Effect of organic solvents on cell stability

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

by

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Abstract

Many existing and novel approaches to bioprocessing using different types of reaction media have evolved in recent years involving biocatalysis of poorly water soluble compounds eg water-miscible cosolvent aqueous, aqueous-organic biphasic, reversed micellar and super critical fluid media. Commercial exploitation of the appropriate media and its engineering characteristics will require detailed knowledge of the media and its engineering characteristics with regards to biocatalyst stability.

This thesis examines the effect of membrane integrity as measured by the capacitance from cell beta-dispersion. It relates this to methods of determining cell viability, reactor mixing, and the effect of organic solvents with similar hydrophobic characteristics, as measured by the log of partitioning (log P) between octanol and water, which has become the standard approach. The effect of organic solvents on endogenous uptake rate is also considered. These factors are related to the transfer of organic solvent into the cell. The test organisms selected were: two yeast strains, a bacteria, a fungus and an animal cells line.

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CHAPTER 1

CHAPTER 1
INTRODUCTION

1.1 Biotransformation

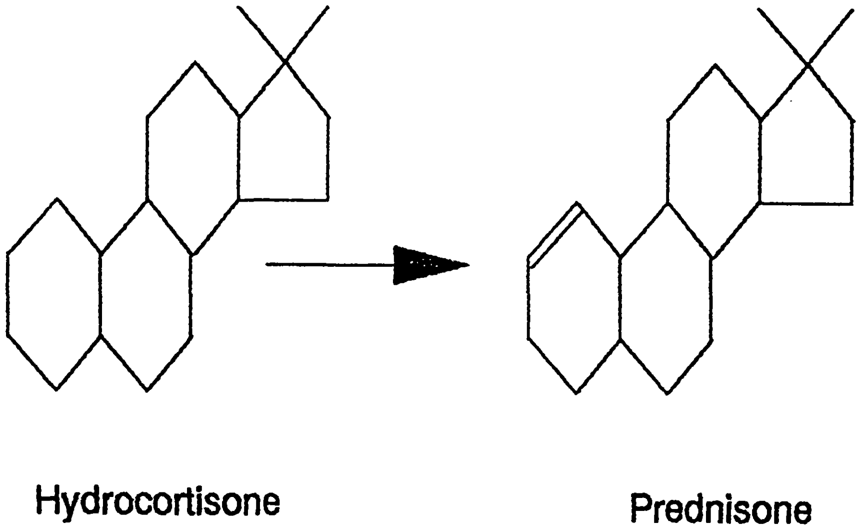
Microorganisms have the versatility to modify a myriad of organic compounds (Kieslich 1984). These properties of biocatalysts are utilised commercially in fermentations and biotransformations.

For centuries certain fermentations have been employed, using multi-stage reaction pathways often with multi-enzyme systems. Biotransformations, in contrast to these processes may be considered as selective, enzymatic modifications of defined pure compounds into a defined, final product. These modifications often involve specific reactions on relatively complex molecules (ie antibiotics). These processes utilise mono-cultures of microorganisms or purified enzymes (Kieslich 1984). The reaction products from cells or cell preparations which are not further metabolised are often detectable in the cell free medium. The microbial transformations cover nearly all chemical reactions (Kieslich 1976) (see table 1.1).

In addition to their usual substrates some enzymes accept 'unnatural compounds' (xenobiotics). Acyloin formation from benzaldehyde and acetaldehyde by Neuberg in 1921 (Kieslich 1984) using yeast was one of the first biotransformations. Today the more commercially significant biotransformations are those involving steroids (eg cortisone to prednisone by *Arthrobacter simplex*) see figure 1.1.

Figure 1.1

Delta-1-dehydrogenation by *Arthrobacter simplex*


Hydrocortisone
Prednisone

(Fukui and Tanaka 1985)

Delta-1-dehydrogenation by *Arthrobacter simplex* showing the high degree of specificity possible from biocatalysts.

TABLE 1.1
Biotransformation properties

Reaction specificity	Usually one specific enzyme is employed, therefore few side products are formed.
Regiospecificity	Substrate molecules are transformed at the same site even if several groups of equivalent or similar reaction sites are present.
Stereospecificity	Enzymes often distinguish between enantiomers of a racemic mixture and can produce an optically active product if the enzyme produces a new asymmetric centre.
Mild reaction conditions	The energy for activation of a chemical reaction is lowered when substrate and enzyme are present. This allows reactions to take place at low temperature, pressure and almost neutral pH, reducing cost, operational problems and allowing the use of substances which are labile to low and high pH values or heat.

Biotransformations, which give high yields are possible (Wang et al 1979) and may be a viable alternative to chemical synthesis (see table 1.2).

TABLE 1.2
Reaction types mediated by microorganisms

Oxidation	Hydroxylation, epoxidation, dehydrogenation of C-C bonds. Oxidation of alcohols and aldehydes, oxidation of alkyl,carboxyalkyl or ketoalkyl chains, oxidative removal of substituents, oxidative deamination, oxidative hetero-functions, oxidative ring fission.
Reduction	Reduction of organic acids, aldehydes ketones and hydrogenation of C=C bonds, reduction of hetero-functions, dehydroxylation, reductive elimination of substituents.
Hydrolysis	Hydrolysis of ester, amines, amides, lactones, ethers, lactams etc. Hydration of C=C bonds and epoxides.
Condensation	O- and N -acylation, glycosylation, esterification, lactonisation, amination.
Isomerisation	Migration of double bonds or oxygen functions, racemisation, rearrangements.

Formation of C-C bonds or hetero-atom bonds.

1.2 Biocatalysts

Biocatalysts can be split into two major groups: Cells and isolated enzymes.

Cells as biocatalysts offer distinct advantages over enzyme systems, since enzymes are often more stable in the 'natural' environment of the cell, where optimal activity may be more easily retained (Tosa 1977). Biotransformations using cells are also able to utilise a wide range of enzymes, each catalysing a specific reaction, often in a complex but well co-ordinated metabolic pathway, with each enzyme specifically acting on a substrate, complete with co-enzyme regeneration system, negating the need for expensive artificial co-enzyme regeneration which would be necessary with an enzyme system.

These biocatalysts can be used in a variety of physiological states, some of which may be difficult to easily separate from enzymic activity (see immobilisation section).

The economics of enzyme purifications, from cell systems, will tend to favour the use of cells as biocatalysts, over purified intra-cellular enzymes (Freeman 1986). Balancing the favourable economics of cell biocatalysts are the problems associated with cells as biocatalysts. These include their relatively complex nature, which gives rise to a number of problems, eg regulation of enzyme synthesis, mutation, removal of cells, cell lysis, release of intracellular materials and permeability of the cell to reactants or co-metabolism.

1.2.1 Micro organisms as biocatalysts

One of the forerunners of biotechnology was Schuetzenbach (Mosbach 1983), who unwittingly used aerobic bacteria as immobilized biocatalysts in his quick vinegar process in 1823. Since Schuetzenbachs' process, it has been recognised that to obtain optimal conditions for a reaction requires careful selection of the organism and optimal reaction conditions.

1.2.1.1 Selection of a biocatalyst

For simplicity, previous workers investigating biotransformations in the organic media have mainly used a single enzyme systems.

Many different processes have evolved for selecting an organism which can catalyse a desired reaction and can tolerate an unusual environment (eg growth in a medium with high solvent content ;Inoue et al 1989). Usually it is necessary to perform a vast number of screens to find a biocatalyst when designing a novel process. The organisms may also be selected from public culture collections.

The empiricism of these methods of selection can be reduced by screening organisms which have already been documented as having the ability to mediate the transformation of structurally related compounds.

Recombinant DNA technology, initially used for expression of foreign proteins is now being recognised for its potential to contribute to the synthesis of other products. In addition to its use for over expression of enzymes, of industrial importance, such as lipases, it has been used to enhance greatly the synthesis of amino acids eg phenylalanine and also novel secondary metabolites such as cephalosporin derivatives. At present, genetic engineering plays only a limited role in physiological adaption of microorganisms, due to a lack of physiological understanding, unlike its diverse role in metabolic pathways.

1.2.2 Biotransformation methods with cell biocatalysts

Two approaches to biotransformation operation can be defined;

1) Using growing cultures.
2) Using 'resting' cells.

An ideal operating system (see figure 1.2) will use 'resting' cells, so that the biotransformation can be optimised independently of the growth phase, so that neither process is compromised, eg Leunberger et al (1987) who used bakers yeast in the biotransformation of carotenoids. However the stability of particular enzymes may be so poor that de novo protein synthesis is needed to maintain the catalytic activity of the cells. If nutrients are added to allow this, then growth may occur eg Birnbaum et al (1983). One solution is to use a cell division cycle (CDC) mutant, which is genetically modified to switch off growth at a specific temperature (Keshavarz et al 1990).

FIGURE 1.2

Ideal process

Optimise growth of biocatalyst → Optimise biotransformation
(Growth process) (Catalytic process)

Timing of substrate addition with respect to microbial growth must also be assessed for maximum yield and induction of any enzymes necessary for the biotransformation.

1.2.3 Biotransformation with enzymes

Enzyme systems may be necessary or preferred to a cell system to carry out a biotransformation:

- If side reactions occur, or if there is product degradation due to other enzyme systems.
- Substrate or product permeation is prevented by cell membrane.
- If enzyme is secreted by the cell and is easily purified or is commercially available.
- Enzyme is from organism which is technically difficult to grow (eg plant or animal tissue) or bacterial and needs to be pyrogen free (eg in pharmaceuticals for injection).

1.3 Two-liquid phase and its rationale

There are many varied biochemical reactions where the reactant(s) and product(s) are poorly water soluble, which are of industrial importance (Lilly 1982, Lilly and Woodley 1985). The exploitation of many of these hydrophobic biological and xenobiotic compounds in biochemical reactions has been hindered by their low solubilities in aqueous reaction media (Carrea 1984). The solubility of these substrates can be raised by the use of surfactants or organic solvents (also see table 1.3).

1.3.1 Water-miscible and water-immiscible organic solvents

Both types of solvent have been used successfully in biotransformations e.g. Widmer (1985), who used a water-miscible solvent in a peptide synthesis and Schwarts and McCoy (1977) who used an immiscible solvent in epoxide production.

Adding a water-miscible organic solvent to the reaction medium in general does not increase the solubility of poorly water-soluble compounds as significantly as a water-immiscible organic solvent (Lilly et al 1987).

1.3.2 Advantages and disadvantages of an organic solvent and a two-liquid-phase system (TLP)

From the biotechnological perspective, there are numerous potential advantages (see table 1.3) to using an organic biphasic medium, as opposed to a purely aqueous medium, when using poorly water soluble compounds.

The use of a second phase may drastically reduce the reaction volume for a given system and increase the product concentration in the reactor, decreasing the down stream processing costs. Where the reactant or product cause inhibition and is able to dissolve in an organic phase, then the addition of a second phase may reduce the inhibitory effect to the biocatalyst by partitioning the inhibitor into the organic phase. An organic solvent may also be used to suppress water-induced hydrolytic reactions in aqueous solutions, using enzymes like lipases, proteases and esterases. These enzymes catalyse hydrolytic reactions, which are of limited commercial interest.

In organic media however, when the water activity is less than one, the enzymes catalyse a variety of synthetic reactions in high yields. These include esterifications, transterifications, interesterifications(acyl exchange), and lactonisation (Zaks and Klibanov 1985).

Other possible advantages to the use of organic solvents may include ease of separation of reactants from the aqueous phase and the biocatalyst.

TABLE 1.3
Possible advantages and disadvantages of organic solvents and a two-liquid phase

1) To raise the starting concentration of substrate in reactor without increasing reaction volume.
2) Reduce reactant /product inhibition/ toxicity to biocatalyst in the aqueous phase.
3) Improving product/biocatalyst removal in continuous or batch operation mode.
4) Reducing contamination of reactor with a competitive organism.
5) Shifting the thermodynamic equilibrium, allowing previously unfavourable reaction to occur(if the water activity is less than one)

A more nebulous advantage, includes the possibility of integration of a biological system into chemical processes.

Disadvantages of two-liquid-phase systems

One of the major disadvantages of using a two-liquid-phase system is the loss of the biocatalyst stability. The destabilising effects are due to the dissolved organic solvent in the aqueous phase and the proposed destabilising effects of the liquid-liquid interface (Bar 1986, Lilly 1986) (further discussed in chapter 2).

1.3.3 Immobilised biocatalysts; cells

Immobilisation involves confining the cell within a matrix, effectively producing a third phase. This may have many benefits in both single aqueous phase reactions and organic solvent two-liquid phase reactor systems.

One of the first examples of intentional immobilization was reported by Hattori and Furwaka (1960). An abundance of systems have been developed since then in an attempt to exploit the properties of immobilisation (Chibata et al 1979, Birnbaum et al 1983, Bucke 1983 and Keshavarz et al 1989).

Often the reusability of a biocatalyst on the industrial scale (Katchalski et al 1982) will determine the economic viability of the process (Chibata et al 1979). This commercial pressure has led to an increase in the immobilisation of biocatalysts. This may have significance for biocatalysts in two-liquid phase for reusability and reversal of the effects of organic solvents (see chapter 3 and 4).

Cell systems can be used in a number of different metabolic and physiological states, eg viable but not growing, growing, metabolically active but unable to grow or metabolically inactive but still catalytically active.

Many of the advantages of immobilisation (see table 1.4) are a direct consequence of the increased size of the biocatalyst, often 1000 times larger than the free calls. This enhances the ease of handling of the biocatalyst, allowing easy recycling and separation of immobilised biocatalyst from the reaction medium, both during the reaction and afterwards, when separation for disposal of the different components of the reaction medium may be important ie reducing the volume of waste, associated with high concentrations of solvent.

The physical confinement of cells may also play a vital role in protecting the catalyst from physical, chemical and microbiological hazards (see section 1.4.3).

The chemical composition of the support matrix can also play a significant role in creating a favourable environment for the biocatalyst, by partitioning effects between the matrix and the reaction medium. The concentration of ethanol experienced in an ethanol fermentation by *Saccharomyces cerevisiae* immobilized in kappa-carrageenan is effectively reduced by the partitioning out of ethanol by the relatively polar gel matrix. The same principle has also be used for other organic solvents (Fukui and Tanaka 1984, Sonomoto et al 1980).

Another interesting aspect of immobilisation is the use of a hydrophobic gel matrix to enrich the immobilised catalyst, by partitioning hydrophobic reactants into the immobilisation matrix, such as in a steroid conversion (Fukui et al 1980).

Clearly the matrix can be used to enhance or retard the transfer of compounds to the biocatalyst. This single feature can make immobilisation of tremendous use in a two-liquid phase system.

Immobilisation may also help to prevent or reduce the formation of stabilised emulsions by confining cells and cell debris, preventing their build up at the liquid-liquid interface, where it may favour the formation of a stabilised emulsion. This might reduce the mass transfer across the liquid-liquid interface (Yeast build up at interface)

TABLE 1.4

Advantages of immobilised cells over free cells.

Physical size of catalyst
when immobilised (0.3 - 3 mm)

1) Catalyst is easy to handle, wash, store and transport.
2) Dosing catalyst is easier in batch process.
3) Easy retrieval of catalyst.
4) May be suitable for continuous process, especially packed beds

Advantages gained by physical confinement of cells to a support.

5) Product not contaminated by free cells if it is in aqueous phase.
6) Cells are protected against pH shock, contact with organic phase and interface in a T.L.P. system.
7) Cellular activities may be stabilised.
8) The viscosity of phase containing biocatalyst is reduced.

Advantages gained by the chemical and physical composition of the support.

9) Partitioning of hydrophobic/hydrophilic compounds into or out of the support depending on the type of matrix

Advantages of re-use

10) Less cell cultivation of biocatalyst need.
11) Less spent on disposal of cells, which is increasingly becoming important, especially concerning the use and disposal of genetically modified organisms (GMO).

1.3.3.1 Limitations of immobilisation

The drawbacks to immobilisation may occur when; the cells are destabilised during the immobilisation procedures (eg elevated temperatures used in kappa-carrageenin or toxic effects of monomers associated with crosslinking the matrix), the product is intracellular or the desired activity is closely associated with growth.

Other problems may arise if the reactant or product has a high molecular weight causing difficulties in mass transfer.

1.4 Design of a biotransformation in a two-liquid-phase system

The use of biocatalysts in two-liquid phase is a relatively new endeavour and the effects of solvent on biocatalysts have in many cases only been confirmed in general terms and still need further elucidation for predictable selection and manipulation.

One of the most important considerations in the design of a biotransformation carried out in a biphasic system is the stability and activity of the biocatalyst (Lilly 1982, Bruce et al 1991). The predictability and selection of a solvent to enhance these characteristics has to some extent been correlated to the hydrophobicity of the solvent as estimated by log P (the log of the partition coefficient of the solvent in normal octanol and water) as outlined by Laane et al (1987) (see section 1.4.3), this has been further advanced by using Seemens (1972) concept of a toxic membrane concentration as applied by Osborne et al (1990).

But the most reliable way to determine the toxic effects of organic solvents on a particular biocatalyst is still to test the solvents individually (Carrea 1984). Immobilisation may increase the biocatalyst stability in a biphasic system (see section 1.4.3).

A fundamental requirement when selecting a suitable organic solvent in the design of biotransformation in addition to retained activity and stability of the biocatalyst is that it solubilises the reactants to a high capacity (see section 1.3.1).

Carrea et al (1975) showed that there is a trade off between the activity and stability against solvent solubilising properties of the organic solvent: The productivity of 20 beta-hydroxysteroid dehydrogenase was greater with solvents which partially destabilised the enzyme, but solubilised large amounts of the reactants, over those which allowed high activity and stability, but solubilised smaller amounts of reactants.

When inhibition occurs with reactant and product, then solvent selection by partition coefficient may become important, by controlling its aqueous concentration.

Additional factors which may influence the choice of the solvent include solvent, selectivity (ability of solvent to remove product over water), flammability, viscosity, cost, nonhazardous, density and waste disposal coupled with solvent recycling (which is becoming of more important).

1.4.1 Biotransformation media

An ideal cell biotransformation is

carried out with a non-growing cell system, where the cells can be grown and then stored ready for use. In these cases where resting cells are used, often only a simple medium is required, consisting of a buffer and simple carbon source.

The specific components in the medium will vary according to the strategy adopted, eg if prolonged viability is required to allow cell regeneration or just to maintain cofactor regeneration. Essential nutrients may also need to be added depending on the specific requirements of the microorganism (see immobilisation).

1.4.2 Structure and function of microorganisms

The most widely used microorganisms for two-liquid phase biotransformations are bacteria and yeast. Gram-negative bacteria are one of the few organisms which have documented evidence of resistance to hydrophobic compounds.

Gram-negative bacteria have thus been of interest in two-liquid phase systems because of their stability in an organic solvent environment. To further consider the general advantages and disadvantages of the use of two-liquid phase, an understanding of their cell physiology is necessary, in particular the structure of the cell envelope.

Gram-negative bacteria have a distinctive outer membrane, the outer leaf of which contains lipopolysaccharides (LPS) a major lipidic molecule. The inner membrane is composed of phospholipids and has no LPS (Lugtenberg 1983). LPS is an amphiphilic molecule which is negatively charged and lends a negative charge to the external surface of the cell (Sherbert 1973). The hydrophobic region of it is termed lipid A which is also an endotoxin. There are also 5-6 fatty acids linked to glucosamine phosphate and a 2-keto-3-deoxyoctanate (KDO) molecule covalently attached to it. Attached to the rough oligosaccharide core are tri-penta saccharides which repeat and are called 'O' antigens (Palva 1980). The LPS is bound to the outer membrane by protein, probably through hydrophobic interactions with lipid A and non-covalent cross bridge interactions with adjacent LPS molecules with divalent cations. The outer membrane itself is attached to the peptinoglycan layer by 'major' proteins. Chelation with EDTA will remove the divalent cations and disrupt the outer membrane.

Some yeasts also have LPS on their cell wall (see section 1.6), these yeasts are also able to utilise hydrocarbons (Kappeli et al 1976). These cells may also have an affinity for the organic solvent interface.

Gram-negative bacteria have hydrophilic and hydrophobic pathways across the outer membrane. By freeze fracturing, electron micrographs (EM) have shown that the outer membrane has structural discontinuities called porins (Lutenberg 1983), which contain both LPS and proteins. These sites are chelation sensitive (EDTA and Ca^{2+} ions influence the number of these sites Lugtenberg 1983). Porins (hydrophilic pathways), are water filled channels, which allow hydrophilic molecules across them depending on the charge at the entrance to the channel (and relative size of the molecule). The overall permeation being dependent on the number of porins. Nakaido (1976) showed that they severely restricted hydrophobic molecules. Hydrophobic compounds are taken passively into the interior of the cell bilipid membrane, while amphiphilic (moderately hydrophobic) molecules are able to transverse the membranes (Sha'afi et al 1971).

Nikaido et al (1976) demonstrated that it is the outermembrane of wild-type *Salmonella typhimurium* and *Escherichia coli* cells which does not allow uptake of hydrophobic or hydrophilic molecules. Symptomatic of the lack of the hydrophobic pathway is the resistance to uptake of hydrophobic agents like actinomycin D, phenol and crystal violet (Nikaido et al 1979). This is a common property of wild type Gram-negative bacteria (Franklin 1981). The poor hydrophobic pathway in Gram-negative bacteria may be the result of the high negative charge along the LPS divalent cation bridging in the outer membrane.

The Gram-positive bacteria have relatively efficient hydrophobic uptake compared to the Gram-negative bacteria (Franklin et al 1981) and are more susceptible to hydrophobic compounds. This feature might be mimicked through use of an immobilisation matrix.

1.4.2.1 Reversal of divalent cation action on outer membrane permeability

When the outer membrane has been permeabilised by chemical agents like polycationic antibiotics or moderately high concentrations of TRIS (Hancock 1984, Goldschitdt 1987), they displace the divalent cations of the LPS (making 'rough mutants'). Gallbrath et al (1977) showed that solvent action could be reversed by addition of Mg^{2+} and Ca^{2+} ions, which may have further advantages in re-use of biocatalysts. This would seem to indicate that a single site is involved in these permeabilisation alterations which is possibly associated with the magnesium binding.

Treatment of the cells with EDTA is accompanied by the loss of large amounts of LPS, however the outer membrane remains intact as with other permeabilising agents (Gilleland 1979).

Several reasons for these effects have been postulated;

1) Removal of the LPS may structurally upset membrane giving rise to non-membrane bilayer,
2) Elastic distortion of the membrane
3) Enhanced fluidity (discussed in Hancock 1984)

However the importance of the outer membrane is clear in Gram-negative bacteria, also that while the membrane is structurally altered it remains intact. This is significant for understanding the field of biocatalysts in organic media and possible avenues for future work in reducing the susceptibility of cells to organic solvents.

1.4.3 Stability and activity of a biocatalyst in a two-liquid-phase biotransformation

Preventing inactivation of a biocatalyst in organic solvents cannot be overcome by a simple selection of an appropriate solvent (Lilly et al 1986). It is only through understanding the mechanism of solvent action that a systematic approach to the use of solvents can be adopted.

While the activity will give an

accurate description of solvent effects on the biotransformation, the activity measured over a time period may also reflect the stability of the cell. For this study it is useful to define activity as the conversion rate and the stability as cell activities and functions which are necessary to support the biotransformation or which enhance it eg the cell membrane in many processes.

Thus, in addition to the activity of the biocatalyst, the stability of the cell must also be studied to understand biotransformations in organic media.

1.4.4 Aqueous-organic interface

The addition of an organic solvent to a reactor may help to overcome the problem of solubility of a poorly water-soluble compound.

Playne and Smith (1983) indicated that above the saturation level of an organic solvent in an aqueous system, organic solvents had an inhibitory effect on extractive fermentations, Cho and Shuler (1986) also showed the negative effects from increased mixing in a two-liquid-phase system.

The transfer of reactant between the aqueous and organic phase can be enhanced by shaking or stirring. While high reaction rates have been achieved by moderate shaking (Cremonsi 1975) the rate of agitation which gives maximum transformation is generally dependent on the nature of the substrate, solvent and biocatalyst.

If the liquid-liquid interfacial area needs to be increased to raise the reaction rate then increasing the agitation rate to accomplish this may have a deleterious influence on the biocatalyst. Many studies have reported the toxicity of organic phase to cells, however few have mentioned the distinction between molecular toxicity and phase toxicity.

Bar (1988), Hocknull and Lilly (1987) suggested that solvents may exhibit phase toxicity without molecular toxicity. They concluded this by comparing high log P solvents which are "non-toxic" in a poorly mixed system, with the same solvent in a well mixed system.

1.4.5 Effects of organic solvents on microorganisms

Organic solvents appear to have their initial effect on the permeability of the cell barrier of Gram-negative bacteria (De Smet et al 1978, Ingram et al 1984). The initial effect is on leakage of mono and divalent ions (Magnesium and Potassium) which is followed by larger molecules (Proteins and RNA , see table 1.8). Monitoring the internal ion concentration of cells may therefore yield significant insight into cell stability in organic media.

