is thought to result in the increased activity observed when the membrane is permeabilised, allowing a more rapid entry of naphthalene into the cell.

4.2.2. Cell structure comparisons

Much of the work into the structure of the LPS of Pseudomonads and Enterobacteria have looked at various strains of *P.aeruginosa*, *E.coli*, and *Salmonella*. Although work has been carried out on the LPS structures of PAC1R [Chester and Meadow,(1975); Koval and Meadow,(1977); Rowe and Meadow,(1983)], no work has been performed on JM107 or UV4. However, the similarities in the LPS units displayed by the Pseudomonads and the Enterobacteria were thought to be sufficiently similar to allow inferences on the structures of the LPS units of the strains used in this study.

In general, *Pseudomonas* display a greater tolerance to many hydrophobic compounds than the enterobacteria. A survey of a number of soil pseudomonads, identified an intrinsically high resistance to several hydrophobic and hydrophilic antibiotics [Fass and Barnisham,(1980)]. We may therefore assume that *P.putida* UV4 would also be likely to produce a more effective hydrophobic barrier than *E.coli*. 

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The influence of the LPS

The greater hydrophobic tolerance exhibited by Gram negative organisms compared with Gram positive organisms [Harrop et al. (1989; 1992)] is thought to result from the presence of the outer membrane. Several features of the LPS units suggest that they play a key role in forming the hydrophobic barrier function of this membrane [Nikaido and Vaara, (1985)]. These include:

i) exclusive localisation of the LPS to the outer membrane;

ii) the outer leaflet of the outer membrane being composed mainly/entirely of LPS, in cells not defective in their LPS;

iii) the highly ordered structure observed in isolated LPS molecules, which is thought to contribute to the restrictive movement of hydrophobic molecules;

iv) the very stable nature of the LPS-LPS interactions;

v) preferential diffusion of hydrophobic probes through phospholipid domains.

The role of LipidA

LipidA is unique to the LPS and differs significantly from the phospholipids of the cytoplasmic membrane. A strong homology exists amongst the lipidA components of Gram negative organisms [Rivera et al. (1988)], with a common β-(1,6)-linked glucosamine disaccharide ‘backbone’ unit to which 6-7 fatty acid residues are bound. These saturated hydrocarbon chains form regular 3D-hexagonal arrays which are thought to contribute to the hydrophobic barrier of the membrane [Labischinski et al. (1985); Kato et al. (1990)]. The specific function of these chains is supported by the observation that the composition of these fatty acids remains constant even at different growth temperatures [Nikaido et al. (1977)]. Furthermore, cells defective in the fatty acid composition of their lipidA, in which unsaturated fatty acids were substituted for the saturated chain, were seen to be less able to retard the movement of hydrophobic compounds [Rosen and Hackette, (1972)].
The fatty acid composition of lipidA from *Pseudomonas* species appears to differ from those of enterobacterial cells in terms of the number of carbon residues present. In enterobacteria 12, 14 and some 16 carbon chains were observed, in contrast to the 10, 12, and 16 carbon chains found in *P. aeruginosa* [Kulshin et al. (1991)].

Similarities in the lipidA exist within a species, with the main variations occurring in the quantity and composition of the fatty acid residues [Kulshin et al. (1991)].

The extent to which these structural differences influence the ability of hydrophobic compounds to traverse the membrane, is unclear, although different rates of diffusion may be expected.

**The role of the polysaccharides**

The highly organised state of the LPS units is thought to arise through interactions between neighbouring LPS units, and between LPS and proteins. The presence of phosphate groups and anionic sugar residues are able to set up ionic interactions with similarly charged groups in adjacent LPS molecules, mediated by divalent cations [Hancock, (1984); Nikaido and Vaara, (1985)]. Addition of chelating agents such as EDTA, increase the permeability of the outer membrane by removing these cations, so reducing the interactions which can be formed [Nikaido and Vaara, (1985)].

A large number of anionic groups exist in the core region of the polysaccharide, with a number of these sugar residues common to both enterobacterial cells and pseudomonads. Ionic interactions mediated by these core saccharides alone would therefore be insufficient to explain the differences in the solvent tolerance observed between PAC1R, UV4 and JM107. Observed variations in the molar ratios of these sugar residues, may partly explain some of the differences in the activity retentions shown.

The outer saccharide chain isolated from a *Salmonella* species was shown to be made up predominantly of neutral sugars, and thought to contribute little to the structuring of the LPS [Kastowsky et al., (1991)]. In contrast, the outer saccharide chains isolated from various *P. aeruginosa* strains identified the presence of several anionic groups, each capable of forming such ionic
interactions. Combined with the greater length of the O-side chain (>30nm) produced by the *Pseudomonas* cells, it would be expected that the resultant hydrophobic barrier produced by *Pseudomonas* would be greater than that produced by the enterobacteria [Lam et al.(1992)].