The successful use of organic solvents in microbial biotransformation maybe limited because they can denature or inactivate the biocatalysts. Brink and Tramper (1987) studied the influence of various biphasic systems on the production of propene oxide by microbial cells. They concluded that when the polarity of the organic solvent was low (using the Hildebrand solubility-parameter delta) delta < -8 and its molecular weight > 150 the biocatalytic rates were high (see table 1.7 for aqueous solubility). Laane et al (1985) commented that the Hildebrand solubility-parameter, delta, was a poor measure of polarity, particularly for apolar solvents and that log P partition coefficient (see table 1.5) was a better indicator of solvent polarity (see fig. 1.6). They also claimed the log P-activity correlations observed for bacterial conversions are a general phenomenon with low activities in log P solvents < 2, and variable between 2 and 4, while high in apolar solvents having log P > 4.

Other parameters have been used to describe solvent hydrophobicity (see table 1.6) but have been unsuccessful in giving a good correlation with activity (Laane et al 1987).

TABLE 1.5

$$\text{Log P of solvent} = \log \frac{([\text{Solvent}] \text{ in octanol})}{([\text{Solvent}] \text{ in water})}$$

The Log P of a solvent is the logarithm **of its partition coefficient between a two phase system of octanol and water (Laane 1985)**

TABLE 1.6

Other parameters that have been used to correlate solvent properties with catalytic activities.

1) Hildebrand solubility-parameter
2) Dye E.T.
3) Dielectric constant
4) Dipole moment

(Laane 1985)

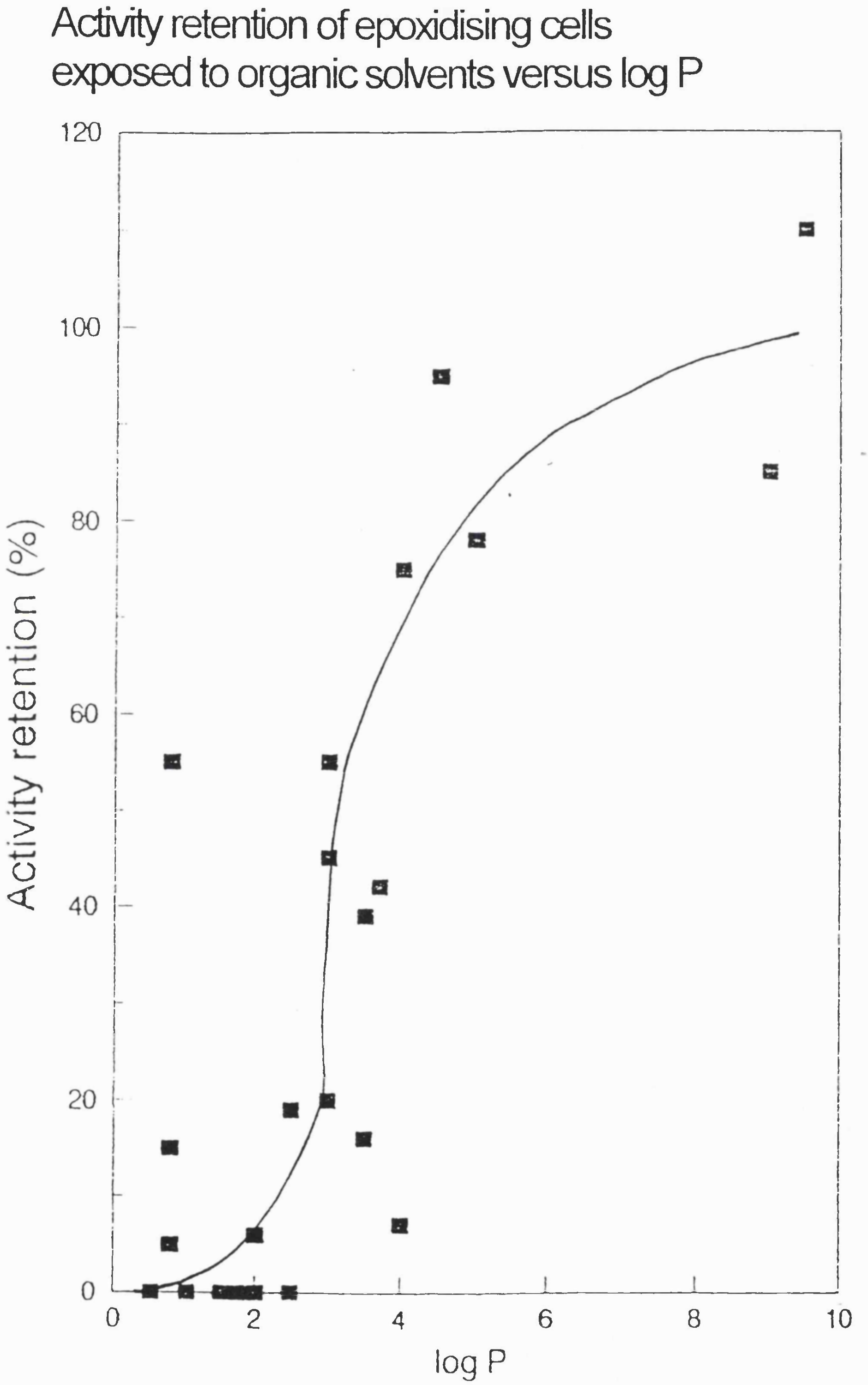
TABLE 1.7

Maximal solubility of organic solvents in water versus log P of organic solvent

Log P	Maximum solubility of organic solvent in water(20 c) (wt %)
log P <= 2	> 0.4
2 < log P < 4	0.04-0.4
Log P >= 4	< 0.04

One of the first attempts to rationalise log P (From Brink and Tramper (1985),Riddick and Bunger (1970))

FIGURE 1.3



Graph of activity retained after challenge against the log P of solvent (from Laane et al 1987)

Log P is a useful tool since it can be calculated for simple molecules by hydrophobic fragmental constants to give the hydrophobicity of an organic solvent (Rekker 1979). More complex molecules can be given a log P by experimental estimation.

Recent work has confirmed that initial organic solvent action on Gram-positive bacteria involves the cell membrane electron transport chain. Also that poor stability with high log P solvents was obtained despite good initial activity (Hocknull and Lilly 1987). Thus good stability is not guaranteed by simple selection of a high log P solvent.

Methods for improving stability are required. Immobilisation has proved successful in many cases (Brink and Tramper 1985, Hocknull and Lilly 1987, Yamane et al 1979). There is no reason to believe that immobilisation will protect a biocatalyst against all solvent inactivation, but it may protect a biocatalyst from "phase" toxicity. Yabannavar et al (1991) noted that while immobilisation can protect against the immiscible solvent phase, the water soluble solvent still caused toxicity to the cells, by the diffusion of the solvent through the matrix.

Thus stabilising a biocatalyst in an aqueous/organic solvent is only going to be possible by understanding and protecting the biocatalyst from the solvent action at the membrane level (with special regards to the protein conformation).

TABLE 1.8

Potential effects of solvents on microorganism stability

1) Loss of cell viability

2) Changes in cell morphology

  a) cytoplasmic shrinkage

  b) loss of membrane organisation

  c) ultrastructure changes

    -displacement of the chromosome toward the cell periphery

    -loss of electron dense material

3) Physiological changes

  a) inhibition of nutrient uptake

  b) inhibition of oxygen uptake

  c) loss of membrane permeability barrier

  d) alteration or inhibition of intermediary metabolism

  e) inhibition of DNA synthesis

(From Lilly et al 1987)

Seeman et al (1980) described the effects of anaesthetics and their partitioning into the membrane of nerve cells, with general partition coefficients five times that of the standard octanol-water partition coefficients. An extension of this work by Osborne (1990) related the partitioning of the organic solvent into the microbial membrane to give a critical level from the aqueous phase of solvent which prevents membrane activities.

While the critical solvent level in a membrane is a useful concept, it is of limited use where the concentration in the organic solvent in the cell membrane is below the critical membrane concentration and when the organic solvent is not present as a two-liquid phase. It is therefore only applicable to co-solvent systems.

If this is just a partitioning effect then optimal activity should be regained if the solvent is partitioned out of the membrane.

Other indications that the solvents primary site of action is at the cell membrane come from the increased fluidity of the cell plasma membranes when treated with solvents such as benzyl alcohol, Gordon et al 1980). Protein conformation is also likely to play a key role in solvent action on the cell membrane (see 1.4.6).

1.4.6 Enzymes in organic solvents

To distinguish between the general effects of organic solvents on cells and enzymes, it is necessary to further consider how enzymes are effected by organic solvents. The full 'natural' catalytic activity of an enzyme is only exhibited when it is in a strictly defined (native) conformation. Since many enzymes including lipases, esterases and dehydrogenases function in a natural hydrophobic environment, usually in the presence of a lipid bilayer, it is not surprising that these enzymes are active in organic solvents.

Enzyme conformation is determined by a

network of both hydrophobic and electrostatic interactions and hydrogen bonds. It has been postulated that a hydration shell is necessary for the enzyme conformation and that strong distortion of the shell or loss of it will upset catalytic properties of the enzyme (Dordick 1989 and Khmelnits et al 1988, provide excellent reviews of this subject).

Since it is thought that the enzyme structures may be distorted by solvents, it is not surprising to find that under these circumstances they accept novel substrates and remain in the conformation from the previous pH, which it last 'saw' and does not change this conformation in the organic solvent since there is no pH in an organic solvent (Zaks and Klibanov 1988, 1986).

A less obvious outcome of enzymes in water-restricted organic solvents is their thermostability (Zaks and Klibanov 1984). This topic is extensively covered in a mini review by Zaks and Russel (1984).

It would seem clear that from this and the lack of evidence from the literature of a correlation of enzyme activity and log P (with the exception of Laane et al 1987), that the initial action of solvents on enzymes and microorganisms may well be different.

1.4.7 Classification of two-liquid-phase systems

To enable a rational approach to be adopted to two-liquid phase Lilly and Woodley (1985) proposed a classification system for reactions involving product and/or reactants which are poorly-water soluble.

Their classification is based initially on the nature of the aqueous phase including the biocatalyst and discriminating with regards to two factors, the reactant concentration profiles and the relative phase volumes. They defined three main concentration profiles (see figure 1.4a). However they did not take into account where the biocatalyst reaction takes place (interface or bulk aqueous) or accumulation of biocatalyst at the liquid-liquid interface.

From these concentration profiles the classification structure may be expanded to take into account the relative phase volumes (see figure 1.4b). Five main reaction systems were classified (omitting those in only aqueous phase).

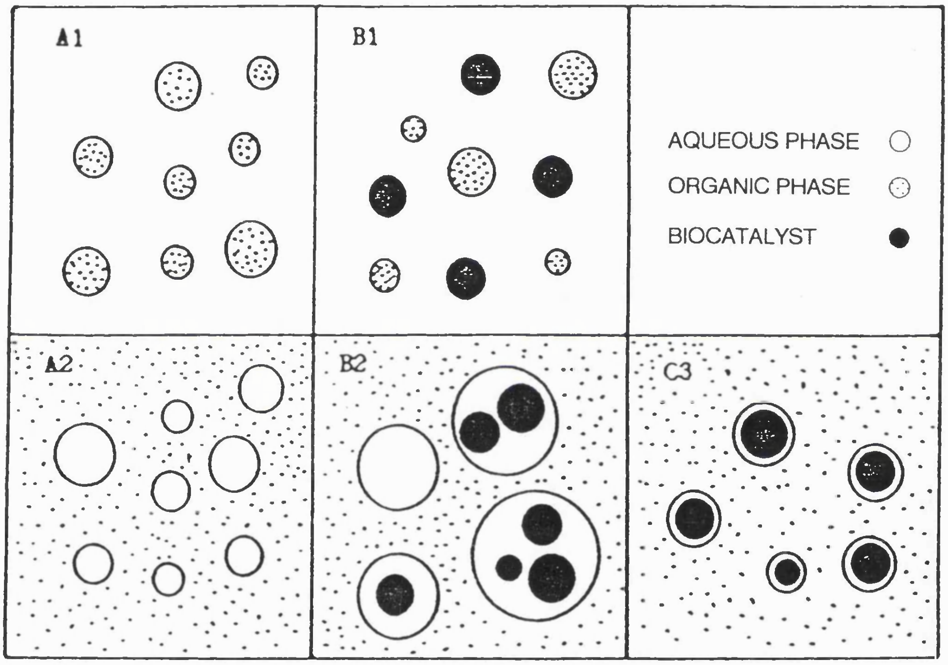
The ratio of the aqueous phase volume (as well as the biocatalyst) compared to relative organic phase determines whether there is a discrete aqueous phase surrounding the biocatalyst.

When four reaction components (eg two reactants and two products) are involved then there are twenty five different possible distributions of reactants and product between the aqueous and the organic phase. Of these only four have no components in the organic phase.

The number of possibilities increases if the physical nature of the components in the reactor is taken into account (gas, liquid or solid).

FIGURE 1.4a

Diagram of reaction system involving organic liquid phase


A1
B1
AQUEOUS PHASE ○
ORGANIC PHASE ◌
BIOCATALYST ●
A2
B2
C3

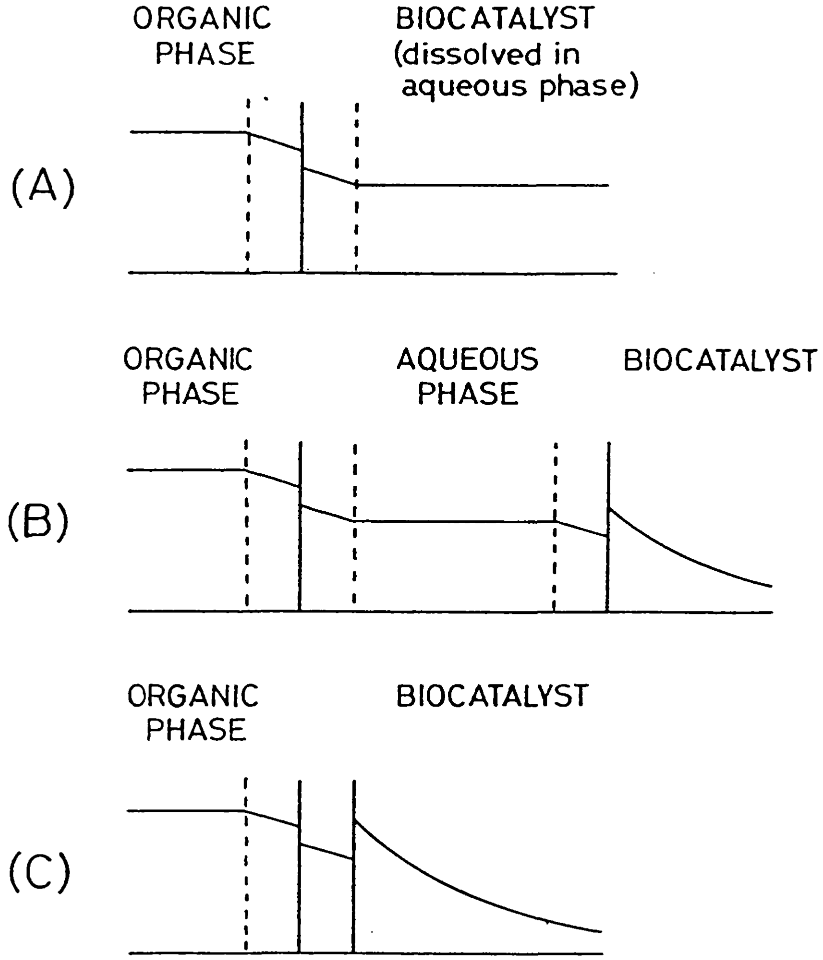
Type 1 discrete aqueous continuous phase

Type 2 discrete aqueous discontinuous phase

Type 3 non-discrete aqueous phase

(From Lilly and Woodley 1985)

FIGURE 1.4b
Concentration profile in two-liquid phase systems


ORGANIC PHASE
BIOCATALYST (dissolved in aqueous phase)
(A)
ORGANIC PHASE
AQUEOUS PHASE
BIOCATALYST
(B)
ORGANIC PHASE
BIOCATALYST
(C)

Liquid/liquid and liquid/solid interfaces are shown by solid lines; films at the interfaces are shown by dashed lines.

(From Lilly and Woodley 1985)

1.5 Selection of a two-liquid-phase system

Yeasts have a great commercial importance, producing products from a diverse range of industries, from the brewing industry to products from the pharmaceutical industry produced by DNA technology. Some of these compounds have application in two-liquid phase, making yeast a worthwhile target for investigation in this study for use in organic media.

An important and interesting aspect to using biocatalytic cells with organic solvents is the stability of the biocatalyst's cell membrane. This can be investigated by measuring the beta-dispersion of yeast cells (see section 1.8) in the presence of organic solvents.

Of the many biotransformations catalysed by yeast, the most easily followed are those with reactants and products which are relatively easy to detect. Naphthalene is an aromatic hydrocarbon which is easily detectable by using an ultra-violet (U.V.) monitor and separated from other aromatic and aliphatic hydrocarbons by high pressure chromatography (HPLC) (Harrop 1990). The yeast *Yarrowia lipolytica* can convert naphthalene to a mixture of 1-naphthol and 4-hydroxylone, both of which can be detected by HPLC (Cerniglia et al 1978,1981). One of the interesting questions about using this system in two-liquid-phase is the behaviour of the ratio of the products to each other when they are removed into the organic phase.

The effect of organic solvents might also be elucidated by comparing cells of *Y.lipolytica* grown on glucose and a hydrocarbon, and then comparing their tolerance to solvents.

1.6 Yeast growth on n-alkanes

In the period preceding 1973, when oil prices soared, hydrocarbons were a relatively cheap carbon source. Industrial processes to produce single cell proteins (usually yeast) from hydrocarbons were developed. In parallel, studies on the mechanism of utilisation of hydrocarbons were undertaken. Since hydrocarbons in fermentations are present as dispersed droplets, these fermentations were early examples of two-liquid phase systems (see Abbott and Glendill 1971).

Little direct evidence is available regarding the mode of assimilation of liquid n-alkanes by microorganisms in biphasic systems (Klug and Markovetz 1971). It is possible that microorganisms may grow entirely upon the dissolved hydrocarbon in the aqueous phase (McAuliffe 1969), as indicated by Chakaravaty et al (1972), who modelled microbial growth and examined growth of microorganisms on solid hydrocarbons. Alternatively, the cells may grow on the surface of the hydrocarbon interface by direct incorporation of the hydrocarbons through the phospholipid cell membrane (McLee and Davis 1972). The most commonly accepted mechanism is " solubilisation" of hydrocarbons as sub-micron droplets caused as release of surfactants by the microorganism (see table 1.9).

Uptake of n-alkanes by yeast

The first step of assimilation is uptake of exogenous alkanes by the cell, the second step involves transport to the site of oxidation.

TABLE 1.9
Uptake mechanisms of alkanes

1) Through direct contact of cell and oil droplet
2) Via accommodated alkane phase (submicron attachment between cell and oil droplet)
3) Solubilised alkanes (dissolved in aqueous phase)

(From Fukui and Tanaka 1981)

In a mixture of hydrocarbons it is often those with a lower molecular weight which are metabolized first. Degradation of all of these n-alkanes is often accompanied by the production of biosurfactants (Abbott and Glendhill 1971)

Teh et al (1974) suggested that lower molecular weight hydrocarbons (>= octane) caused rapid inhibition of glucose incorporation and considerable loss of potassium and protein from the cells of *Cladosporium resiniae*, suggesting a limited disorganisation of the cell membrane was the cause, resulting in a rapid loss of selection of permeability of the cell. This contradicts Gill and Ratledge (1973) who proposed that it was due to accumulation of fatty acids and other metabolites.

Teh et al (1974) also showed the lower molecular weight hydrocarbons failed to remove significant quantities of tri glycerides, free fatty acids, hydrocarbons, sterols or phospholipids which where extracted by lipid solvents (eg chloroform-methanol). This suggest that the integrity of the membrane is probably maintained to a large extent in the presence of short chain n-alkanes.

On alkane grown *Candida tropicalis* slime-like out growths are formed during the early growth phase. These growths reach the cell membrane through electron dense channels, which may allow migration of alkanes through these channels to the endoplasmic reticulum, the site of alkane hydroxylation. The slime-like protrusions or outgrowths are not alkanes or lipids with simple attachment to the cell, since treatment with detergents and organic solvents cannot remove them (Fukui and Tanaka 1981). They are arrangements of LPS, which have been identified as mannan composed of approximately four percent covalently linked fatty acid (Kappeli and Fiechter 1978) and are located on the cell wall. They enable alkane microemulsions to adhere to the cell through a non-enzymic affinity.

1.7 Transport systems in yeast

Cell membranes are typically the first major organelle to come into contact with an organic solvent in a two-liquid phase system. One of the major functions of a membrane is to promote transport of certain molecules. If the membrane function becomes impaired or enhanced, all of the membrane's diverse transport processes would be affected. This would have a dramatic effect on the cell processes.

Transport systems are also of major importance in biotechnology, and especially to biotransformation in two-liquid phases (TLP). In biotransformation, it is likely that a xenobiotic substrate or product involved in the biotransformation will need to cross the cell membrane to obtain a high yield of product. To accomplish this the effect of organic solvents on the transport of molecules through the cell membrane also needs to be appreciated.

The primary effect of solvents has been demonstrated to act on the cell membrane function by Ingram et al (1984), who showed that ethanol toxicity prevented glucose transport systems in yeast.

Types of transport systems in yeast (small molecules or ion transport in microbial cells)

Simple diffusion

This is diffusion of molecules down the concentration gradient without carriers participating eg O_2, CO_2, water and not sugars.

Facilitated diffusion

Stereo-specific proteins conducting

molecules across the membrane down the concentration gradient. Inhibited by structural analogues.

Both *Saccharomyces cerevisiae* and *Zymonas mobilis* which convert monosaccharides to ethanol, transport monosaccharides by facilitated diffusion.

Active transport

Expenditure of energy is necessary to overcome high internal chemical gradients. Two classes of active transport have been identified.

1) Chemiosmotic activity

Where protons are extruded (primary active transport) creating a chemical gradient of hydrogen ions and an electrical potential (negative inside the cell). The substrate is then carried in across the membrane with a proton. Secondary active transport is where the substrate moves across the cell membrane with the afore-mentioned proton.

The primary electrochemical gradient can be produced in two ways.

a) electron transport system operation respiration (or anaerobic respiration). In bacteria electron transport is arranged in the cytoplasmic membrane and operates by flavoproteins and quinones, accepting both electron and hydrogen ions when they are reduced and oxidised. They are located between carriers which transfer only electrons.

b) Extrusion of hydrogen ions through membrane bound ATPase at the expense of ATP hydrolysis (Used by obligate fermentative bacteria that do not respire).

Eukaryotes (fungi and yeast) also transport amino acids and sugars by chemosmotic coupling (Goffeau et al 1981)), but they are unlike the prokaryotes in that their electron transport system is located in the mitochondrial membrane. Mitochondria generate ATP, which is hydrolysed by cytoplasmic bound ATPase to produce the primary electrochemical gradient used for active transport (Goffeau et al 1981)

2) Direct chemical energy coupling

The essential feature is of energisation of active transport by phosphate bond energy. The binding protein in the periplasmic space is required as well as the participation of a membrane-bound permease. But only Gram-negative bacteria have a periplasmic space between cytoplasmic membrane and outer membrane. The binding protein in the periplasmic space may be lost by osmotic shock (Stinson 1977).

Group translocation

Transport substrates are chemically altered during transport process. Phosphoenolpyruvate (PEP) is the best known group translocation system; Many proteins are involved as the PEP transfers phosphate groups to a number of sugars as they traverse the cytoplasmic membrane. They are integral membrane proteins and cytoplasmic proteins.

1.8 Organic solvent tolerance and adaption

Many organisms find three to four percent concentration of ethanol toxic, however many yeasts do not find this concentration toxic and may retain their viability in such environments.

Unlike other organisms Gray (1941) showed that ethanol tolerance is not confined to any one genus or species of yeast. Troyer (1955) confirmed this and showed that methanol is generally less toxic than ethanol which fits the general trend of log P and cell activity (see chapter 1.4.5)

As the plasma membrane of the cell is the first sensitive organelle to make contact with ethanol from a solution, and ethanol is an amphipathic compound, the lipid composition may have an important role in ethanol tolerance and therefore also tolerance to other solvents.

The threshold for cell stability, when challenged with organic solvent, can be modified by nutritional requirements. Thomas et al (1978) showed that plasma membrane lipid composition affected tolerance in *Saccharomyces cerevisiae*, by exploiting the anaerobically-induced requirement of this organism, for sterol and unsaturated fatty acid (Andreasen and Steir 1953, 1954) both requirements are fairly non-specific, as they are not required for a functional metabolism (Light et al 1962, Hassack and Rose 1976).

Alteration in levels of saturated and unsaturated phospholipid chains alters cell stability in the presence of organic solvents (Vollherst-scheck et al 1984, Sinesky 1974, Thomas 1978). Hayashida et al (1974) reported sake' yeast (*Saccharomyces cerevisiae* strain) acquired enhanced ethanol tolerance when grown in the presence of *Aspergillus oryzae* fractional envelopes containing unsaturated fatty-acyl residues.

Hitzeman et al (1986) indicated that organic solvents like ethanol are anchored in the hydrophilic region, and not dependent on bilayer symmetry or the presence of other membrane components such as proteins.

Another line of research was followed by Park et al (1988) who showed an adaption to solvents by the addition of a non-toxic solvent to the broth of a *Saccharomyces cerevisiae* fermentation. This resulted in a reduced hydrophobicity adaption of the cell surface resulting in a greater tolerance to another more toxic solvent. An extension of this work to cover other solvents in general, would have great significance to biocatalysis in two-liquid phase.

1.9 Dielectric spectroscopy

The dielectric spectroscopy and passive properties of cells and tissues have long been known to change significantly with cell death (Shwan (1957),Aldey (1981),Harris et al (1985) and Pethrig et al (1987)). This provides the basis for a useful non-invasive approach to cell death.

Dielectric properties of cells

In contrast to simple ionic solutions, any cell with an intact membrane, especially microbial cells will have general electrical properties which are frequency dependent i.e. that their permittivity increases and the conductivity decreases as the frequency used for measurement (fm) is lowered.

When passive electrical properties measured are frequency dependent, the characteristic is termed dielectric dispersion. Schwan (1957) termed the dispersion alpha, beta and gamma.

The beta-dispersion occurs in the range of ca. 0.1-100 MHz (radio frequencies of the electromagnetic spectrum), and is the region this study is primarily concerned with.

Beta-dispersion

The beta-dispersion is reported to be derived from the highly conductive internal protoplasm of the cell (thought to only weakly vary with culture conditions (Markx et al 1991)), which are separated by the poorly-conducting cytoplasmic membrane. Viability of the cells is thought to be dependent on the relative impermeability of the membrane to ions (Mackey 1980).

Analogy to an electrical capacitor

The membrane may be considered as analogous to the plates of an electrical capacitor. A "static" build up of charge at low frequencies can be detected as a macroscopic capacitance due to the low frequency induced field, by a Maxwell-Wagner type mechanism (Harris et al (1985),Schwan (1983) and Zimmermann (1982)).

Membrane charge

The capacitance of a cell with a beta-dispersion can be measured between two plane-parallel electrodes. From equations developed by Maxwell and refined by Fricke (1925) it has become possible to calculate membrane capacitance per unit area (Cm) (Markx et al (1990), Markx et al (1991), also see theory section). The experiments by Fricke (1925) gave a value generally accepted today of ca. 1 micro Farrad, and since the membrane was known to be of a lipid nature, with a dielectric permittivity in the range of 3-10 it was calculated that from the slab equation that the membrane thickness was in the range of 10-3nm.

Microelectrodes

It has been possible to measure the charge per unit area in large cells Fernandez et al (1984). Microelectrodes have also found great use with black lipid membrane (BLM). These are formed by the painting of hydrocarbon and compounds of interest over a small hole (Coster et al 1974), or by assembling them by solvent free methods (Yoshida 1989). The BLM physical arrangement allows direct measurement of the membrane permittivity.

Whole cells

Studies on whole cells includes the effect of drugs and chemicals on the substructure of the membrane (Yoshida et al 1989 and Stoicheva et al 1989) and their interaction with membrane charging. It also has uses with immobilised cells (Lovitt et al 1986). Further reviews include Harris et al (1985).

The bugmeter

This equipment was designed by Kell (1990) to produced a signal which can be used to provide a measure of cell membrane intactness. The macroscopic signal is derived from the beta-dispersion (Schwan 1957), which occurs at certain frequencies and is caused by the charging of the cell membrane. The bugmeter can monitor the beta-dispersion and therefore be used to provide a signal to monitor intactness of biological membranes (Pethig 1979 and Kell 1978).

Kell (1987) and Harris (1987) considered that this technique has application in determining biocompatability or otherwise of organic solvents. Stoicheva (1989) demonstrated the compatibility of this technique with several organic solvents.

Solvents effect the cell at their membrane, causing a leakage of ions from the cell into the suspending solution. Since the ions which are monitored are effectively impermeable to the cells cytoplasmic membrane, their leakage is likely to be through the protein channels which traverse the lipid bilayer. These are protein channels which cross the membrane and allow ion transfer (and therefore ion gradients to be maintained) between the cell and the surrounding medium. Organic solvents partition into the cell membrane, which may also cause an increase in its fluidity (Gordon et al 1980). The solvents also increase the membrane volume (Ashcroft 1977a,b) as shown by the rise in capacitance (Stoicheva et al 1989). This causes the trans-membrane proteins to alter their conformation and allow ions across the membrane (not all of these proteins are necessarily associated with maintaining the ion gradients).

1.10 Theory of operation of the bugmeter

1.10.1 The electrical properties of the system

When an electrical alternating current is passed between two electrodes, the characteristic 'passive' electrical properties of the system can be measured by two variables : electrical capacitance G (units Farads (F)) and conductance C (Siemens (s)). In this work biological readings (pF) and (mS) are used throughout unless otherwise stated.

For two plates of area A and separated by distance d the capacitance and conductance measured will vary as a function of both A and d.

These readings can be further characterised by its permittivity E' and its conductivity s' which are related to C and G by :

$$s' = G\ (d/A) \qquad -(1)$$

$$E' = C\ (d/AE_0) \qquad -(2)$$

Where :

E_0 is known as the permittivity of free space and is equal to the capacitance of a 1 cm cube which by experiment is found to be $8.854 * 10^{-14}$ F/cm. (d/A) is known as the cell constant (K) and has units of cm^{-1}. Thus the units of s' are mS/cm and E' is dimensionless. The value of E' for water at 298K is 78.4 and thus from equation (2) for a cell which has unit dimensions (for which K= 1cm^{-1}) it has a capacitance of $6.93*10^{-12}$pF.

Due to probe geometry variables A and d will not change during assay and it is suitable to talk in terms of gauged readings of C and G separately.

The effective cell constant of the 25 mm-type bugmeter electrode ('probe constant') is often in the range of 0.8cm^{-1}, and may be determined by measuring the conductivity of a solution of known conductivity (10mM KCl which at 298K has conductivity of approximately 1.413 mS/cm).

$$\text{Cell constant} = \frac{\text{Conductivity of test solution}}{\text{Bugmeter conductivity reading}}$$

The effective cell probe constant will vary on each range of the bugmeter.

1.10.2 The beta-dispersion

Ionic solutions have a passive electrical property as do cell suspensions, the latter have frequency dependent dielectric properties, this characteristic is the dielectric dispersion which is present in all biological cells with a cell membrane. The magnitude of this beta-dispersion is dependent on the poorly conducting lipid membrane separating the conducting cytoplasmic phase from the conducting extra cellular phase (this also includes the cell wall).

The electrical capacitance of the lipid membrane area is in the region of 0.5 - 1.0 micro Farads/ cm^2 and may well be independent of the nature of cells considered.

The current carried in the cytoplasmic and the extracelluar milieu are ionic and must reach the cytoplasmic membrane before charging it up. At high measurable frequencies of detection, the magnitude of beta-dispersion (the capacitance) decreases as the frequency is raised and conversely increases as the frequency of measurement is lowered. The time taken to charge up the membrane is the time constant (T) which depends on the internal (s'_i) and external (s'_o) conductivities and will also be reflected in the mean cell radius r, as well as the particular membrane capacitance per unit area (Cm).

$$T = r\ Cm[\{1/s'_i\} + \{1/2s'_o\}] \qquad -(2)$$

When capacitance measurement is plotted against frequency, a two plateau graph is obtained, and measurement on or near the low frequency plateau gives a means for gaining an estimate of biomass.

The frequency at which half this beta-dispersion effect is obtained is referred to as characteristic frequency (fc)(see chapter 3).

$$fc = 1/(2\ pi\ T) \qquad -(4)$$

Consequently a medium containing cells will have larger capacitance at a measuring frequency than medium without cells due to the ability of a membrane to receive charge.

1.10.3 The importance of the critical frequency to biomass measurements

The position of critical frequency (fc) is dependent on the conductivity of the medium in which the cells are suspended. If the conductivity of the medium is increased then the fc will move to a higher frequency. If the conductance falls then fc value will move to a lower frequency.

While the conductance charge in the medium does have an affect on fc forcing it either up or down, it does not have an effect on the capacitance as seen at the measuring frequency (fm), which will not change.

1.10.4 Alpha dispersion

At frequencies below that of the beta-dispersion some cells show an alpha dispersion , especially Gram-positive bacteria. This is thought to be due to ionic process within the cell envelope. The nature of the cell wall may change under differing environmental conditions and is therefore a less desirable monitoring parameter than the beta-dispersion.

1.10.5 Cell volume interference

The ratio of membrane enclosed to volume (relative density) of microbial cell is not thought to vary greatly dependent on physiological status. The volume fraction P is thus dimensionless as it is volume (ml) contained within the cell cytoplasmic membrane per volume of cell suspension.

1.10.6 Conductivity

The energy of excitation from an electric field can be stored only as capacitance (C) and can only be dissipated by conductance (G). Thus any decrease in C must be accompanied by an increase in G.

The measurement of beta-dispersion can also give a measurement of the electrical conductance of the system. It will be frequency-dependent, increasing with a rise in frequency. The conductivity being lower in the system than in the background medium.

In fermentation conditions the conductance would be expected to change throughout a fermentation with the addition of acid and base along with the production and uptake of charged metabolites. In the system chosen here the effect on E′ as measured at the frequency of choice will be minimal.

$$s'_L = s'_o \ (1-P)/(1+[P/2]) \qquad -(6)$$

s'_L conductivity at low frequency, s′o conductivity of suspending medium.

1.11 The aims of this project:

1) To confirm the use of this relatively new tool as a non-invasive means of monitoring cell viability.

2) To demonstrate involvement of the cell membrane when the cell is challenged with an organic solvent.

3) To use this technique on a range of organisms.

4) To investigate the effect of solvent structure on the cell membrane by monitoring the cell beta-dispersion and cell activity.

5) To investigate the effect of sub and super saturation of the aqueous phase with organic solvent.

CHAPTER 2

CHAPTER 2

MATERIALS AND METHODS

2.1 Beta-dispersion measurement

The instrumentation was supplied by Aber Instruments, it gives a continuous reading of both capacitance and conductance, which were recorded on a chart recorder (Lloyd instruments). These values were used to construct data points for graphic representation.

2.1.1 Operation

Three factors determine the suitability of the beta-dispersion meter for use in a particular system:

1) Medium conductance
2) Cell size
3) Biomass concentration

1) Medium conductance

The standard Bugmeter (216) has two ranges of sensitivity "lo" range and "hi" less sensitive range. On the high range the instrument is optimal between 0.1 to 1 MHz with conductance having minimal effect up to 10 mS. The sensitivity was approximately half that of the low range.

The "low" range was selected for use throughout these experiments. For technical reasons supplied by the manufacturers an upper limit of approximately 8mS is recommended. The measured conductance of the medium was set at approximately 2mS.

2) and 3) Cell size and biomass

The level of reading is dependent on the magnitude of the charge per unit biomass and the capacitance measurement errors or noise.

The output from the bugmeter was also dependent on cell size (see chapter 3). For **non-rodent hybridoma** cells, 1 mg/ml (w/w) of cells (1-10 micrometers) changed the permittivity in the region of 1pF.

Setting up

Before measurements commenced the probe electrode pins were checked to ensure that they were clean. The probe was placed approximately 20mm from the bottom of the challenge tank (220 ml volume). The position of the probe was not changed throughout the assay. The challenge tank was maintained at a constant temperature by means of a water jacket surrounding the challenge tank and linked to a water bath.

Care was taken to ensure that no bubbles were entrained between the probe pins.

The capacitance was backed off prior to the addition of biomass. The capacitance settings of output and display set to delta capacitance. The delta capacitance could then be backed off to zero. The one second time constant was found to be suitable giving low noise and rapid response. 0.4 MHz was chosen as the measuring frequency (see chapter 3) challenge tank volumes:

Volume of beaker	150 ml
Volume of buffer	100ml
Volume of cells paste ca.	20ml

(These values were used unless otherwise stated)

The cells were kept in suspension by stirring with a magnetic bar and bottom magnetic stirrer from Ikamag Reo (FSA), which maintains a constant rpm value by opto-electric control once set. The rpm value used are given in the results (also see section 2.3). The magnetic bar speed was set by using a tachometer from FSA.

Further instrument settings

The clean pulse switch was set to off during experiment.

Frequency operation: A manual internal frequency was chosen.

2.1.2 Probe

The probe used was 25mm port type, made of resin, which encased four gold pin electrodes. To avoid any growth of cells on the electrode surface the probe pulse-clean was occasionally used in the manual mode. This involved passing a voltage of less than 10 volts through each pin. Only infrequent cleaning was found necessary, and a cleaning pulse was not used during the experiments.

To prevent any interaction of the probe with the second liquid phases added the probe was sheathed in a glass cylinder which fitted over the probe and was flush with the surface which the electrodes project. In all cases the probe was below the surface of the aqueous phase.

2.2 Cells, growth and harvesting

2.2.1 Strains

Saccharomyces cerevisiae isolated from bakers yeast from Distiller Company Ltd.(DCL) onto nutrient agar, identified by microscopic and biochemical test. The DCL yeast block was used in preference to growing fresh batches of yeast, since it maintained its viability for several weeks and could be obtained readily without the need for a fermentation.

Yarrowia lipolytica ATTC NO.46482

Pseudomonas putida UV4: donated by ICI.
P.putida UV4 mutant is known to hydroxylate naphthalene to naphthalene-1,2-dihydro-1,2-diol and to lack the gene necessary to break it down any further (see Harrop 1990).

Penicillium chrysogenum P1 : Panlabs Inc.,Fayetteville, NY,USA.

Animal cells :A rodent hybridoma cell line (monoclonal antibody raised against human blood group B). Culture donated by J.Waite.

2.2.2 Culture maintenance

Saccharomyces cerevisiae and Yarrowia lipolytica

Master culture cells were maintained at -20°C in 75% glycerol and phosphate buffer at pH 7.0, working cultures were stored at 4°C on nutrient agar plates. *Saccharomyces cerevisiae* from DCL was stored at 4°C for up to three weeks prior to being discarded. The culture was pure when diluted (to 30-300 CFU per plate) in sterile buffer and streaked out onto nutrient agar plates.

Pseudomonas putida UV4

Master cultures were maintained at -20°C in 50 mM phosphate buffer at pH 7.0, with 10%w/v glycerol. Working cultures were maintained on nutrient agar plates at 4°C.

Penicillium chrysogenum P1

The spores were maintained in 70% (v/v) glycerol to suspension buffer at -20°C as a master culture. The cells were removed and plated onto nutrient agar plates to revive the cells. Plate cultures for spore inoculum were store at 4°C for 1-2 weeks prior to use.

Animal cell

Storage :Cells stored in liquid nitrogen at -70 °C

Maintenance :After cell growth the cells were used immediately (within a day before the medium pH indicator changed colour)

2.2.3 Growth of *Y.lipolytica* and *S.cerevisiae*

2 litre MBR reactor

Cells were regularly grown in an MBR bioreactor (1.9L working volume, 2.5L total volume). The glass reactor measured 25cm by 12.5 wide. Agitation was by two six bladed-turbine impellers 5cm wide. Stirrer speed was maintained to give a dissolved oxygen tension (DOT) in the solution of greater than 40% of air saturation. The fermenter was sterilised in situ at 121°C for 20 minutes. Temperature was maintained by a water jacket and the pH was corrected by addition of 3M KOH. Air was sparged into the reactor by a Charles Austen air pump. The BCS BioPC package (BCS software) for monitoring fermentation parameters was operated on a Tandon PC.

Y.lipolytica and S.cerevisiae

Selection of colonies for hydroxylation activity of naphthalene, through screening by the ability of organisms to covert indole to indigo. Detection of activity was from nutrient agar plates containing indole (0.01% w/w), and incubated at 28°C or 24-48 hours. Neither strain showed an ability to hydroxylate the indole as indicated by a change colour.

Inoculum was taken from agar plates grown overnight then used to inoculate a 250ml baffled shake flask containing 20 ml of sterile medium. This was then incubated at 200 rpm at 28°C for 24 hours before inoculating the reactor.

Fermentation medium was Yeast Nitrogen Base (YNB,Difco) and 50mM phosphate buffer pH as required, which was sterilised at 121°C for 15 minutes in the reactor or filter sterilised for use in baffled shake flasks, when growing the inoculum. pH control in the reactor was by addition of 3M KOH and HCl. Sterile glucose (20g) in solution was added continuously over 24 hours to promote accurate control of the fermentation; the glucose was not in excess during the experiment. Polypropylene glycol MW 2000 (2ml) was added to prevent foaming.

Harvesting of cells

Cells were harvested from the fermenter and then sedimented by centrifugation in 250 ml pots at 10,000 rpm for 15 minutes at 4°C, in high speed MSE centrifuge. The supernatant was decanted and the cell paste stored at 4°C (the cells were used within 48 hours)

2.2.4 Growth of *P.putida*

Fermentation media

The hydroxylation enzyme(s) are located on a constitutive gene, but the activity is inhibited in the presence of high concentrations of glucose by catabolite repression and is therefore grown under glucose limitation.

Selection of colonies for activity was from nutrient agar plates containing indole (0.01% w/w), and incubated at 28°C or 24-48 hours. Active colonies appeared dark blue due to the hydroxylation of indigo by the microbial dioxygenase. These colonies were grown in unbaffled shake flasks containing 50ml of sterile defined salts medium (see below) at pH 7.0 containing 1g of glucose (20g/L), and incubated on a rotary shaker (28°C 150 rpm).This culture was used to inoculate the reactor (Harrop 1990).

The defined salts medium was used for growth in fermentations, based upon Harrop (1990). The medium was prepared for carbon limiting growth.

Source	Stock molarity	mls added
Phosphorus	$NaH_2PO_4.2H_2O$ / 2M	5.0
Nitrogen	NH_4Cl / 4M	20.5
Potassium	KCl /2M	5.0
Sodium	$Na_2SO_4.10H_2O$ / 1M	2.0
Chelating agent	Citric acid / 1M	2.0
Magnesium	$MgCl_2$ / 0.25M	5.0
Calcium	CaCl2 / 0.02M	1.0
Trace metals	(see below)	5.0
Molybdenum	Na_2MoO_4 / .001M	0.1

Trace metals per 5 L:

Metal	Quantity
HCL concentrated	5ml
ZnO	2.04g
$FeCl_3.6H_2O$	27g
$MnCl_2.4H_2O$	10g
$CuCl_2.2H_2O$	0.85g
$CoCl_2.6H_2O$	2.38g
H_3BO_4	0.31g

Polypropylene glycol MW 2000 (2ml) was added to prevent foaming. Glucose was continuously added (12.5% ,w/w) to the fermentation during growth providing a 2g glucose pulse per hour.

In the shake flasks phosphate buffer to 50mM was added at pH 7.0 for pH control. Bacteria were harvested in the same way as for *S.cerevisiae* (see 2.2.1)

2.2.5 Growth of *P.chrysogenum* P1

Inoculum was from spores produced on agar plates (Keshavarz 1989). A spore count of greater than 10^9 spores per ml in the inoculum broth was used. The inoculum was grown in a 2L unbaffled shake flask containing 200ml of growth medium (as production medium with the addition of 50mM phosphate buffer pH 6.5). The flask was stoppered lightly with sterile cotton wool and then incubated at 26°C, on an orbital shaker at 150 rpm until sufficient biomass had been produced (approximately 20g/L).

The reactor containing 1L of sterile medium was inoculated with 10% (v/v) inoculum broth.

Production medium was as follows:

Components	g/L
Sucrose	10
Lactose	100
KH_2PO_4	7.5
Na_2SO_4	1
$FeSO_4$	0.18
$CaCl_2$	0.05
$MnSO_4.5H_2O$	0.05
$CuSO_4.5H_2O$	0.008
EDTA	0.55
$MgSO_4.7H_2O$	0.7
NOPCO antifoam	1ml/L

The pH was controlled to 6.5 by addition of 2M H_2SO_4 and NH_4OH, the temperature was maintained at 26°C and the culture agitated at 400-800rpm, with an air flow ranging from 0.5vvm to 1vvm.

Sterile phenoxyacetic acid (POA) was added at 1g/L to the reactor. After the mycelial cells had reached 5g/L of biomass, 200ml aliquotes of the culture were aseptically decanted into 2L unbaffled sterile shake flasks (this allowed an even distribution of biomass in the same growth state to promote an accurate reflection of any subsequent treatment in each of the test flasks). After the addition of the cell broth any organic solvent, where applicable was added to the flasks. The flask were incubated at 26°C, on an orbital shaker at 150 rpm, for 180 hours. Samples were removed by aseptic technique for assay. The samples were spun at 2000rpm in 20ml universal bottles before decanting the supernatants which were then frozen at -20oC and stored until assayed by HPLC.

Dry weights

5ml samples of whole broth were removed for dry weight estimation (see dry weights).

2.2.6 Growth of animal cells

The stock cell line were rejuvenated from -70°C by slowly warming to 36oC and were then grown in roller bottles. All transfers were done in laminar flow cabinet.

Medium and method

Chemically defined serum free medium (supplied by J Waite) was used. The medium was sterilised by vacuum filtration through 0.2 micro meter Millipore pak 40 (Millipore). The cells were rejuvenated and added to the medium (pH 7.1) and incubated at 36.4°C at 2 rpm (Gallenkamp plus series), in 2 liter Bibi roller bottles supplied through ICN Flow (Spiers 1985).

Once grown the hybridoma cell stock culture was stored at 36.4 °C and 5% CO_2 in air was gassed into the flasks for daily pH control. The cells were diluted three times a week with fresh medium to 10^5 cells per litre.

Harvesting cells

The cell contents from the spinner flasks were centrifuged at 2000 rpm for 4 min. The supernatants were decanted and the cells were then carefully resuspended in 0.02M Phosphate (5ml) buffer (pH 7.0) by gentle rolling. The cells were then carefully added to the challenge tank.

2.2.7 Growth in the presence of organic solvent

Y.lipolytica was grown in the presence of a number of alkanes to determine the solvent log P value below which no growth occurred.

The medium for growth was yeast nitrogen base (YNB from Difco) made up at 0.67%(w\w) concentration and filter sterilised through Gibco (FSA) filter sterile filter with no glucose supplement.

Cells from an overnight culture were inoculated into sterile test tubes which were stoppered with sterile cotton wool to prevent aerial contamination, but allow free passage of air. 2ml of an alkane from the series pentane-octane were carefully added to form a second liquid phase, except for the control which contained no solvent. The tubes were shaken at 28°C,and 200rpm overnight. The tubes were sampled in a sterile fashion by microscopic examination for growth. If no growth had occurred the tubes were reincubated. No growth was recorded if cells failed to grow after 48 hours.

2.3 Organic solvent challenge with cells

2.3.1 Beta-dispersion

2.3.1.1 Challenge tank in beta-dispersion type experiment

The instrumentation was set up as described in section 2.1 .The tank dimensions for these experiments were 5cm by 7cm. The tank was covered to reduce excessive solvent evaporation. All experiments were carried out in a fume cupboard.

The volume additions were as outlined in 'set up'. Samples were removed from the challenge tank by pasteur pipette for microscopic examination, or by syringe for the estimation of the OUR using the Clarke oxygen electrode.

S.cerevisiae, Y.lipolytica and P.putida

The challenge tank was arranged as shown in figure 2.1, The probe was mounted vertically as described in set up section. The magnetic bar used was one of two sizes, either 3cm (large) in length operated at 500rpm used in dispersed organic in aqueous experiments or 1.5cm (small) in length operated at 250rpm, used in lewis cell arrangements, see individual experiments for any deviations in details.

Suspension buffer for beta-dispersion assay

The suspension buffer was 50mM potassium phosphate buffer pH 7.0. The buffers were made up by using solutions of the dipotassium hydrogen and potassium dihydrogen orthophosphate (50mM) to the pH 7.0 measured by pH meter (Pye unicam).

Animal cells

20 ml of 0.02 M phosphate buffer pH 7.0 was added to the challenge tank. The suspension buffer was maintained at 37°C and stirred by a magnetic bar (1cm by 0.2cm) at 50rpm (measured). A base trace was obtained before addition of cells. A lower biomass of animal cells was added to the challenge tank (see results) than in the yeast experiments.

2.3.1.2 One volume challenge

The suspension buffer was added to the challenge chamber then left to equilibrate until a steady signal was obtained, ensuring that no bubbles were entrained between the electrode pins. The cells were then added from the stock suspension of cells to the required starting capacitance (20pF unless otherwise stated). The reading was stable within one minute. The challenge organic solvent was added to the cells suspension carefully by slow addition at the edge of the tank and aqueous surface by Gilson pipette.

2.3.1.3 Second-liquid phase with aqueous saturated and unsaturated

The aqueous buffer was saturated outside the challenge tank in a glass measuring cylinder of appropriate size (100ml to 1000ml volume) with a PTFE and glass side arm positioned such that it is near the bottom of the cylinder, allowing the decanting of the aqueous without disturbing any second-liquid phase layered on top of the aqueous. The suspension buffer was added with a magnetic bar. A second liquid phase of the required organic solvent was then layered onto the aqueous phase, by very carefully pouring it down the inside of the cylinder, so that the organic aqueous interface remained unbroken. The cylinder was then placed on a magnetic stirrer and the agitation of the magnetic bar set so that the interface was not disrupted. The temperature control was provided by a water bath hose encircling the cylinder, set to the required temperature of the solvent challenge.