However, the extreme solvent tolerance exhibited by PAC1R, is not easy to consolidate by any one of the mechanisms mentioned. Formation of an effective hydrophobic barrier is likely to be due to a combination of factors, including the extent of LPS-LPS interactions, the nature of the lipidA, the presence of charged residues in the O-side chain, the presence of membrane stabilising proteins, and the ability of porins to exclude hydrophobic molecules, amongst others. No definitive mechanism was tested during these studies. However, the comparative activities produced by PAC1R(pSS2) and PAC610(pSS2) using naphthalene suggest that the interactions set up amongst the O-side chain residues within and between neighbouring LPS molecules significantly contribute to the ordered structure of the LPS. In addition, the presence of extensive sugar residues on the O-side chain may create a highly hydrated surface around the cell surface, preventing the incursion of the hydrophobic solvent molecules. The contribution of any one single component of the LPS to the barrier property of the outer membrane cannot be identified positively. The structure of the membrane is determined by a number of interactions, within the LPS, between LPS and between LPS and proteins. The difference in the activity demonstrated by PAC1R(pSS2) and PAC610(pSS2) in utilising naphthalene suggests that the O-antigenic side chains may play a significant part in this role. However, the possible effect of an increased phospholipid content, in the outer leaflet of the outer membrane, must be taken into account, in the LPS mutant PAC610. Differences in the O-side chain compositions of JM107 and UV4 may contribute to some of the differences observed between the solvent tolerances of these organisms. Further studies looking at the compositions of the O-side chains and correlating them with solvent diffusion would need to be carried in order to determine any relationship with solvent tolerance.
4.3. PROPOSED MECHANISM SOLVENT ACTION UPON WHOLE CELLS

Although many studies looking at the disruptive effects of solvents upon biocatalysts have noted the resultant damage caused to cells, elucidation of a mode of action by solvents has been hampered by the variety of observed effects [Bar,(1986); Lilly,(1982)]. The distinction between disruptive effects due to dissolved solvent and that occurring at the liquid:liquid interface must be made if any pattern of solvent damage can begin to be distinguished. From the studies on the solvent tolerance shown by *P.putida* UV4, *E.coli* JM107 and *P.aeruginosa* PAC1R, the following mode of solvent disruption has been proposed (Fig 4.3.): solvent dissolves into the outer membrane at a rate determined by the hydrophobicity and concentration of the solvent, and cell surface structure. Accumulation of solvent is likely to occur in the interior of the membrane where the non-polar medium of the lipid molecules allows stable maintenance of the hydrophobic molecules. Studies into the action of anaesthetics suggests that they act at intramembrane locations, either within the lipid bilayer, itself, the protein-lipid interfaces, or the hydrophobic regions of integral membrane proteins [Schneider,(1968)]. The subsequent increase in membrane volume was observed to be significantly greater than could occupied by the solvent [Seeman,(1972)]. The increase in membrane volume was suggested to occur due to disorganisation of the lipid structure by the solvent. Decreased hydrophobic interactions between lipids would lower the van der Waals interactions between acyl chains and hydrophobic protein surfaces. As a result, the space occupied by each lipid molecule would increase, reducing the permeability barrier of the membrane.

Poorly water miscible solvents would be retained within the outer membrane bilayer. In contrast, more hydrophilic molecules are likely to transfer into the periplasm where they may directly interact with many of the periplasmic proteins, causing their inactivation. Further diffusion into the cytoplasmic membrane is likely to cause disruption of many membrane associated functions. The close association between membrane proteins and lipid
fluidity has been described [Laurerio-Dias and Peinado,(1982); Leao and van Unden,(1982); Grisham and Barnett,(1973); Gordon et al.(1980)], and it is the disruption of these functions which has been directly associated with loss of activity in biocatalysts [Hocknull,(1990)].

If cells are exposed to high concentrations of solvent, membrane components may be completely removed from the cell. The cloudy supernatant formed after cells are exposed to pentanol saturated buffer may constitute an emulsion of such membrane components dissolved in solvent. Witholt et al (1990), observed loss of both outer membrane and cytoplasmic membrane components, and the formation of protein free vesicles, after exposure to solvent. Models of interaction of solvents with cells, in which outer membrane components are partitioned into the apolar solvent forming outer membrane vesicles were proposed in this study, to account for the results observed, and may be used to explain the loss of activity upon exposure to pentanol saturated buffer and hexane:buffer two liquid phase systems.