The aqueous was then transferred (after 72 hours for equilibrium) to the challenge tank in a glass beaker and an immiscible solvent was then added by careful layering, if required for the experiment.

2.3.2 Biotransformation

P.putida

Buffer media

The biotransformations were carried out in 50mM potassium phosphate buffer pH 7.0. The buffers were made up by using solutions of the dipotassium hydrogen and potassium dihydrogen orthophosphates (50mM) to give pH 7.0 measured by pH meter.

75ml aerated stirred tank reactor

Biotransformations were performed in glass reactors (working volume 60ml, see figure 2.2) stirred at 800 rpm. The temperature was maintained at 28°C by partial immersion in a water bath. The aeration was controlled by a needle valve attached to a flow regulator (0-100 cc/min).

The cell concentration in the reactors was kept below 0.5g dry cell weight per litre of aqueous phase to avoid oxygen limitations (Harrop 1990).

Two liquid phase system

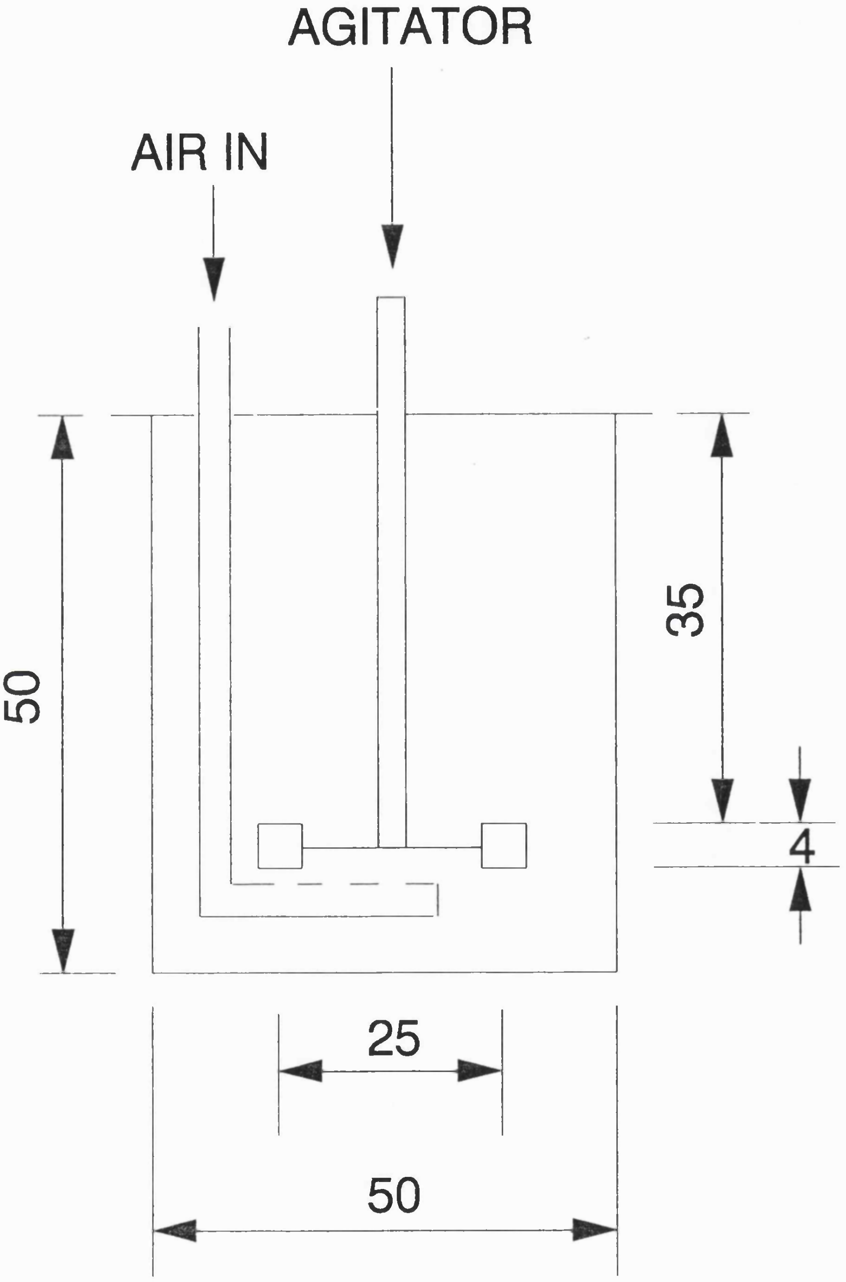
Biotransformations were carried out at a phase ratio of 1:1, organic solvent to phosphate buffer, unless otherwise specified. Glucose was added to 8 g/L of the aqueous phases and naphthalene was added to a concentration of 80-100g/L organic phase. This concentration allowed maximal conversion rate in the reactor (see Harrop 1990). Solvents were allowed to equilibrate for 60 minutes before the addition of cells. The 5ml cell addition was considered as belonging to the aqueous phase, bring the working volume to 60ml.

Y.lipolytica

The cells were grown as described previously at a pH 6.5 and pH 7.0 to alter the activity of the cells in a similar manner discovered to raise the activity of *P.putida* (Brazier 1989). The biotransformation was carried out as for *P.putida*.

Figure 2.2

Biotransformation reactor


AGITATOR
AIR IN
50
35
4
25
50

75ml biotransformation reactor
(units in mm)

2.3.3 Microslide organic solvent challenge

Cells mixed with methylene blue were added to a glass slide (haemocytometer) with two longitudinal wells next to one another, counter sunk so that a cover slip bridges both wells, which were both below the level of the cover-slip. To the second deeper well, an organic solvent could be carefully added without disturbing the cells. A cover slip can then be added. The slide was then observed under a microscope (Olympus BH-2) with attached camera (Olympus OM-2N), to note the change in permeability of the cells and their oxidation potential by their staining over a recorded time period.

2.3.4 Adhesion to organic solvent interface

Cells to be tested were suspended in 5ml aliquots of phosphate buffer 50mM, pH 7.0, in test tubes of approximately 1cm diameter. 1ml of test organic solvent was then added to the cell suspension, which was then mixed vigorously for 60 seconds. The suspension was then allowed to settle for 10 minutes before an aqueous sample was removed and the absorbency read against the corresponding blank. This method was an adaptation from Rosenberg et al (1980), Lichtenberg et al (1985) and Harrop (1990).

2.4 Oxygen uptake rate (OUR)

One of the most successful electrodes for biochemical work has proved to be the Clark electrode membrane covered (Lessler 1989), its chief advantage being the isolation of the electrode current.

The Clark oxygen electrode consisted of an electrode covered with a 25 micron thick Teflon membrane stretched over a minimal amount of electrolyte (a saturated solution of potassium chloride). This is covered with a fine high quality filter paper with a small hole made in its centre. This was held in place by a rubber 'O' ring which sealed the membrane over the electrode. The assay chamber was made of glass (with an integral water jacket for temperature regulation) which fitted over the electrode and sealed onto the 'O' ring, which formed the base of the assay chamber. A piston with a sealing 'O' ring was inserted into the top of the electrode, the piston had a fine hole bored through its centre to allow trapped air to exit when the assay chamber was sealed.

The volume of the assay chamber was 8ml in total, the working volume of the chamber was 6.5ml. Samples of 100 microlitres were used for the assay of cell OUR. The samples were kept in suspension by a needle magnetic bar encased in a sealed glass sleeve placed over the electrode and operated by a magnetic stirrer, integral to the electrode base. Full scale deflection (FSD) was set to 5mV and zero at 0mV.

The suspension buffer was potassium phosphate 50mM at pH 7.0, made up as a stock solution and equilibrated at 26°C prior to use. 6.5ml suspension buffer was added and equilibrated (a steady base line obtained) before the addition of cells. The signal was recorded on a chart recorder (Lloyd).

The Clarke electrode was run with buffer until it equilibrated. After a steady baseline had been achieved showing the stability of the reading, solvents were added as a control to observe any change in reading due to the solvent itself. None was observed with any of the solvents used.

The membrane was replaced daily. Samples were introduced by a syringe (Hamilton 200 microlitre) with integral needle through the fine hole bored into the to sealing bung. The syringe was washed out between each sample and then rinsed out with suspension buffer.

2.5 High Pressure Liquid Chromatography

2.5.1 Normal phase

Sample preparation for HPLC

Samples of 200 micro litres were removed from the stirred tank reactors into 30 ml solvent resistant glass screw-top bottles. These contained 5ml of 1,2-dichloroethane which was vortexed with the sample for 10 seconds to allow substrate to partition into the 1,2-dichloroethane. This was then sealed into glass vials and used to load onto the HPLC column. Samples containing air from bubbles from with in the reactor were replaced and taken again.

High pressure liquid chromatography (HPLC) was used to determine the levels of naphthalene (Substrate) and any product formed such as naphthalene-1,2-dihydro-1,2-diol during the biotransformation (see results).

Analysis was done with a Milton Roy CM 4000 multiple solvent delivery pump in series with a Milton Roy UV Spectro monitor 3000 variable detector set to 254 nm. The separation was achieved by a Techsil 10 CN 25 cm column from HPLC Technology. The mobile phase was 1,2-dichloro-ethane Hipersolv for HPLC (BDH) with 2% Methanol (v/v) HPLC solvent (FSA). The flow rate was set at 1ml per minute. Solvent was degassed by sonication in a water bath for 20 min. Samples were loaded onto the column automatically by a Promis 11 autosampler, using a flush volume of 90 micro litres to prevent carry over.

The column retention times were found to be 3.2 and 4.1 min for naphthalene and naphthalene-1,2-dihydro-1,2-diol. Standard curves were produced from product concentration versus peak area arbitrary absorbency units (AU).

2.5.2 Reverse phase

Assay for naphthalene and its products

Analysis done on a Milton Roy CM 4000 multiple solvent delivery pump in series with a Milton Roy UV Spectro monitor 3000 variable detector set to 254nm. The separation was achieved by a C18 micro Bondapack, 30 cm column from HPLC Technology. The mobile phase was run by gradient,50% methanol:water to 95% methanol:water. The flow rate was set at 0.4ml per minute. Solvent was degassed by sonication in a water bath for 20 min. Samples were loaded (10 microlitre) onto the column automatically by a Promis 11 autosampler, using a flush volume of 90 micro litres to prevent carry over. Standards were run individually to estimate peak retention times. Method modified from Cerniglia et al (1981,1979)

Assay for penicillin V

Samples were thawed before loading onto the column. Analysis was performed on an Milton Roy CM 4000 multiple solvent delivery pump in series with a Milton Roy UV Spectro monitor 3000 variable detector set to 220nm. The separation was achieved by a C8, spherisorb, 25 cm column from HPLC Technology. The mobile phase was acetonitrile (200ml) Hipersolv for HPLC (BDH) with deionised water (300ml) plus phosphate buffer 0.15M pH 5.0 (500ml) (FSA). The flow rate was set at 1.5ml per minute. Solvent was degassed by sonication in a water bath for 20 min. Samples were loaded (10 microlitre) onto the column automatically by a Promis 11 autosampler, using a flush volume of 90 micro litres to prevent carry over. Standards were run individually to estimate peak retention times. Method derived from Miners (1985) and Meffin (1980).

2.6 Viability assay

2.6.1 Methylene blue

Since the 1920's methylene blue has been used as the standard method for the estimation of yeast viability. With only slight modification over the years it gives an accurate method for cell viability (reference for assay Lee et al 1981).

Methylene blue viability assay

Materials

Methylene blue 0.025% (w/w)

Ringers' salt solution:

	g/100ml
Sodium chloride	0.9
Potassium chloride	0.042
Calcium chloride	0.048
Sodium hydrogen carbonate	0.02
Glucose (when added)	1.0

Methylene blue and Ringers' solution were mixed to make a methylene blue solution.

Method

Yeast cells suspensions of concentration 2-4*10^8 cells per ml diluted with 'Ringers' solution when necessary. Then 0.1ml of diluted cells were mixed with 0.9ml of methylene blue solution. The solution was loaded onto a counting chamber and a standard counting procedure was followed, for viable cells and non-viable cells (blue).

$$\text{Percentage viability} = \frac{(\text{number of colourless cells})*100}{(\text{number of colourless cells})+(\text{blue cells})}$$

When treated with methylene blue the cells remained viable for more than 2 hours. Cells were counted within 10 minutes of sampling throughout the experiment.

2.6.2 Trypan blue

Trypan blue assay was used to determine animal cell culture viability (Murhammer et al 1988).

Samples were removed (100 micro litres) at different intervals and mixed with an equal volume of trypan blue 0.5% (w/v). The total number of cells and the number of non-viable cells (blue) were counted within five minutes of removal onto a haemocytometer.

2.7 Cell quantum determination

2.7.1 Cell counting

Cells were counted in a bacterial counting cell (FSA) by microscopic examination, using the usual counting technique.

2.7.2 Optical density determination

Optical density was measured at 600nm unless otherwise stated, using a pye unicam PU 8600 UV/VIS spectrometer. The culture samples were measured in a 1 cm path length cuvette, against a water blank. Samples were diluted into the range of 0-0.5 AU., using phosphate buffer pH 7.0. Some of the solvents were able to turn the plastic cuvettes opaque, quartz cuvettes were used when OD measurements were made in the presence of organic solvents.

2.7.3 Dry weight determinations

Dry weights were carried out by filtration on Whatman membrane cellulose nitrate filters, 47mm diameter, 0.2 micro meter pore size. Papers were dried over night in a 90°C oven before being removed to a desiccator to cool before being weighed. The same procedure was used for papers plus filtered cells.

2.7.4 Glucose determination

Glucose presence was determined by use of glucose clini-stick (FSA).

2.8 Chemicals and equipment

All chemicals were supplied from Sigma at analytical grade unless otherwise stated. For list of equipment and companies see appendix I.

CHAPTER 3

CHAPTER 3
RESULTS

3.0 Organisms selected

Two eukaryotic organisms were initially selected (*S.cerevisiae* and *Y.lipolytica*) and a Gram-negative bacterium (*P.putida*). The aim was to compare and contrast these organisms using their beta-dispersion and naphthalene hydroxylation by both *Y.lipolytica* and *P.putida*, which in their wild type can also grow on hydrocarbons. The *P.putida* mutant chosen (UV4) is biochemically modified so that it can no longer grow on the hydrocarbons. Animal cells were also used to compare the yeast cells to another eukaryotic cell without a cell wall.

The focus of this project was to observe any underlying trends in cell stability when challenged with an organic solvent media and to outline important parameters involved in determining that stability.

3.1 Introduction to experimental approach

In choosing an experimental approach, it is necessary to define toxicity of an organic solvent in terms of the physical parameters influencing the cell stability. These include the following:

-Aqueous organic solvent concentration
-Second liquid phase
-Type of organic solvent
-Type of organism

-Agitation conditions
-Time(s) taken as analysis point(s)

It has already been reported in the literature that the first site of action of organic solvents is probably the first organelle it comes into contact with, i.e. the cytoplasmic membrane (Hoek 1988,Ingram 1984, and Tsuchiya 1987). This project focuses on organic solvent effects on the cell membrane.

When developing a method it is important to set the working parameters so that variations in the selected parameter will enable unambiguous and reproducible results to be obtained.

This project is aimed at identifying the underlying trends of biocatalyst stability in organic solvents. To this end a novel approach has been adopted:- the exploitation of the cell beta-dispersion, which can be detected in all living cells with a cytoplasmic membrane. To establish the validity of this process it was compared against other assays and used to further explore basic underlying trends which have been established in the literature.

A brief overview of the development of the experimental approach is given in Chapter 4.1

3.2.1 Calculation of the probe constant

The effective cell constant of the standard 25mm type four electrode probe is usually around 0.8cm^{-1} (Kell 1990). This value can be determined by measuring the conductance of a solution of known conductivity. A solution of 10mM KCl at 298K has a conductivity of approximately 1.413mS/cm. Since :

$$\text{Cell constant} = \frac{\text{Conductivity of test solution}}{\text{Bugmeter conductance reading}}$$

This constant will vary for the different ranges used. For the probe used here the cell constant was:

0.868cm^{-1}

This is well within the expected range of a probe according to the manual.

3.2.2 Cross talk of probe

A cross talk (interference of the probe with itself) plot will show the linearity of response characteristics of the probe used.

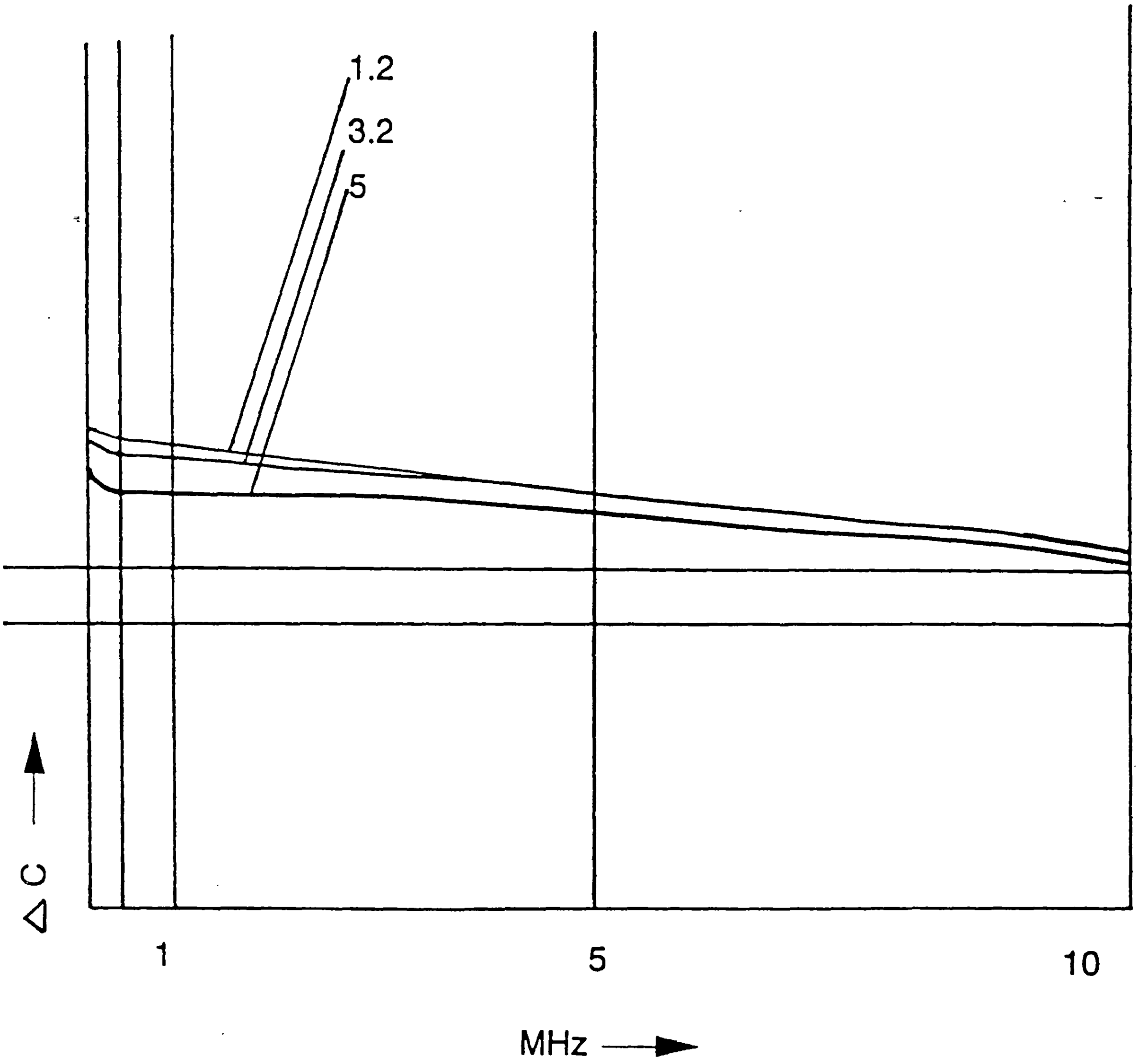
The readings for the probe used (figure 3.1) are taken in a low ionic solution of potassium chloride (-3pF) without the addition of cells (the parallel bar is a delta 2pF reading, also shown are three values of G for probe).

A one second time response was selected on the frequency selector, which was set on low level (sensitive level) to give a high attenuation response level in relation to the conditions used.

The graph for the probe is displayed on log linear axis (figure 3.1). Since the cross talk is characteristic of the probe itself and relates to any self interference the graph type is referred to as cross talk i.e. where a small fraction of the conductance signal erroneously adds to or subtracts from the capacitance reading. With this probe G can be seen to make relatively small changes.

FIGURE 3.1

Cross talk of probe



Graph of capacitance against frequency to illustrate cross talk of the bugmeter probe, in KCL solution.

3.3 Dielectric characteristics of study organisms

Plots of cell capacitance versus log of frequency (figure 3.2 and 3.3) give an inverted sigmoidal type curve. At the critical frequency (fc) value the data shows a clear increase with conductance. The measured frequency (fm) value over this range remains constant only varying by a fraction of a capacitance unit over the whole range. Thus over the range of frequencies used the critical frequency is dependent on conductance of the medium while the measuring frequency is independent (Note that these values were recorded on the same batch of yeast).

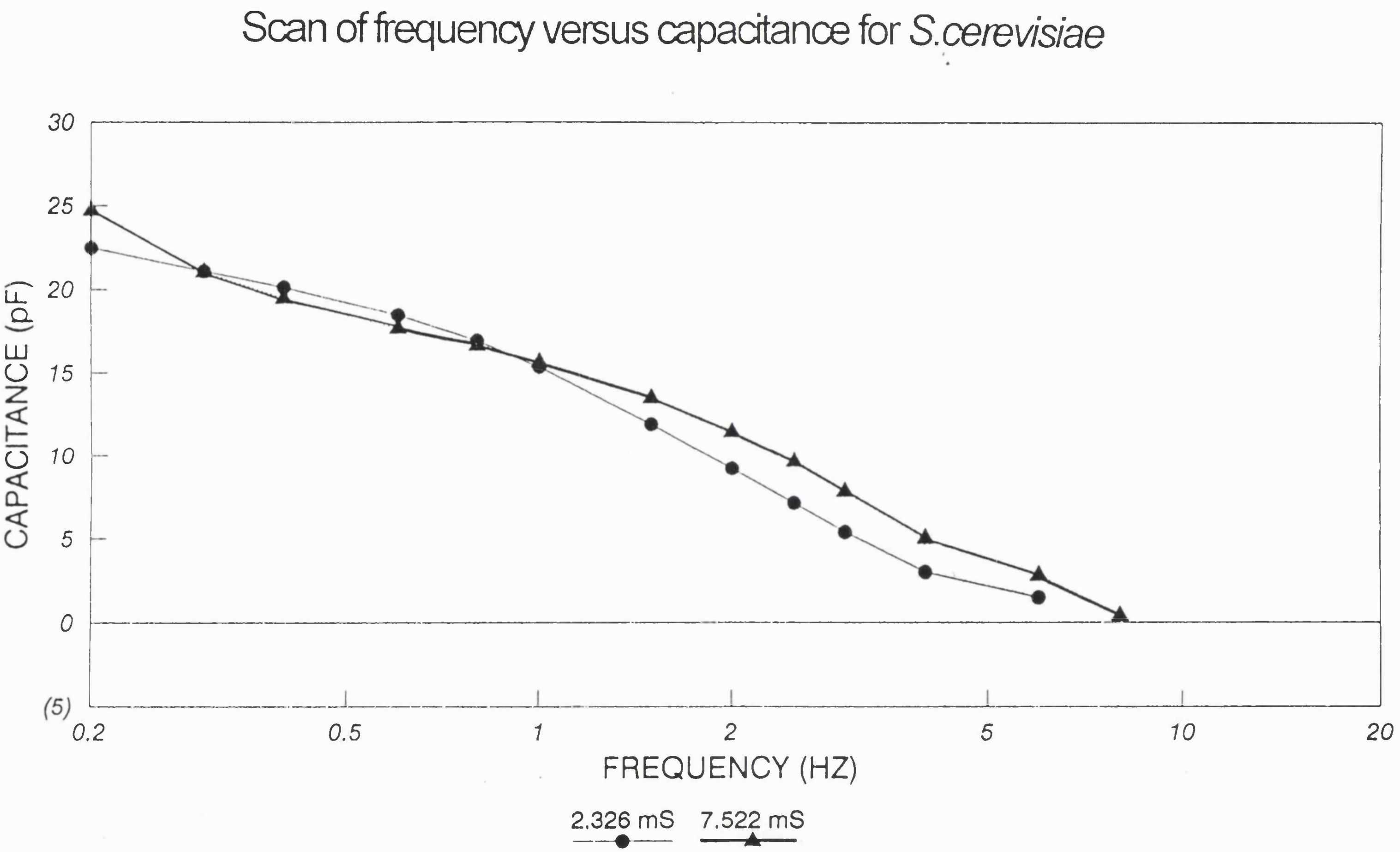
The phosphate buffer plus suspended yeast gave a recorded conductance of 2.36mS. After addition of 1ml of octanol the conductance increased by 0.8mS from its original value, well within the acceptable limits for accurate fm readings. Figure 3.2 demonstrates that the cell beta-dispersion as measured by capacitance could be followed in a wide range of solutions with varying conductivities, without affecting the capacitance measured at the frequency fm.

No alpha-dispersion is present in the trace, which indicates that this yeast has a poorly charged cell envelope as described by other workers (Harris and Kell 1985).

Frequency scan versus capacitance with varying biomass concentrations

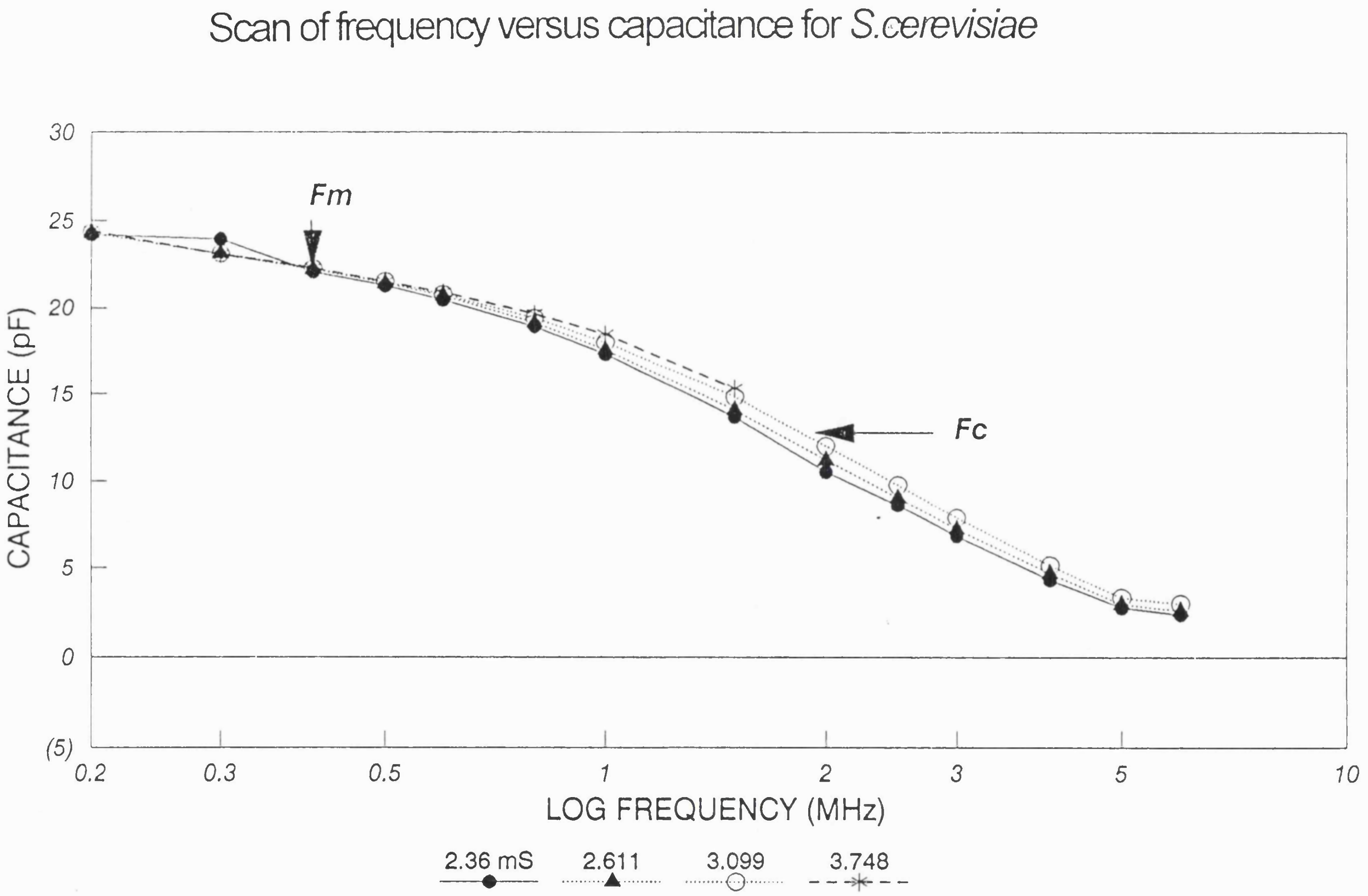
The frequency scan (figure 3.4) illustrates the fundamental concept behind the beta-dispersion measurements. A linear correlation between the recorded capacitance and the biomass (dry weight) was obtained at fm and fc. This is in response to the capacitance stored in an intact cytoplasmic membrane capable of being polarised when subjected to a low frequency electric field (see chapter 1).

FIGURE 3.2



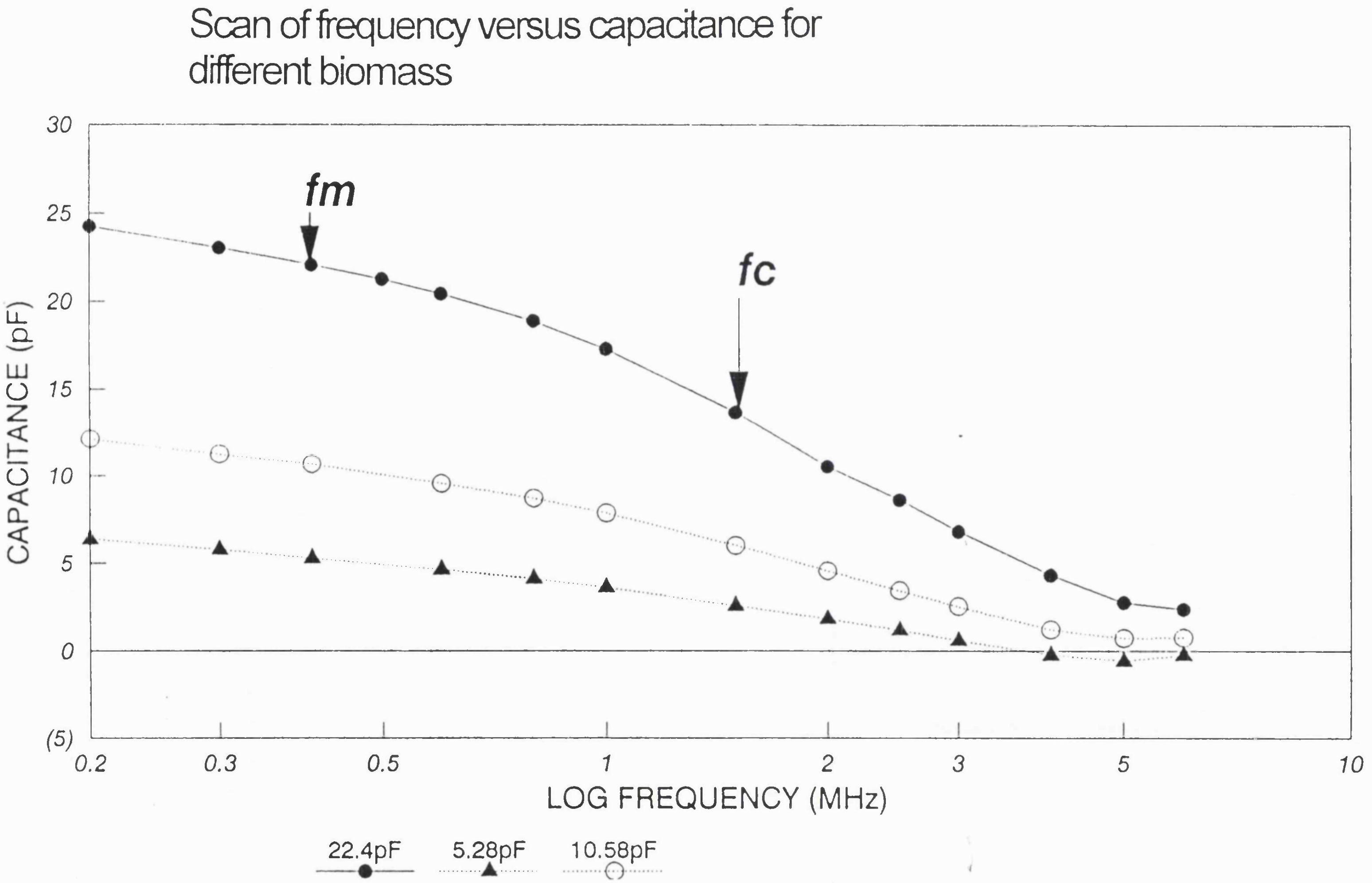
Graph of frequency scan for *S.cerevisiae* suspended in phosphate buffer at two conductivities

FIGURE 3.3



Graph of frequency scan for *S.cerevisiae* suspended in phosphate buffer at several different conductivities

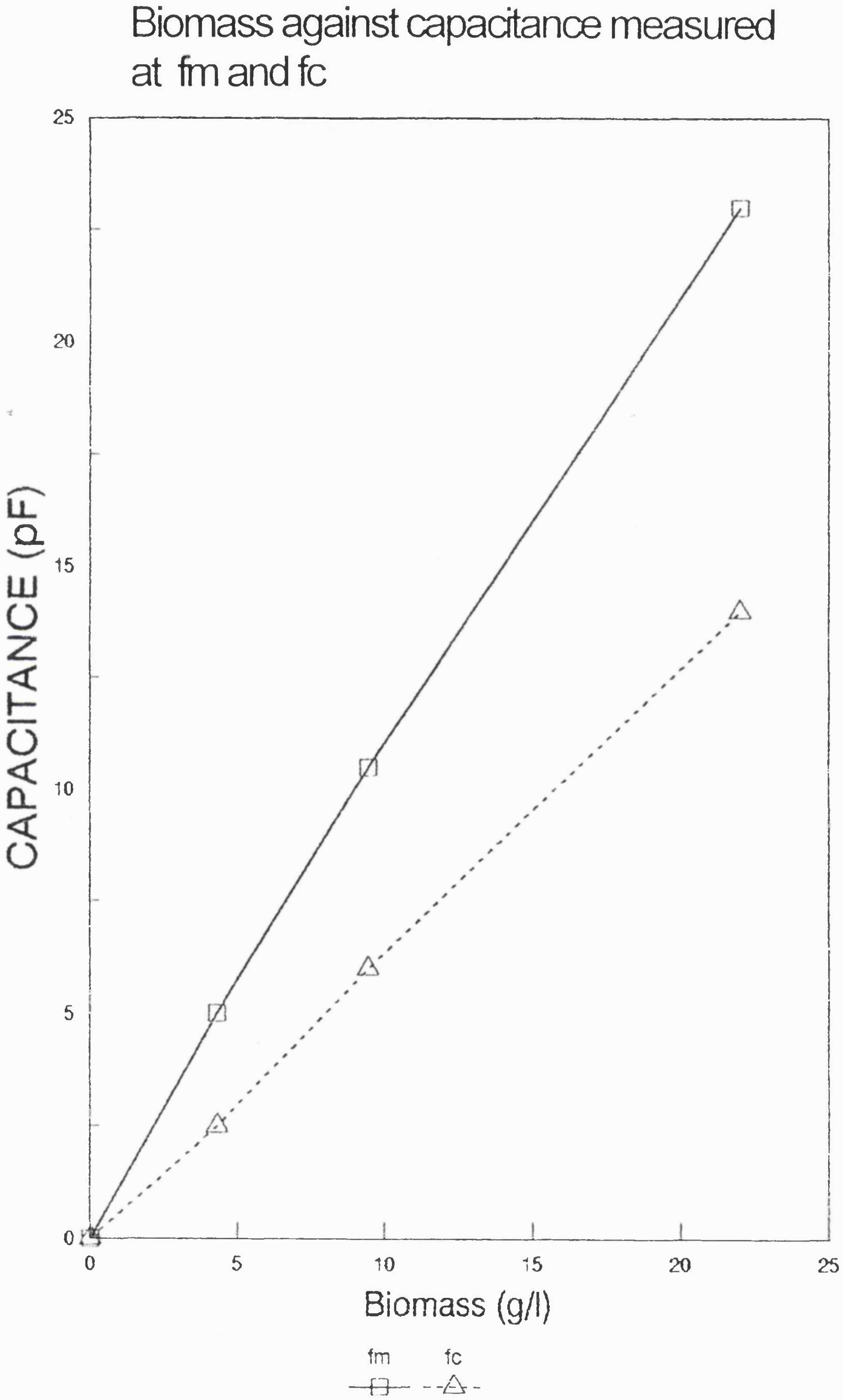
FIGURE 3.4



Graph of frequency scan for *S.cerevisiae* at three biomass concentrations

It is also apparent that the recorded measurements of fc against biomass may give a good correlation, but will be subject to interference from the suspension conductivity, see figure 3.4.1.

FIGURE 3.4.1



Correlation of capacitance at fm and fc against dry weight for *S.cerevisiae* suspended in buffer.

Scan of frequency versus capacitance for octanol treated *Sacchromyces cerevisiae*

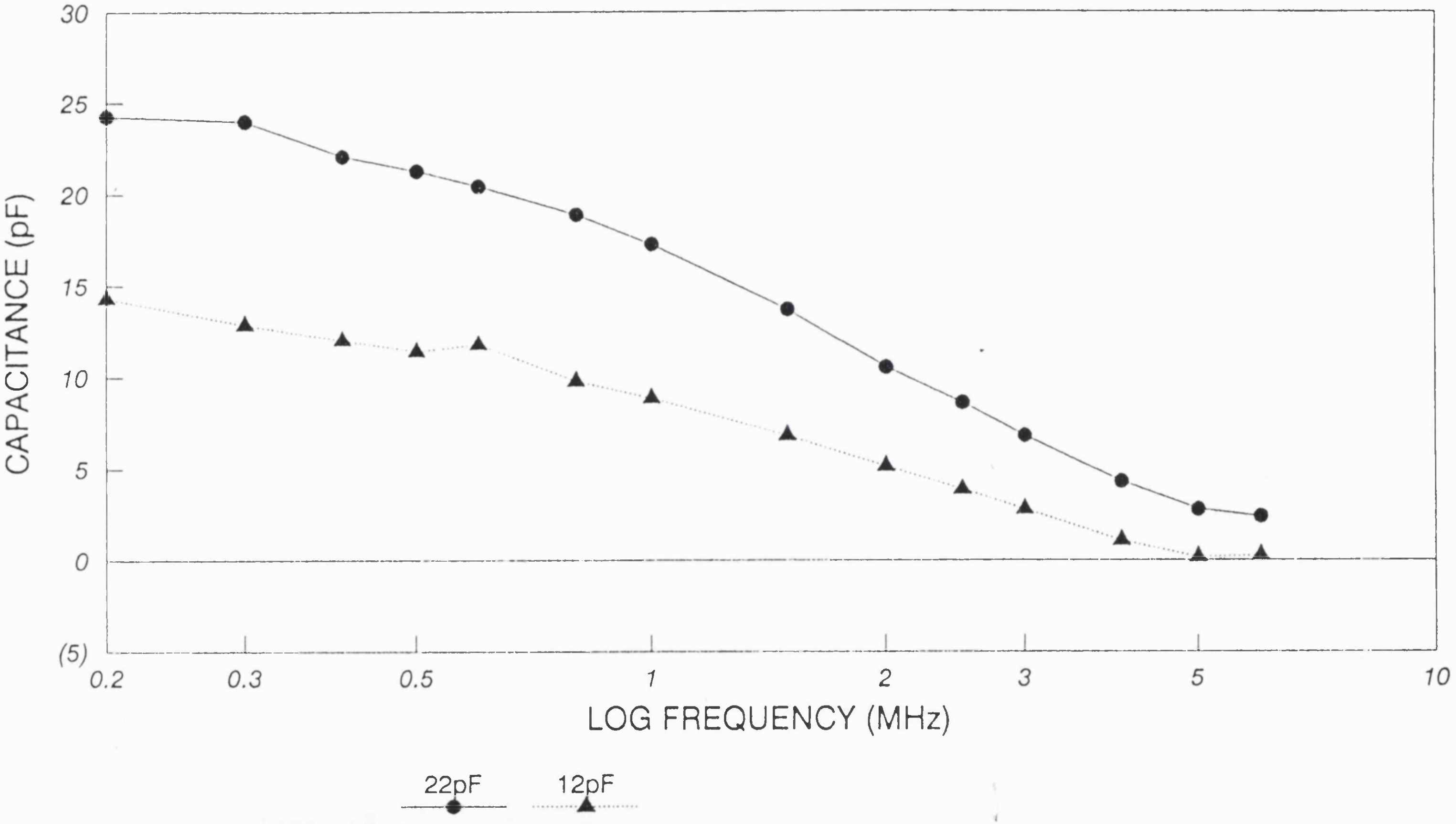
Cells taken prior to solvent addition have a characteristic beta-dispersion (as described earlier) over a range of frequencies. In order to show that the addition of an organic solvent does not interfere with this characteristic, further frequency scans were necessary to enable this technique for comparing solvent toxicity.

This can be carried out as previously described with the addition of a solvent. The solvent chosen as a typical test case was octanol (log P 2.9), it has a relatively high aqueous solubility (4.13mM), and is considered a toxic solvent by many workers.

The solvent addition was made subsequent to obtaining a steady base line, after the addition of buffer and cells (20pF) from the stock reservoirs. They were then allowed to equilibrate. The addition time was marked as zero logged hours, just prior to the frequency scan. After the addition of octanol a drop in the capacitance occurred, at a value approximately equal to capacitance fm/ 2 (at t=0) a further scan was taken (figure 3.5).

The inverted sigmoidal curve characteristic of the beta-dispersion at fm/2 (at t=0) was still present and unaffected after solvent addition, although it induced a drop in the recorded capacitance reading.

FIGURE 3.5



Scan of frequency versus capacitance for *S.cerevisiae* challenged with 1-octanol

Graph of frequency scan of *S.cerevisiae* in buffer during octanol challenge

Size of cell versus the cell permittivity

From an earlier graph the correlation between capacitance and a defined number of cells was established. In figure 3.6 a correlation can also be agreed between diameter and permittivity (Data from Kell 1990).

Frequency scan of *Yarrowia lipolytica* versus capacitance

Figure 3.7 shows the frequency scan of *Y.lipolytica* suspended in an aqueous buffer. This frequency scan is characteristic of the species of yeast examined.

Since fm of the two yeasts is conveniently close, 0.4MHz can be used in both cases. Figure 3.8 gives the frequency scan at two different biomass concentrations.

Comparison of *Y.lipolytica* versus *S.cerevisiae* frequency scan

A clear distinction is illustrated between these two yeasts in (figure 3.9) their respective frequency profiles. Neither yeast shows any deviation from the characteristic inverted sigmoidal curve.

FIGURE 3.6

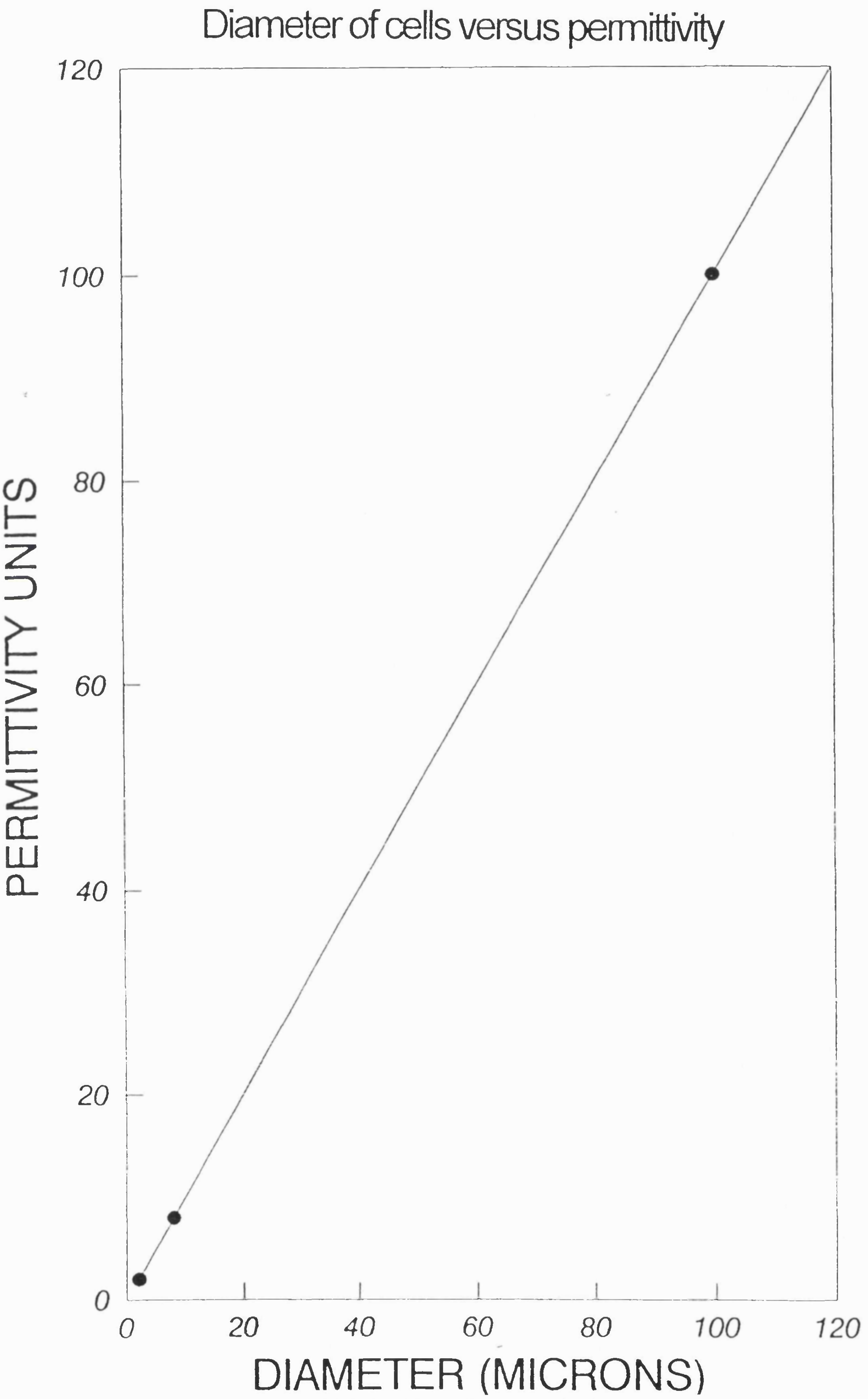
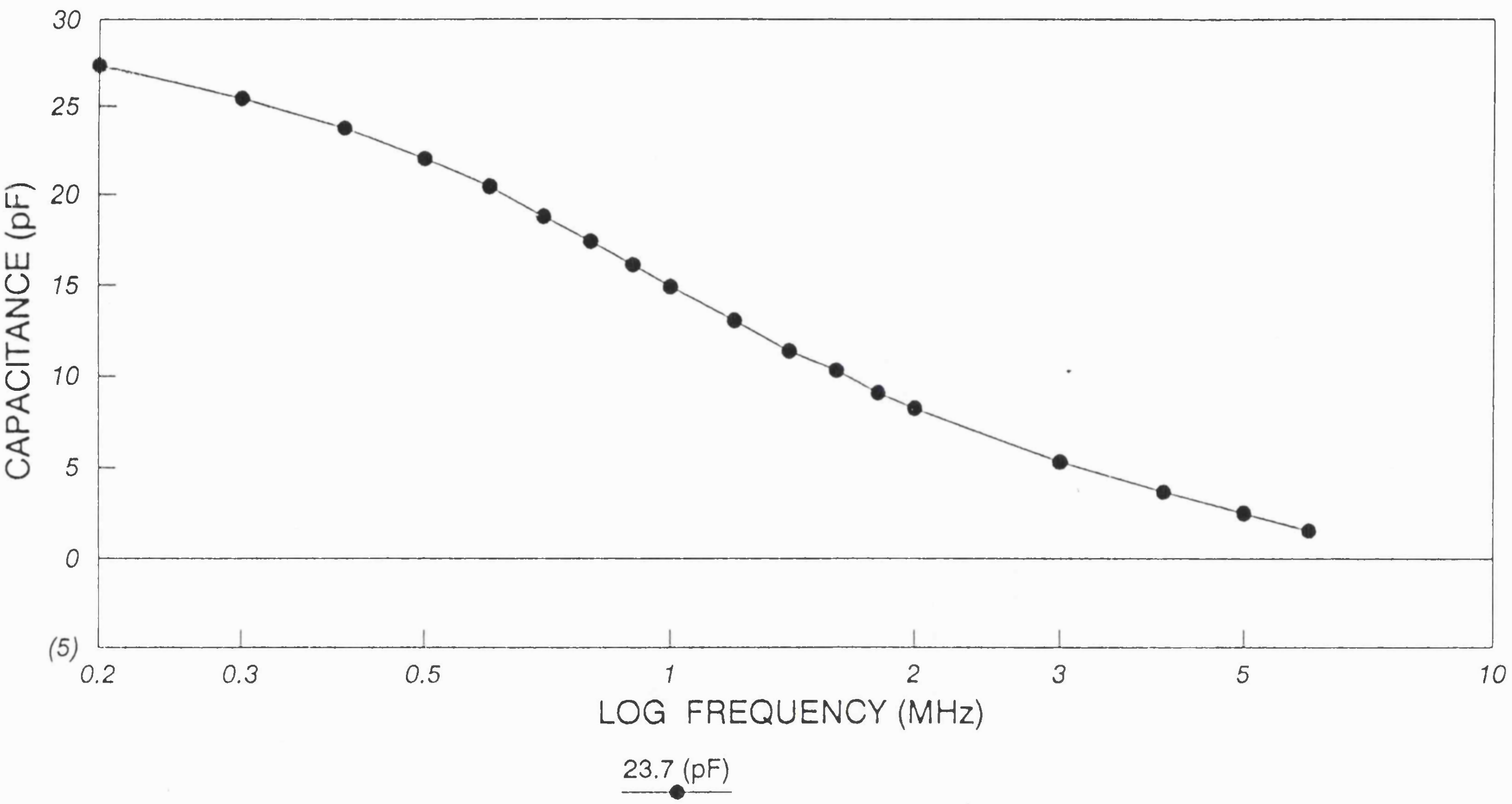


Figure of permittivity (as relates to capacitance) against cell diameter.