4.4. IMPLICATIONS FOR INDUSTRY

Observations relating solvent tolerance in terms of solvent hydrophobicity and presentation of the organic phase, have been made previously, and used as guidelines to develop biotransformation schemes. Further considerations, now appear to be required, to take into account:

i) the progressive denaturation of cells in solvents, in a bimodal manner, (initial rapid loss of activity, followed by a continued loss of activity at a slower rate);

ii) the nature of the cell surface structure.

The level of solvent tolerance afforded by the Pseudomonas species, appeared to be greater than that shown by E.coli JM107. This was particularly so in the presence of ethanol, where even levels of <1%v/v were thought to contribute to the significant loss of activity.
outer saccharides core region lipidA

Phospolipid

periplasm (+enzymes)

peptidoglycan

1) Cells retain highly ordered structure in absence of solvent

2) Diffusion of solvent into the outer membrane results in disruption of the membrane structure

3) Further diffusion of solvent into periplasm and the inner membrane allows direct and indirect interaction with cellular proteins, destabilising the cell.

Fig 4.3. Proposed mechanism of solvent action upon the cell.
In general, *Pseudomonas* have a relatively high intrinsic resistance to a number of hydrophobic compounds. However, the patterns of solvent tolerance afforded by strain PAC1R, were quite remarkable. Although this was thought to contribute to the low level of naphthalene uptake, partial permeabilisation by dissolved levels of solvent were observed to increase the activity by over 2.5fold. This may imply that limited permeabilisation of PAC1R in a two-liquid phase system with suitable solvents, may actually increase the amount of product which can be obtained. Biotransformations may then be carried out over a period of time, as defined by a predetermined level of activity retention which the cells are required to maintain. Further work would need to be carried out in two-liquid phase systems, in order to make this a viable option.

For large scale biotransformations, the use of *P.aeruginosa* is effectively preclude by its pathogenicity. *P.putida* or other soil *Pseudomonas* species are more suitable, in this respect. The isolation of highly toluene tolerant *P.putida* strains, unable to utilise toluene as a carbon source, may prove to be suitable organisms, for this purpose [Inoue and Horikoshi,(1989); Inoue et al.(1991)]. Incorporation of plasmids encoding the required enzymes, such as pSS2, may enable such strains to mimic the solvent tolerance shown by PAC1R (the isolation of a toluene tolerant *E.coli* strain, by the same group, implies that these cells may produce a higher tolerance to a number of solvents than the JM107 strain used in these studies [Aono et al.(1991;1992)]. High level expression vectors utilising strong promoters, such as Plac, Plac and the λPL and λPR, have been constructed for expression in a variety of Gram negative hosts, and shown to produce activity levels in *Pseudomonas* equivalent to or greater than that produced in *E.coli*. [Bagdasarian et al.(1981;1983); Rangwala et al.(1991)]

Overall therefore, from these studies, it would appear that *Pseudomonas* may provide a more efficient biocatalytic system than *E.coli*, for use in the presence of solvents.
5. CONCLUSIONS

1) Recombinant DNA technology can be used to enable synthesis of active naphthalene dioxygenase isolated from a *P. putida* strain, in both *E. coli* JM107 and *P. aeruginosa* strains PAC1R and PAC610.

2) A difference in the rate of biotransformation of naphthalene between PAC1R(pSS2) and PAC610(pSS2) is possibly due to an inability to take up the substrate, by PAC1R. Partial permeabilisation of the outer membrane may enable uptake to occur more readily.

3) The rate of biotransformation of naphthalene by UV4, can be increased by presenting cells with the naphthalene predissolved in ethanol.

4) The solvent exposure systems established in this work are able to define patterns in the loss of activity resulting from the interactions of cells with dissolved levels of solvent.

5) Solvent disruption of a cell is dependent upon:
   i) Solvent hydrophobicity: solvents of low hydrophobicity bring about a greater rate of loss of activity compared with more hydrophobic solvents.
   ii) Solvent presentation: cells exposed to solvent at the liquid:liquid interface are more readily inactivated than if solvent is presented dissolved in the aqueous phase.
   iii) Time of exposure: rapid loss of activity occurs over the initial two to four hours, followed by a continued decline in activity level at a lower rate.
   iv) Cell surface structure: the rate of loss of activity of cells exposed to dissolved levels of hexane and tetradecane is dependent upon the cell surface structure, and is directly influenced by the nature of the outer membrane.