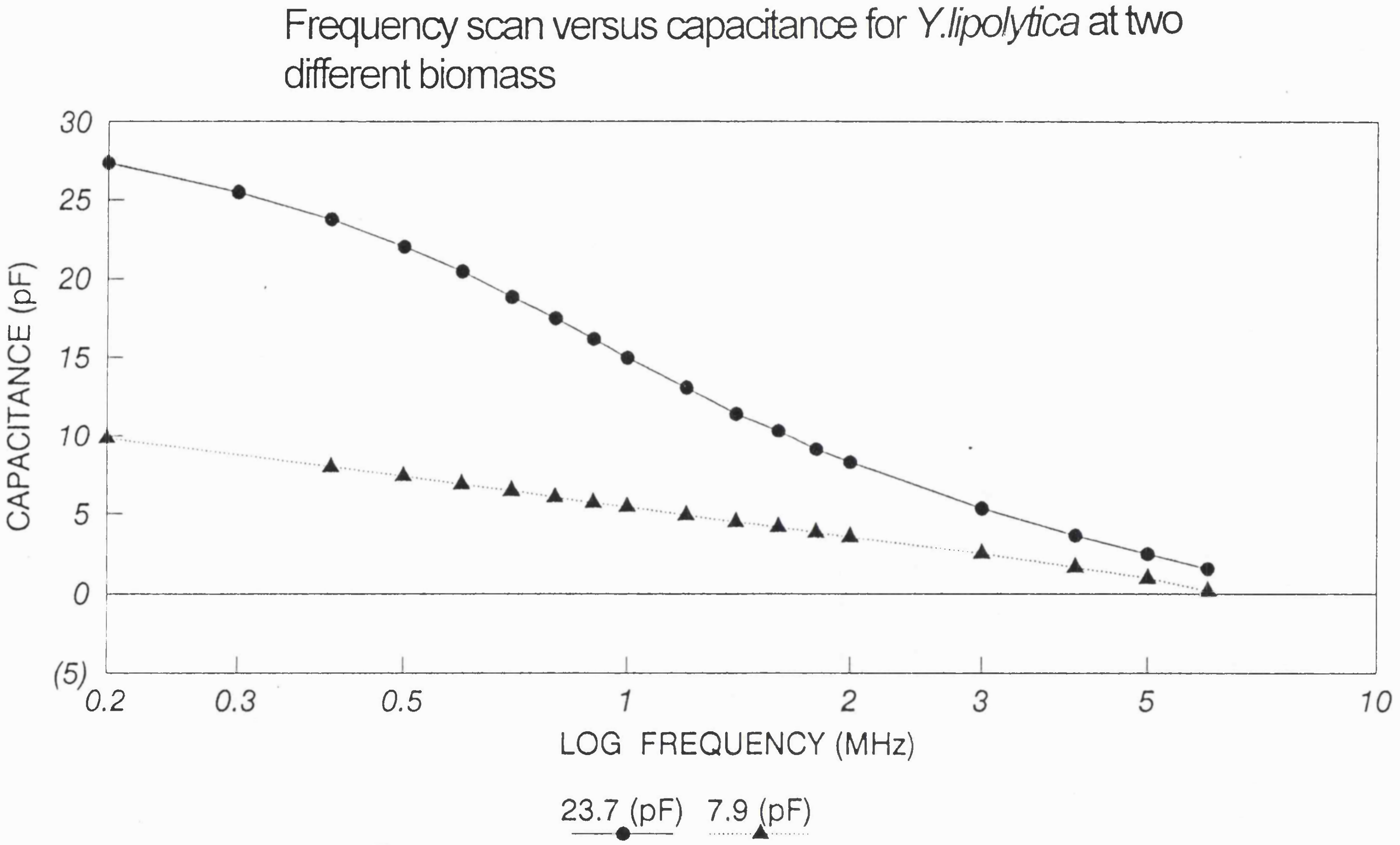
FIGURE 3.7

Frequency scan versus capacitance for *Y.lipolytica*



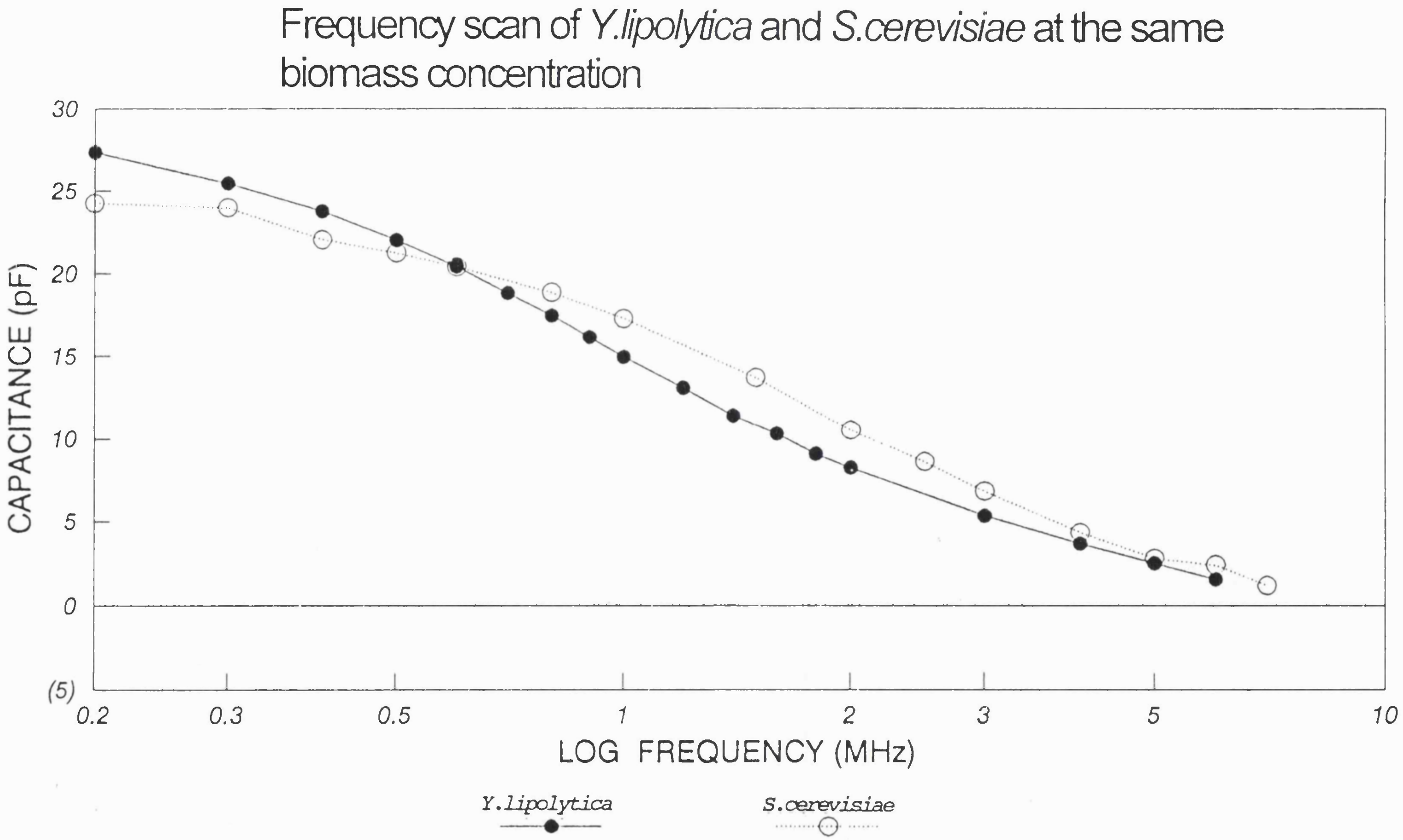
Graph of frequency scan for *Y.lipolytica* suspended in buffer

FIGURE 3.8


Frequency scan versus capacitance for *Y.lipolytica* at two different biomass

Frequency scan versus capacitance for *Y.lipolytica* in buffer at two biomass concentrations

FIGURE 3.9



Frequency scan of *Y.lipolytica* and *S.cerevisiae* at the same biomass concentration

Frequency scan versus capacitance for *Y.lipolytica* and *S.cerevisiae* in buffer at the same biomass concentration.

P.putida frequency curve

Pseudomonas putida also gives the typical beta-dispersion curve, as shown in figure 3.10 on an expanded y-axis curve.

Both the *Y.lipolytica* and the *P.putida* organisms show an inflection upwards at the lower dispersion frequencies, which is absent in *S.cerevisiae* traces. This is a trend that is often associated with traces from Gram-positive bacteria, described as an alpha-dispersion (Kell 1990), and is related to the charged molecules incorporated into the Gram-positive cell wall.

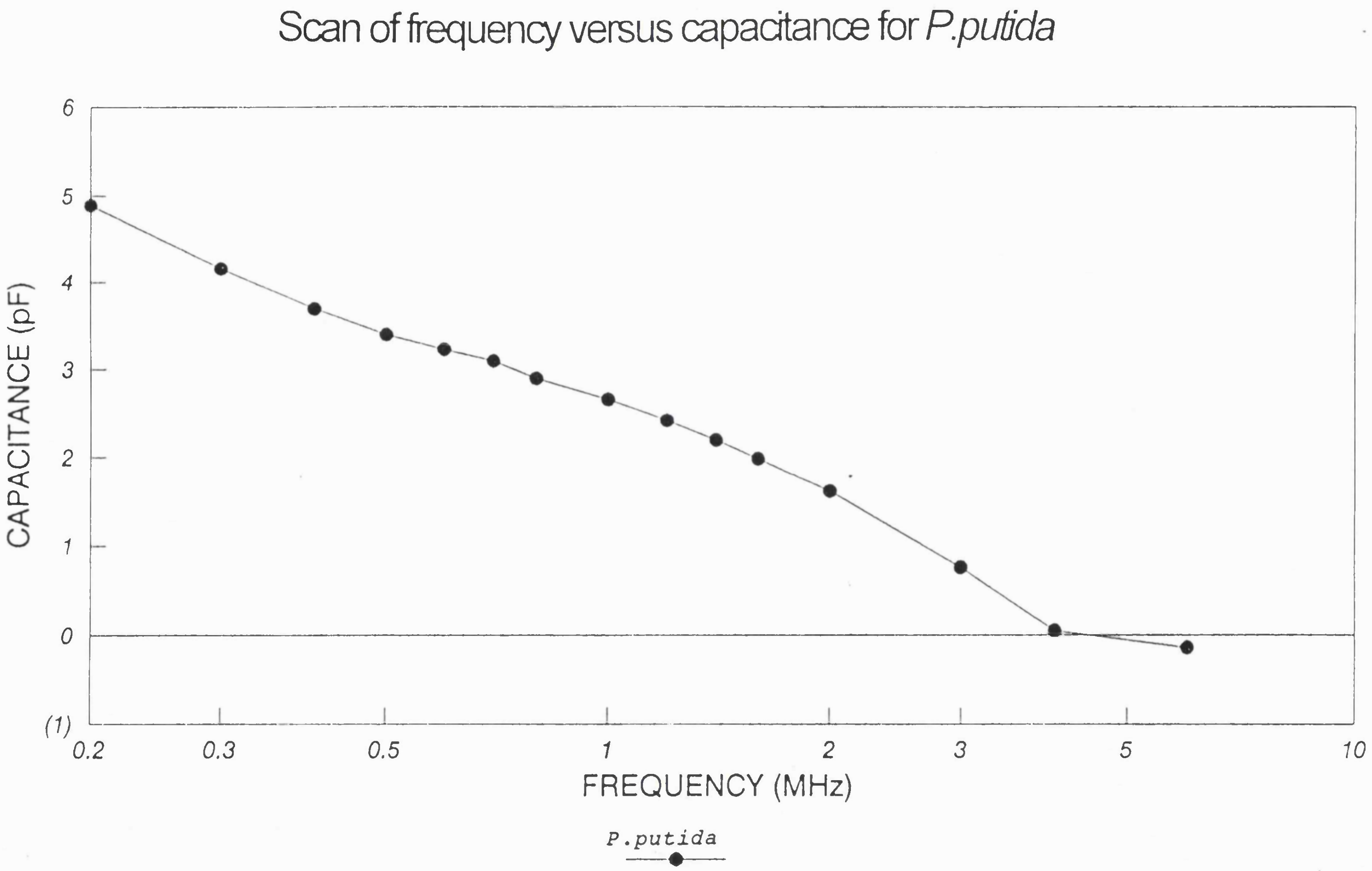
These two organisms have cell walls which are adapted to a two-liquid phase environment, which maybe reflected in their distinctive frequency scans.

3.4 Standard curve of capacitance versus biomass (dry weight)

To establish a correlation between the measured capacitance and cell biomass (dry weights) and optical density at 600nm (OD600). Values were plotted for a range of biomass.

The *S.cerevisiae* cells gave a linear relationship with OD600, dry weight (g/l) and the recorded delta capacitance measured at fm 0.4MHz, as described in figures 3.11 and 3.12. The readings were taken while the cells were stirred in phosphate buffer (10mM) at pH 7.0, in the challenge tank (this medium will not support growth).

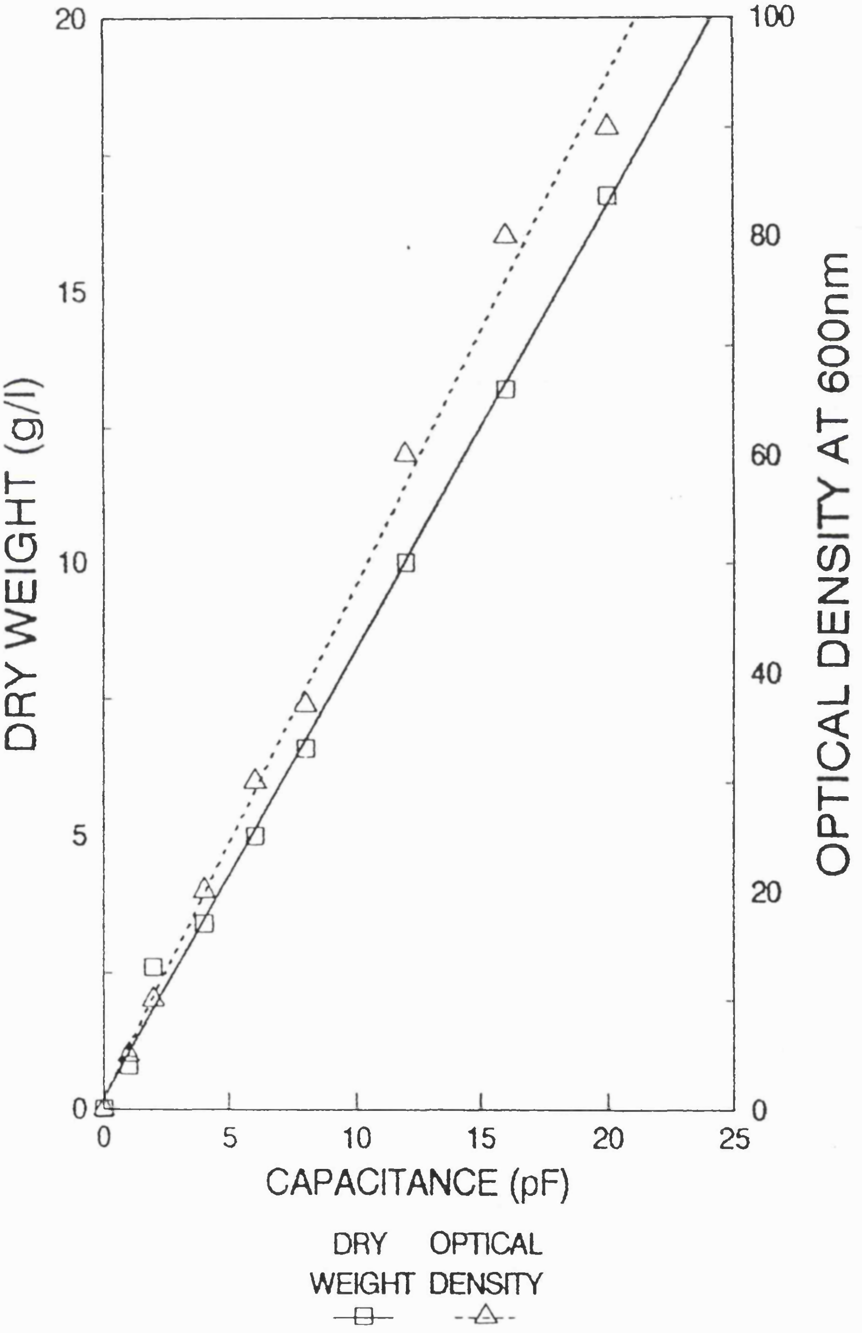
FIGURE 3.10



Frequency scan versus capacitance for *P.putida* in buffer.

FIGURE 3.11

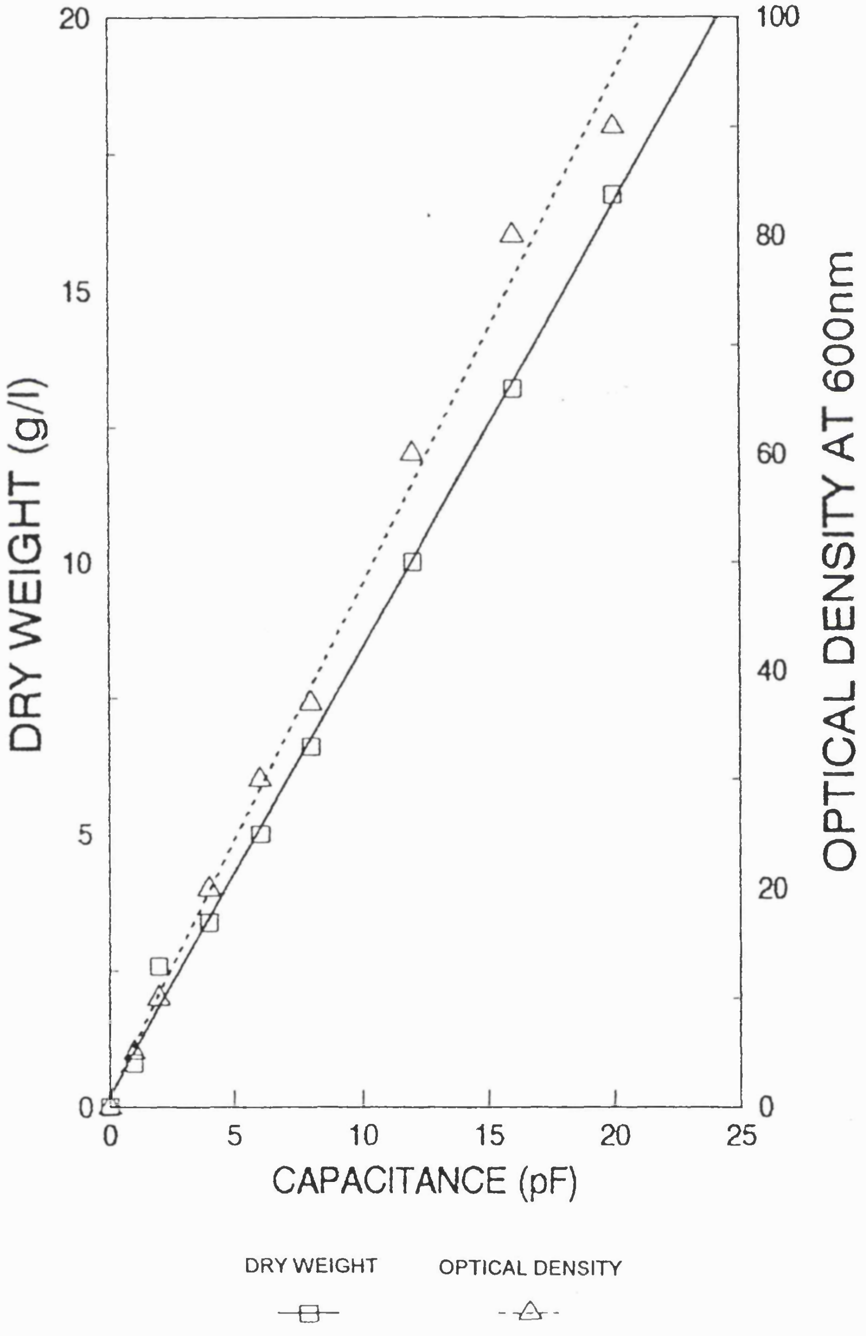
Standard curve of capacitance versus dry weight and optical density for *S.cerevisiae*



Graph of dry weight and optical density against capacitance for *S.cerevisiae*

FIGURE 3.12

Standard curve of capacitance versus dry weight and optical density for *Y.lipolytica*



Graph of dry weight and optical density against capacitance for *Y.lipolytica*

Whilst the OD600 readings had to be suitably diluted before reading and the dry weights had to be read after 24 hours drying, the capacitance readings were instantaneous.

The capacitance of *Y.lipolytica* cells gave a linear correlation with OD600 and dry weight. A cell suspension of 13.5 g/l dry weight gave a delta capacitance of approximately 16pF, which is almost the same for *S.cerevisiae* at the same cell concentration. The corresponding curves for *P.putida* are shown in figures 3.13, 3.14.

3.5 Solvent addition to cell suspensions

Effect of the aqueous/organic interface

To determine any effect of cell adsorption onto the aqueous/organic interface, the location of the cells was investigated by microscopic examination and by monitoring any removal of cells from the bulk aqueous phase.

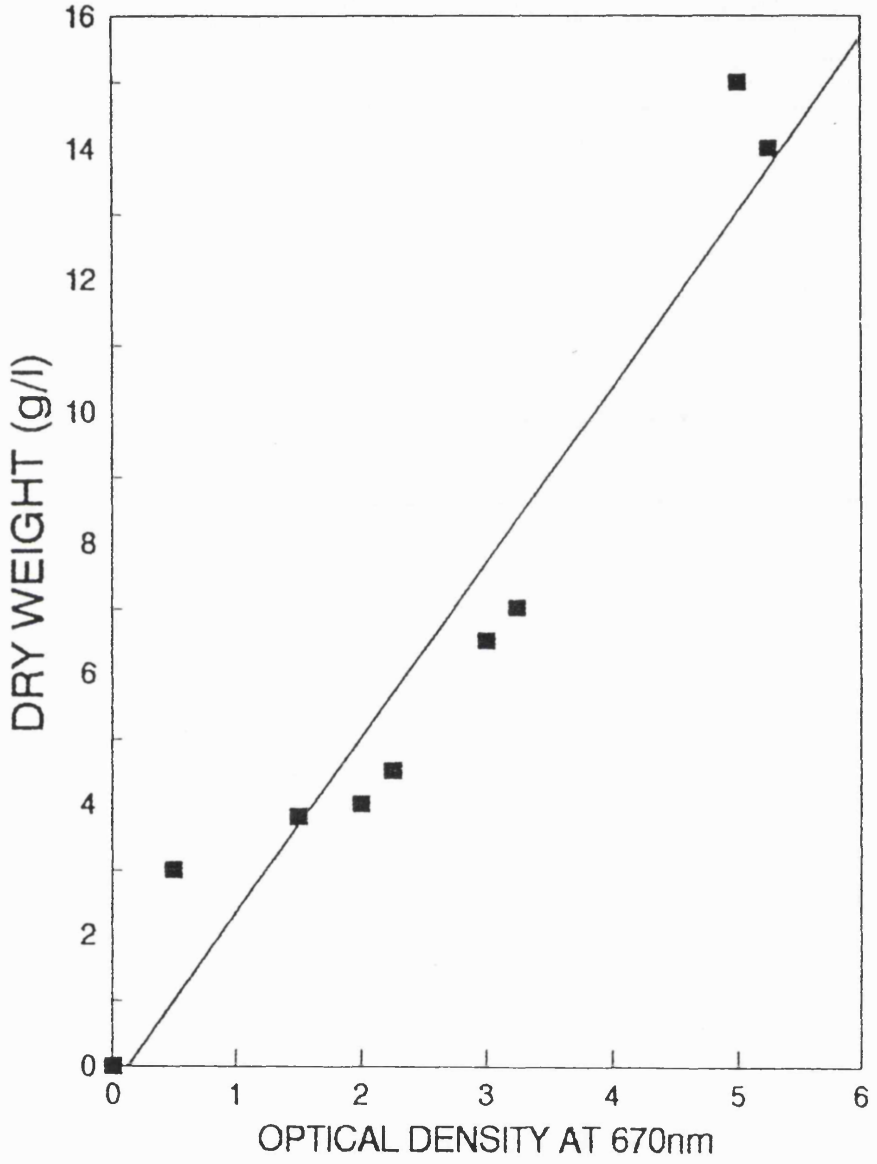
Cell suspensions were challenged with a series of aliphatic solvents, present as a two liquid phase system (see chapter 2). The the removal of cells from the aqueous was determined by following the optical density and microscopic examination. Adsorption of cells onto the solvent interface indicates the hydrophobicity of the cell wall. Figure 3.15 and 3.16 show the hydrophobic nature of *P.putida* and *Y.lipolytica* cell walls. Growth on hydrocarbons did not significantly alter the cells ability to absorb onto the aqueous/organic interface. The *S.cerevisiae* cells were not adsorbed onto the interface. They were therefore suitable as an initial test organism with organic media, since inclusion of cells which are be removed onto the interface, would cause unnecessary complication.