6) i) The solvent tolerance of *P. aeruginosa* PAC1R>P. putida UV4>E. coli JM107; ii) the solvent tolerance of PAC1R>PAC610.
6. FUTURE WORK

1) Earlier work studying the effect of the outer membrane in protecting the cell against solvents, compared steroid $\Delta^1$-dehydrogenation by *A. simplex* with naphthalene hydroxylation by *P. putida* UV4. Although this provides some information about the influence of the cell wall structure upon stability, it does not take into consideration the different growth, harvesting, and biotransformation conditions utilised in the two studies. Although the actual extent to which these may have influenced the final stability of the cells is unknown, the environmental conditions in which cells are grown, are known to influence the compositions of lipids, LPS and the peptidoglycan. Comparison of the same reaction carried out in both Gram negative and Gram positive cells, cultivated under standardised conditions, may indicate more clearly, the influence of the cell wall in providing protection against hydrophobic compounds. The *nahA* gene isolated in this work, may be used for this purpose by incorporating it into a number of plasmid vectors able to express in Gram positive cells [see Gruss and Ehrlich,(1989) for review]. Alternatively, broad host range vectors able to replicate in both Gram positive and Gram negative cells have been constructed [Trieu-Cuot et al.,(1990); Trieu-Cuot et al. (1991)], and could be employed to express *nahA* in both cell types, although differences in the level of expression in the two cell types may preclude this strategy.

2) Comparison of the solvent stability of different organisms, has demonstrated that amongst Gram negative cells, variations in the composition of the outer membranes, may lead to significant differences in the solvent tolerance of the cell. Only a limited number of organisms were studied here, and comparison of a greater variety of cells may reveal differences in solvent tolerances within and between species. Plasmid pSS2, may be used for this purpose, as it is able to replicate in a variety of host cells. The nature of the cell is an important determinant in the overall reaction design process, since neither fermentation biotrans-
formation, nor downstream processing can be considered individually [Lilly,(1982)]. It may be possible to tailor specific cell-solvent combinations for a given reaction, using either broad-host range plasmids, or cassettes incorporating the gene of interest, which can be inserted into a specific plasmid.

3) The lipopolysaccharide moiety of the outer membrane has been implicated as the main factor in providing a barrier against hydrophobic compounds. Studies into the extent to which each component of this tripartite molecule contributes to the protection of the cell, may be carried out using the family of *P. aeruginosa* cells derived from PAC1R, differing in their outer membrane composition. Limited work using PAC610 and PAC1R, has shown that significant differences may arise due to the presence of the O-side chain. Expression of plasmid pSS2 in these organisms and comparison of their solvent stabilities, may help to elucidate the extent to which each component, or combination of components, affords protection to the cell. However, this must take into consideration differences in the amount of phospholipid and protein which may also result with formation of defective LPS.

4) Environmental conditions are known to influence the nature of cellular components, often producing changes designed to enable cell survival in the given environment. *P. putida* UV4 may have produced the levels of solvent tolerance observed through adaptation to the high hydrocarbon content of its original environment. Adaptation to octane present in the growth medium by a *Pseudomonas oleovorans* strain, was reported to have occurred after 10-15 cell divisions, enabling continued growth in the presence of the solvent [Preusting et al.(1991)]. A *P. putida*, *P. aeruginosa* and an *E.coli* strain resistant to high levels of toluene, but unable to utilise toluene as a carbon source, have been isolated [Inoue and Horikoshi,(1989); Inoue et al. (1991); Aono et al.(1991;1992)]. These may represent an adaptation of the cells to the solvent, with cultivation in the presence of toluene occurring over a one week period. The possibility, therefore, lies in selection of solvent tolerant cells, by
exposing cells to a limited amount of solvent during growth and isolating solvent tolerant strains.

5) The NAD/NADH co-factor recycling step of a co-factor dependent reaction is subject to disruption by solvents, upon diffusion into the cell, and intercalation into the inner membrane, in particular [Hocknull and Lilly,(1988)]. Removal of the dependency upon the membrane to recycle NADH using a coupled reaction to produce the NADH, may enhance the stability of the cell. Formate dehydrogenase conversion of formate has been used successfully in \textit{vitro} to maintain the conversion of ketones to lactones, using such a coupled enzyme system [Grogan et al.,(1992)]. Expression of formate dehydrogenase genes in \textit{vivo} together with \textit{nah}A, may increase the cell stability in solvents.
7. APPENDIX

Aldrich, Gillingham, Dorset, U.K.

BDH, Poole, Dorset, U.K.

Beckman, Palo Alto, California, U.S.A.

Cecil Instruments, Cambridge, U.K.

Difco, Detroit, Michigan, U.S.A.

Fisons, Loughborough, Leicestershire, U.K.

Gibco BRL, Paisley, U.K.

HPLC Technology, Macclesfield, Cheshire, U.K.

LDC/Milton Roy, Riviera Beach, Florida, U.S.A.

MSE Scientific Instruments, Crawley, Sussex, U.K.

Northumbrian Biotechnology Ltd, Cramlington, Northumberland, U.K.

Pharmacia, Milton Keynes, U.K.

Sigma, Poole, Dorset, U.K.

Sorvall-DuPont, Newtown, Connecticut, U.S.A.

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