Growth of Y.lipolytica on alkanes

The *Y.lipolytica* showed growth on the alkanes in the series above pentane, when incubated with a carbon deficient medium. This medium did not support growth on its own.

FIGURE 3.13

Standard curve of *P.putida* dry weight versus optical density (670nm)

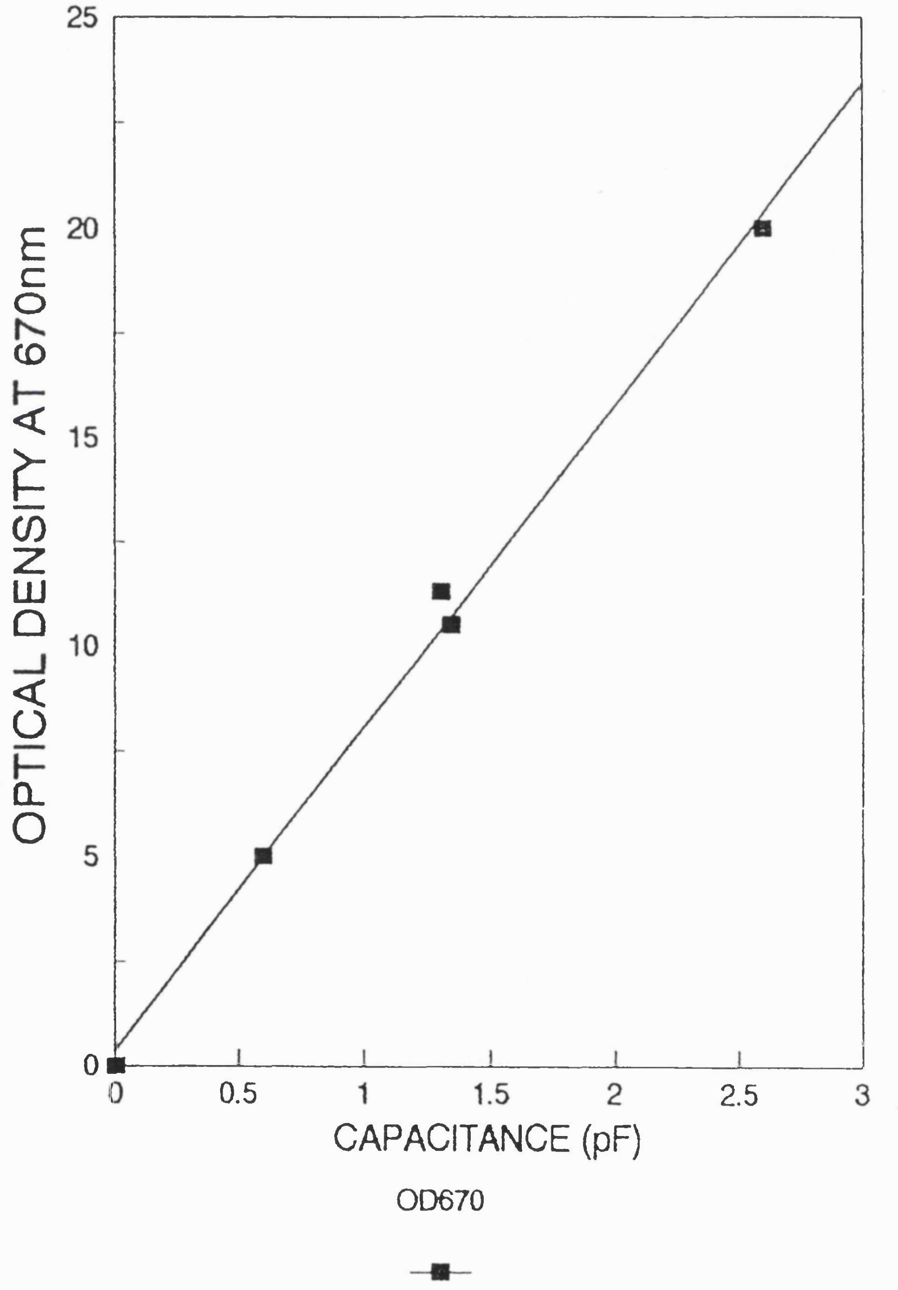


Graph of dry weight against optical density for *P.putida*

(BRAZIER 1989)

FIGURE 3.14

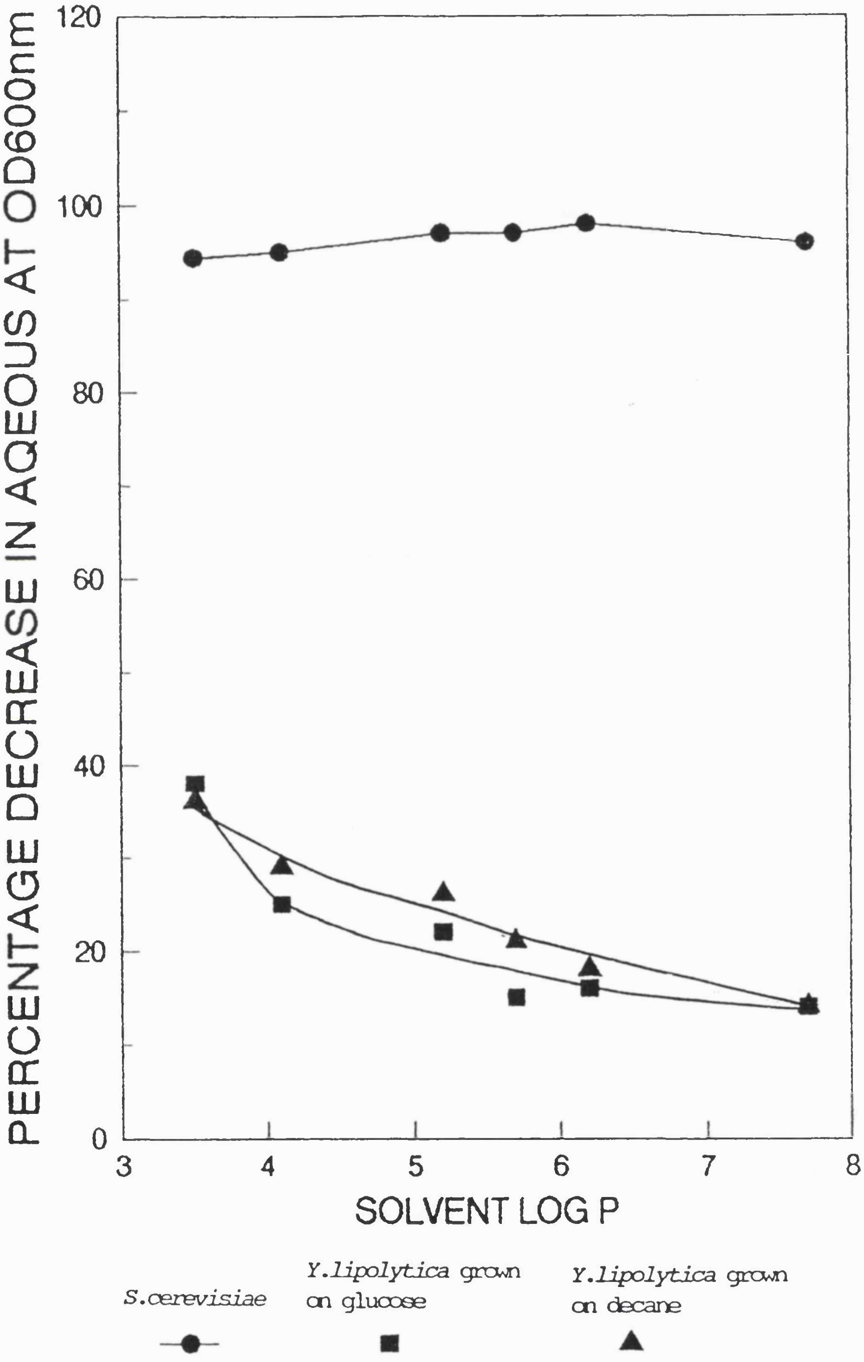
Standard curve of *P.putida* wet weight versus capacitance



Standard curve of optical density in buffer against capacitance for *P.putida*

FIGURE 3.15

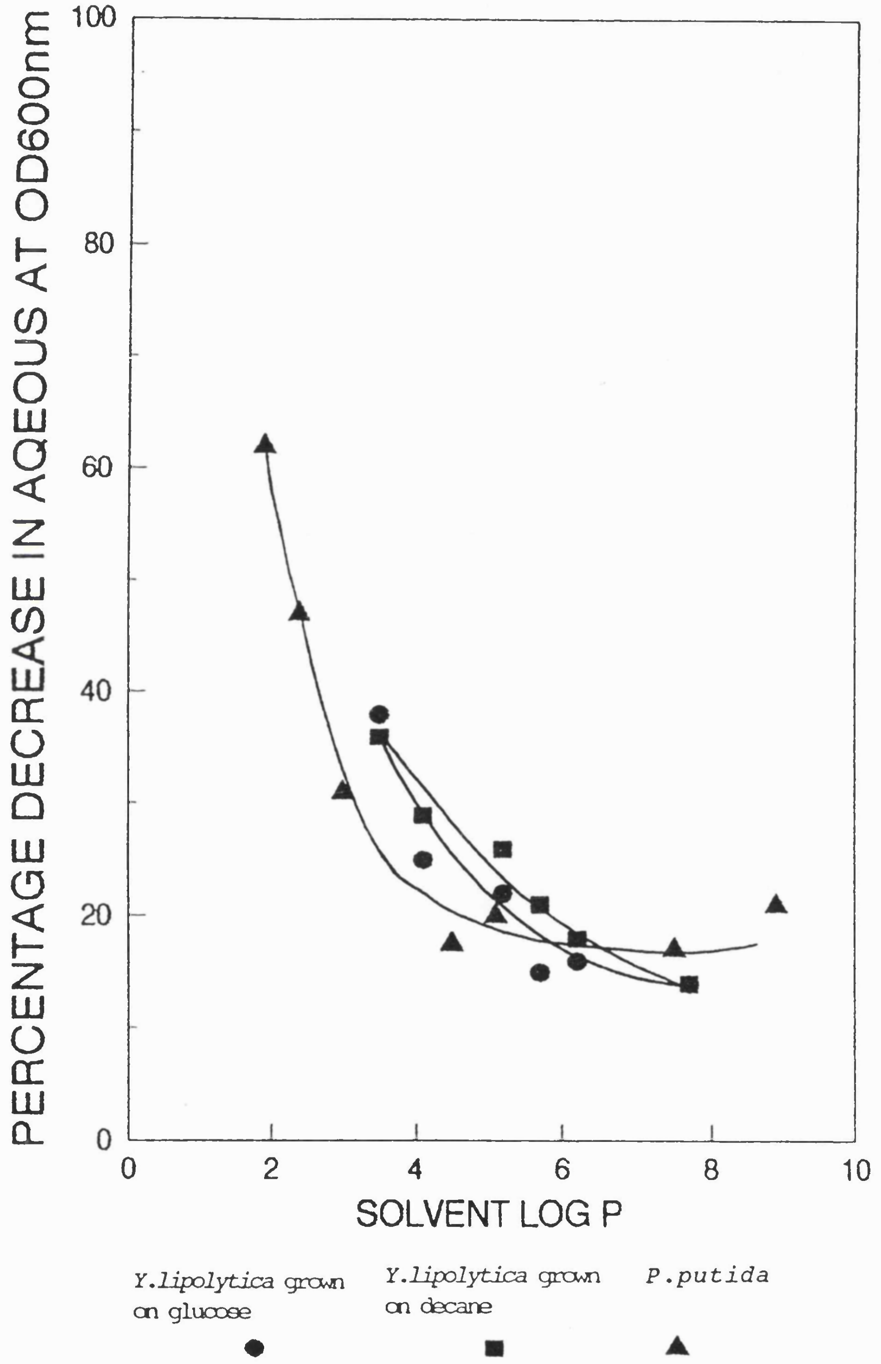
Adsorption of *S.cerevisiae* and *Y.lipolytica* onto a second-liquid interface



Adsorption of washed cells, suspended in buffer, onto the aqueous/organic interface.

FIGURE 3.16

Adsorption of *Y.lipolytica* and *P.putida* onto a second-liquid interface



Adsorption of washed cells, suspended in buffer, onto the aqueous/organic interface.

Beta-dispersion

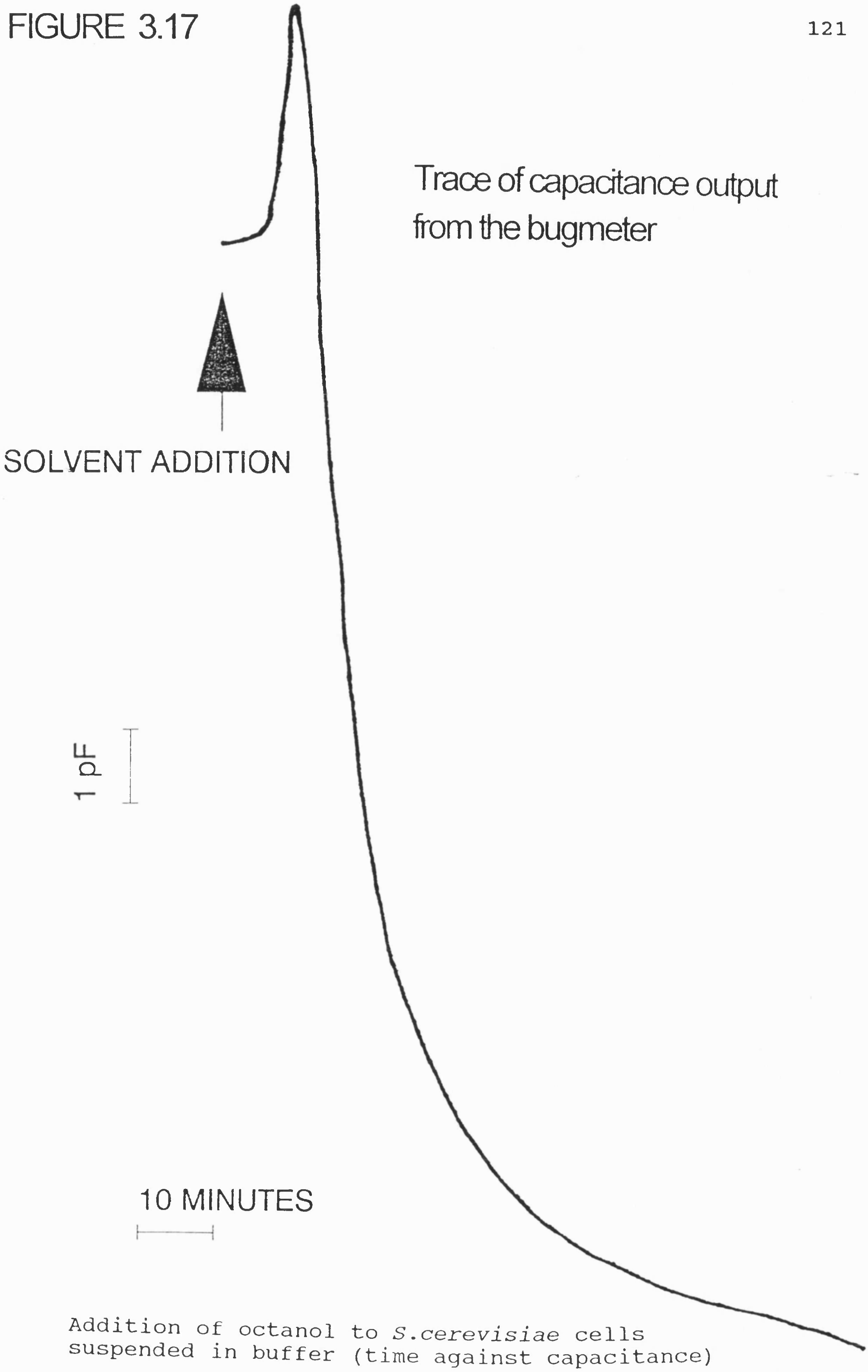
Addition of solvent (octanol) to aqueous buffer, with no cells gave no change in the capacitance greater than 3% over 72 hours.

Figure 3.17 shows the trace of 0.5ml addition of octanol to a stable suspension of *S.cerevisiae* suspended in phosphate buffer, stirred at 500rpm (fm was set to 0.4MHz). At zero logged hours (addition point of the organic solvent) the delta capacitance was recorded as 20pF, the conductance reading at 2.3mS.

The read out is a continuous trace of capacitance against time and is therefore a real time recording of the state of the dielectric properties of the cells throughout the challenge time. The trace tailed off at approximately zero delta capacitance units. The conductance by this time had risen to 3.4mS, well within the acceptable limits determined in the frequency scan figures 3.1-10. The cause of the rise in conductance is attributable to ion movement through the cytoplasmic membrane.

From the addition of solvent a slow rise in capacitance occurred which gradually increased, peaking at approximately 20 percent above the delta 20pF level recorded at zero logged hours. After peaking there is a rapid drop in the capacitance reading, where the rate of change is at its fastest. Then the rate gradually decreasing until a value of approximately 30 percent of the initial value is reached, where the drop is much slower.

FIGURE 3.17



Addition of octanol to *S.cerevisiae* cells suspended in buffer (time against capacitance)

As indicated in the introduction the reading of capacitance gives an indication of the integrity of the cytoplasmic membrane and its ability to become charged. For the purpose of this study it is important to know how reproducible these traces are.

3.6 Reproducibility of the beta-dispersion technique

Readings of capacitance measure a concentration of charged membrane per unit area (Cm), in a volume of the suspended cells. The readings in such circumstances are not dependent on any other biological parameter. A stable organism will be unaffected by stirrer speed as long as the cells are kept in suspension and air bubbles are not introduced, both would cause an erratic and reduced value of E′ detected by a corresponding variation in the recorded value of capacitance.

Effects of stirrer on probe reading

Initial problems were encountered when trying to obtain reproducible graphs. Problems encountered included maintaining a reproducible stirrer speed which did not interfere with the electronic signal, initially this appeared to be easily solved. The first attempts to get a consistent signal involved stirring a salt suspension of KCl in glass vessel driven by an overhead motor. Interference from the electromagnetic motor appeared to induce an erratic base line in the beta-dispersion signal. Several attempts were made to shield the probe by placing metal plates between the probe and the electric motor. When a 2L MBR reactor was used, erratic reading were also recorded. While larger metal sheets appeared to reduce the interference slightly, none allowed a satisfactory signal to be maintained. From these experiments the best result might be obtained if the probe were totally enclosed in a metal case. The simplest way of achieving this would be to enclose the stirrer itself (the manufacturers now also recommend earthing the probe to further improve the signal quality when using motors).

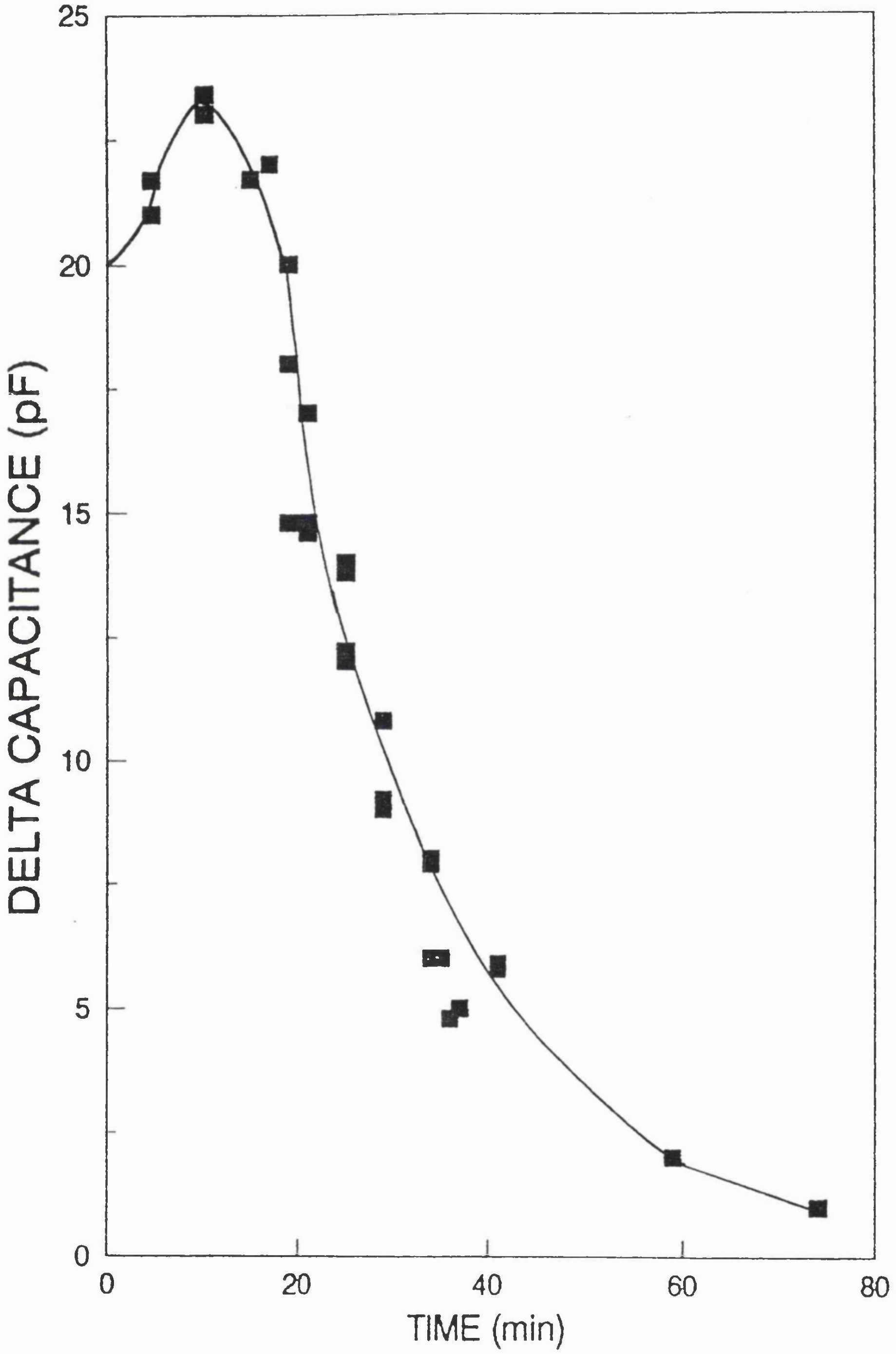
Using a magnetic bottom-stirrer (separated from the probe by a metal plate), proved very successful for eliminating motor interference, although the lack of speed regulation was a problem (most stirrers have a large margin of variation when set). This became apparent when reproducibility was tested over a number of successive experiments, this was overcome by using a self regulating stirrer.

To determine the reproducibility of the this method when used in conjunction with organic solvents, a series of experiments were carried out with different stocks of cells in the same challenge tank, and at the same stirrer speed. Figure 3.18 combines data points from four experiments in which 1ml of 1-octanol was added to *S.cerevisiae* in suspension at an initial value of 20pF.

These cells were removed from a stock solution over a 24 hours period. This illustrates that this method is accurate for cells made up in stock solution for at least up to 24 hours and for different batches of cells. This need for a stable test organism justified the inclusion of the *S.cerevisiae* strain in the selected organisms.

FIGURE 3.18

Reproducibility of beta dispersion readings with solvent addition



Graph of delta capacitance against time during four experiments, where 1ml of octanol was added to *S.cerevisiae* in buffer

3.7 Correlation of capacitance to cell viability

Cell viability

Cell viability as a concept is very ill-defined. The cell viability and the processes leading to it are fundamental to biotechnology. Loss of cell viability in some cell biotransformation processes can result in decreased productivity and is therefore of specific interest.

One measure of viability has been described as whether the cells are respiring. Eukaryotic organisms have the components of the electron transport chain localised in subcellular organelles, while the prokaryotes have these processes located in the cytoplasmic membrane. Failure of these processes is of specific interest to the bioprocessing considered here (outlined in chapter 1).

Cell viability as measured by methylene blue (MB)

To further correlate capacitance to cell viability, capacitance was compared to cell viability using the methylene blue dye technique.

Any intensely coloured dyestuff that behaves as a reversible redox couple may be used as a redox indicator, if its oxidised and reduced forms are differently coloured. The colour must be sufficiently intense that the dye can be used in small enough concentrations that its addition does not significantly change the redox potential.

The yeast ethanol fermentations have

been the centre of research for over 100 years. The methylene blue viability test was developed for use on yeast in ethanol producing fermentations. This assay is therefore likely to be of use in a wider study of other organic solvents and their effect on cell viability.

Since the early 1920's methylene blue has been used as the standard for estimation of yeast viability. This method has been modified and developed, since the original procedures over estimated cell viability and were only accurate for populations of greater than 90 percent viability (Chilver et al 1977 and Parkkinnen 1976). More recent modification to the method of methylene blue stain and the ionic composition of the stain solution have resulted in an apparently accurate method (Jones 1987 I and II).

Methylene blue is an auto-oxidisable dye, which on entry into the cytoplasmic membrane of a living cell is reduced to the leuco-form (colourless). The staining of a cell is then the imbalance ratio of dye influx and its rate of reduction within the cytoplasm. Living cells which have a damaged membrane may stain partially blue (Lee 1981).

$$\underset{\text{(BLUE)}}{MB} + 2H+ + 2e- = \underset{\text{(COLOURLESS)}}{MBH_2}$$

(Morris 1978)

Methylene blue itself may have some effect on the membranes, depolarising neural membranes via the suppression of Ca^{+2} dependent K^+ conductance. This may be linked to the improved accuracy of the methylene blue stain with yeast cells when higher concentrations of Ca^{+2} added (Lee 1981).

Two different strategies were adopted when developing this technique; one was as described in the literature (Jones 1987 I and II), the second was a modification, were the optical density of the cell free solution was monitored (results not shown). The latter did not produce reliable, reproducible results, noted by other workers (results not published).

When monitoring the number of viable cells by the former technique, the methylene blue dye assay gave constant readings over a one hour period before the addition of solvent. During this time no significant change in capacitance was recorded (<2 percent).

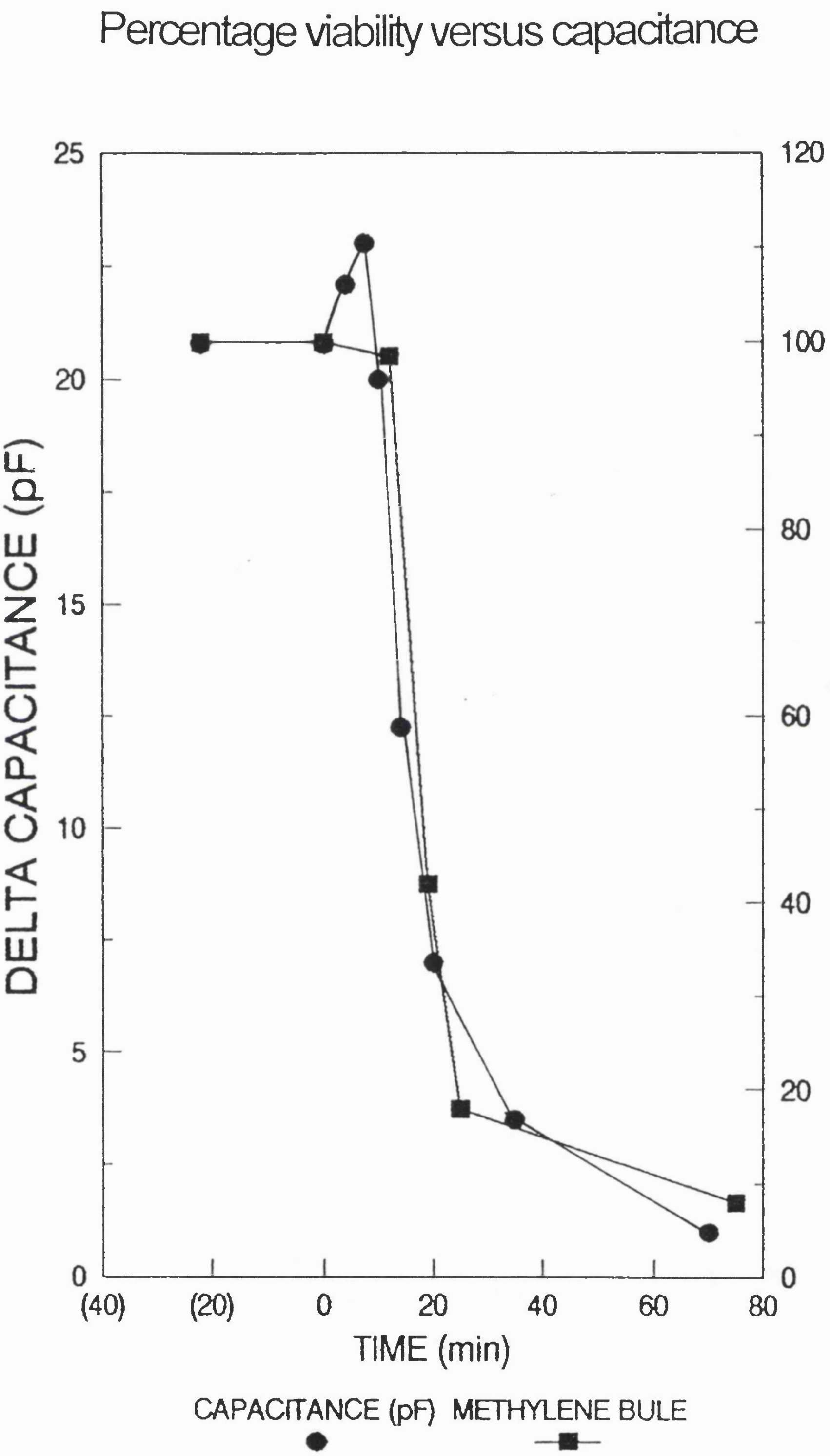
Methylene blue versus capacitance

To validate the dielectric response cells challenged with octanol were removed at time intervals for determination of viability by the methylene blue dye assay.

Overall there was a good agreement between the methylene blue assay and the recorded capacitance (figure 3.19 and 3.20) although discrepancies occur at two points. The first occurs immediately after the solvent addition, where the capacitance shows a rise. During this period the methylene blue assay shows no such rise. Since the cells cannot be more than 100 percent viable, this is inconsistent and is attributed to the effect of the solvent on the cell membrane beta-dispersion (see section 3.18).

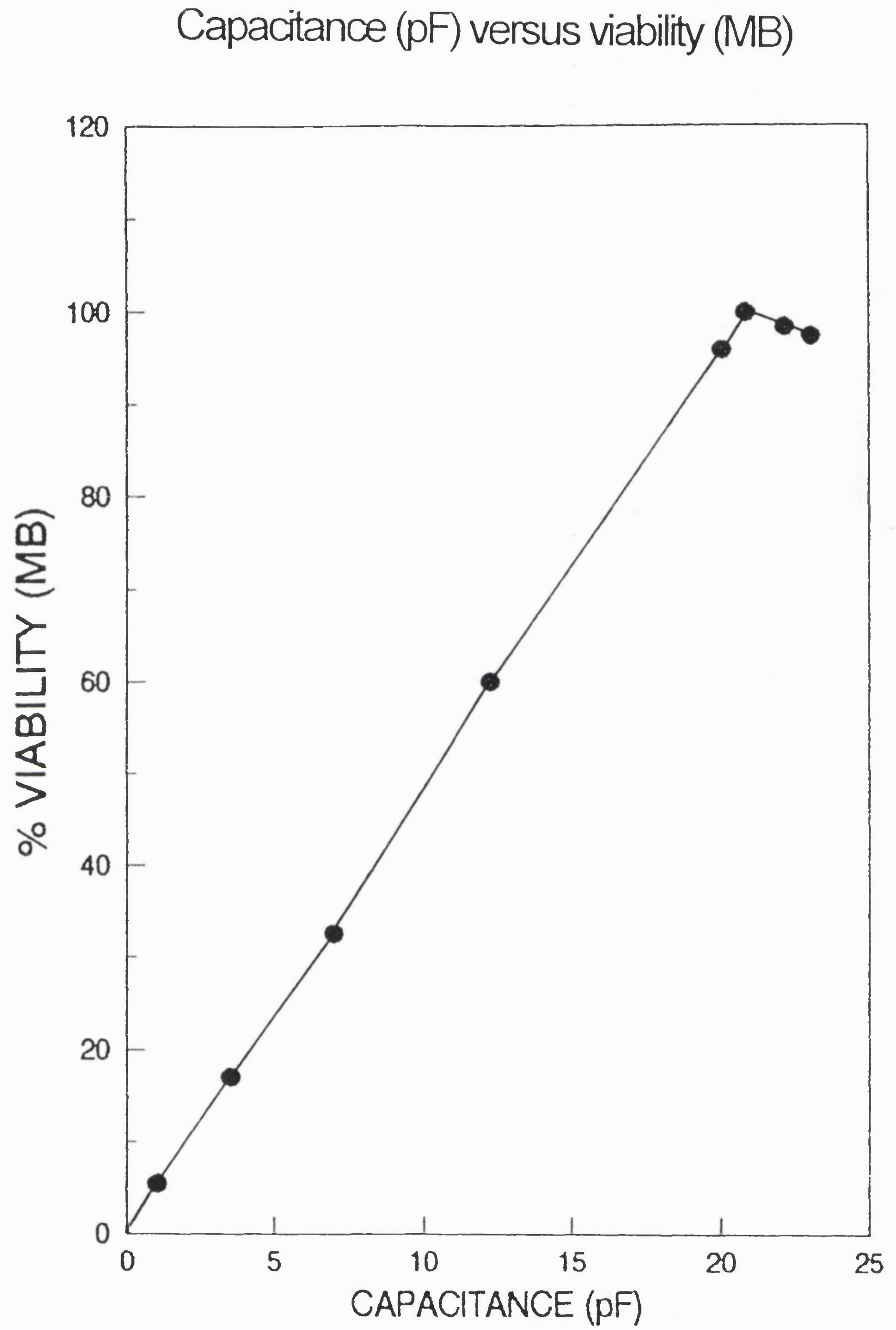
The second deviation occurs toward the lower values of viability (below 20 percent). This can be attributed to the experimental method, since the number of cells viable at this stage becomes very low. At low viability there exists a state in the staining technique where the cells stain light blue, and the differentiation between a 100% viable cell and a totally nonviable one becomes very important, but very difficult to ascribe accurately, this may lead to experimental error.

FIGURE 3.19



Capacitance and methylene blue(MB) viability
against time, following 1ml addition of octanol *iae*
to *S.cerevisiae* in buffer

FIGURE 3.20



Cell viability(MB) against capacitance for *S.cerevisiae* (were capacitance =<100%, see figure 3.19)

3.8 Rise in capacitance when cells are challenged with organic solvents

When octanol is added to a suspension of cells, which are stirred (at a constant temperature), the recorded capacitance (fm 0.4 MHz) rises (see figure 3.22.1) initially before dropping. Since the rise in the reading is not due to the solvent per se (see previous section frequency scan), it must be the result of the interaction of the solvent with the cell. The beta-dispersion is the result of a functioning membrane, surrounded by an ionic solution, measured at a frequency (fm).

The rise in capacitance appears indicative of the toxic nature of the solvent. As the concentration of solvent is increased, it could be reasonably assumed that there is also a rise in the transfer rate of solvent into the cell. This also implies that an improvement in the mixing would increase the toxicity of an organic solvent (see section 3.10).

It has long been known that alcohols partition into the membrane of cells. Seeman (1972) demonstrated an increase in the volume of erythrocyte cytoplasmic membrane when exposed to solvents. Ashcroft et al (1977a,b) also showed that certain solvents increase the membrane thickness, Stoicheva et al (1989) also reported this correlation. It would also be reasonable to assume that an increase in the ion concentration within the cell could also account for this rise in capacitance reading. This raises the question of whether all solvents have the same effect. If the rise in capacitance is the result of an increase in ions within the cell, does this state confer any beneficial effect on the cell (see section 3.14).

It should be noted that *S.cerevisiae* does not oxidise octanol.

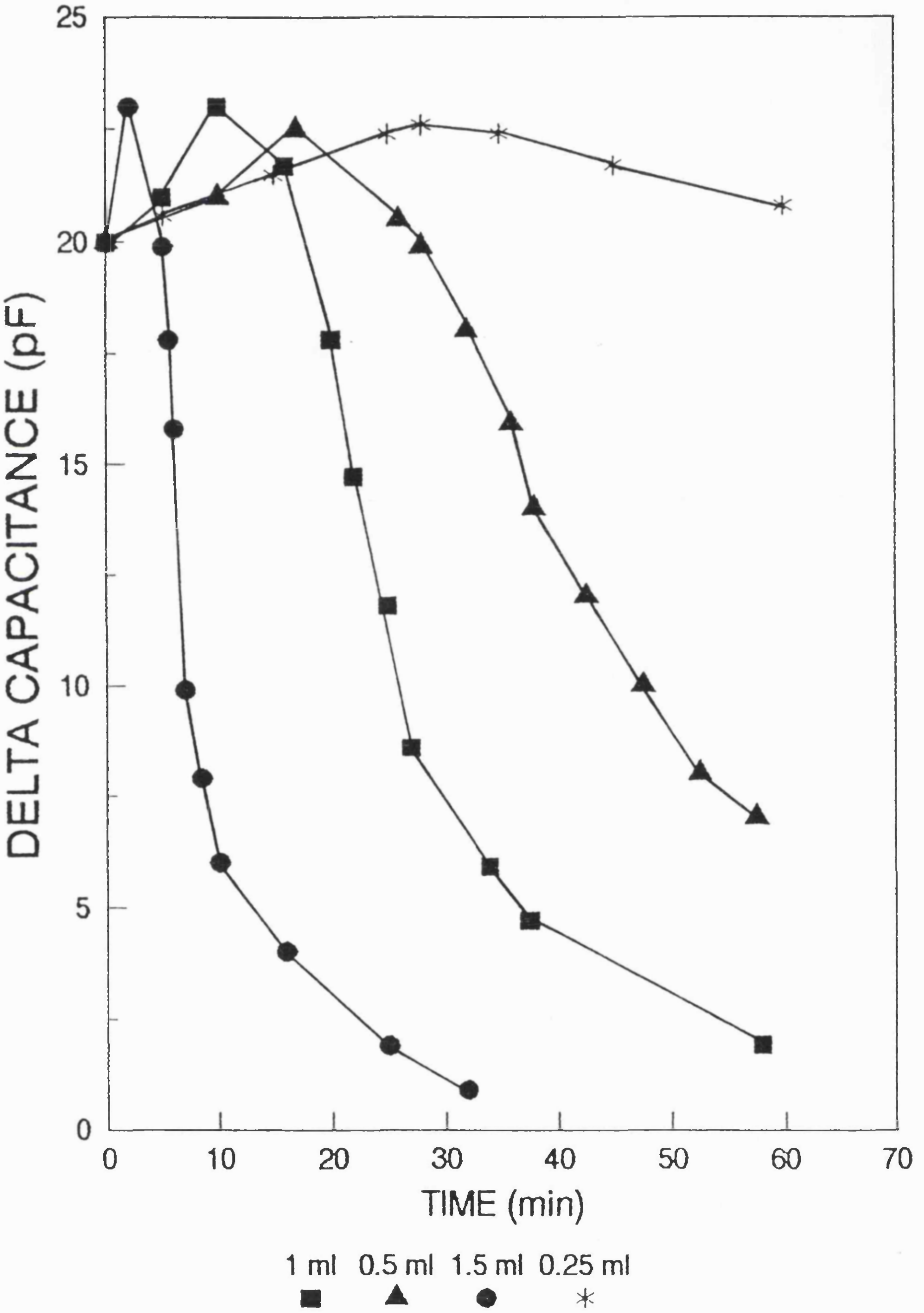
3.9 Effect of aliphatic alcohol addition to *S.cerevisiae*

Volume addition of 1-octanol

Increased addition of organic solvent (octanol) showed a corresponding increase in the toxicity of the solvent to the cells (see figure 3.21), described by the drop in capacitance trace with time.

FIGURE 3.21

Volume addition of 1-octanol against capacitance for *S.cerevisiae*



Capacitance against time for *S.cerevisiae* suspended in buffer and challenged with additions of octanol

The rise in capacitance noted earlier was of different magnitude for each solvent quantum. It did not directly relate to the solvent quantum added, although for the initial low volume additions the rise does increase, but reaching its maximum gradually (see figure 3.22.1). For these graphs the drop in capacitance is initially very fast compared to the tailing drop which approximates to linear.

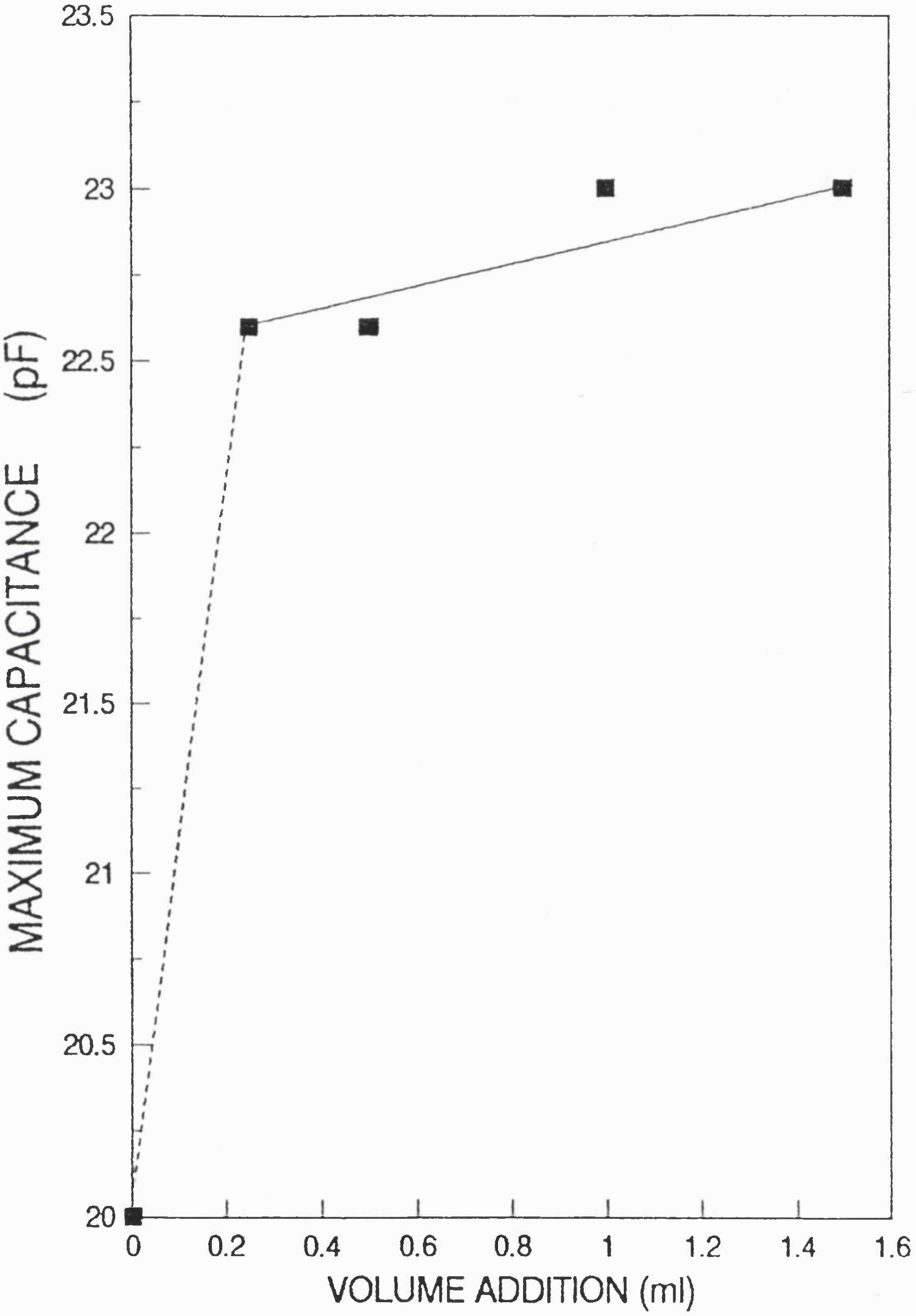
Volume addition (1ml) of aliphatic alcohols

Volume for volume (1ml), additions of the linear alcohols show an increase in toxicity the shorter the chain alcohol (**Figure** 3.23). For this low volume addition the shortest of these alcohols (1-pentanol), which should be one of the most toxic solvents, here, is the least toxic. The choice of this particular volume means that toxic levels can be reached with all of the solvents present with the exception of pentanol. This solvent has the highest aqueous phase saturation level of these solvents. A higher or lower volume addition will alter the apparent toxicity of the solvents (moving the curves to the left and right respective).

This approach has the distinct disadvantage of allowing the variation in several differing parameters : the second-liquid phase area and the molarity. This highlights some of the important parameters which need to be considered in the nature of this work.

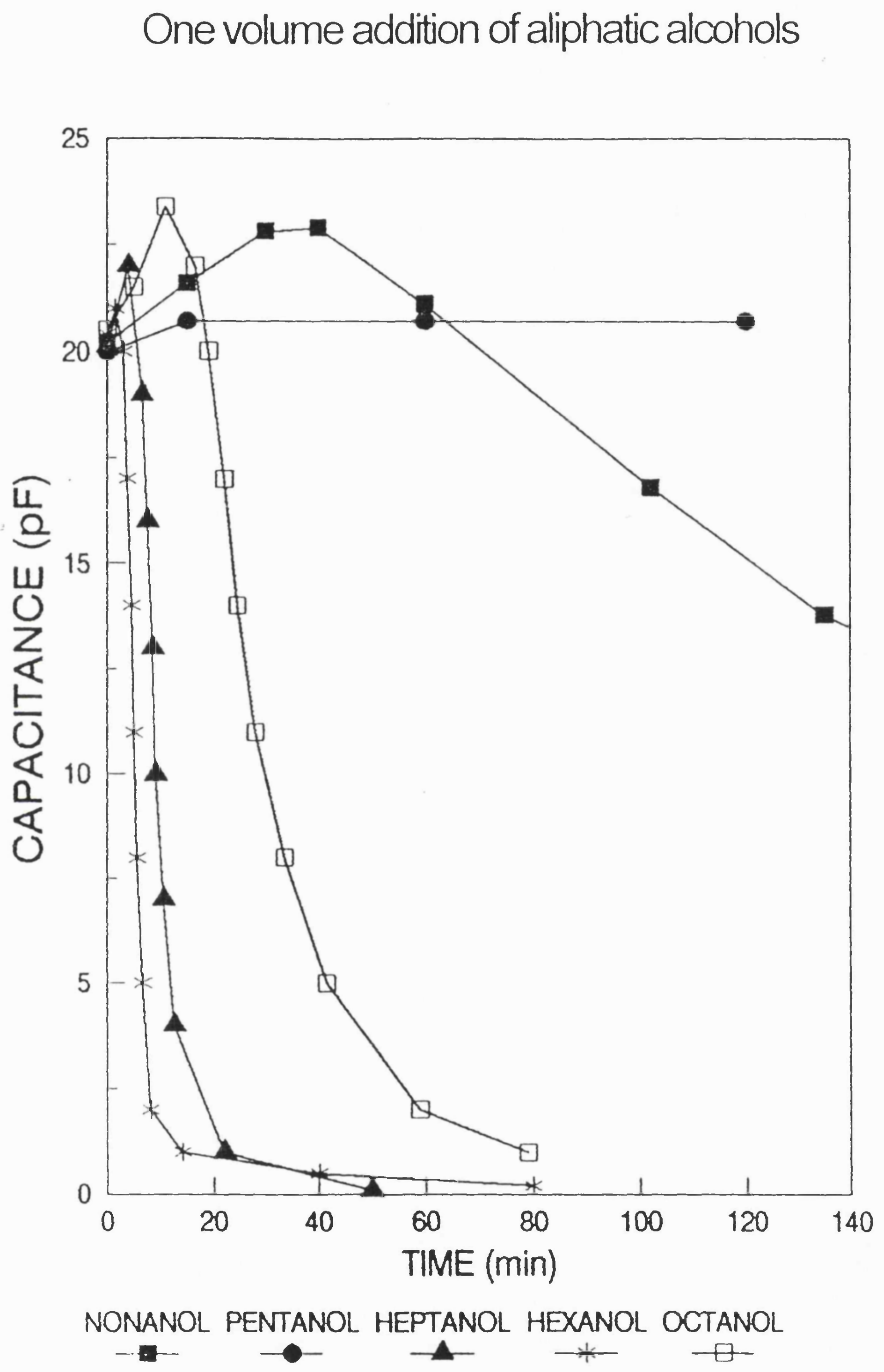
FIGURE 3.22

Peak rise in capacitance after solvent addition for *S.cerevisiae*



Peak rise in capacitance from *S.cerevisiae* suspended in buffer and challenged with volume additions of octanol.

FIGURE 3.23



Graph of capacitance against time for 1mequivalent of aliphatic alcohols to *S.cerevisiae* suended in buffer

From the literature it is clear the degree of aqueous saturation with organic solvent, and the 'phase' effect are important. The phase effect may be attributed to the physical presence of an organic/aqueous interface phase or to an increased aqueous saturation by the continued resaturation of the aqueous phase from the bulk organic. The stirrer type and degree of agitation will alter the mixing patterns, as well as any shear effects caused at the organic and aqueous interface. Differentiation of these two variables will help to unravel the 'phase effect'.

3.10 Effect of agitation on organic solvent toxicity

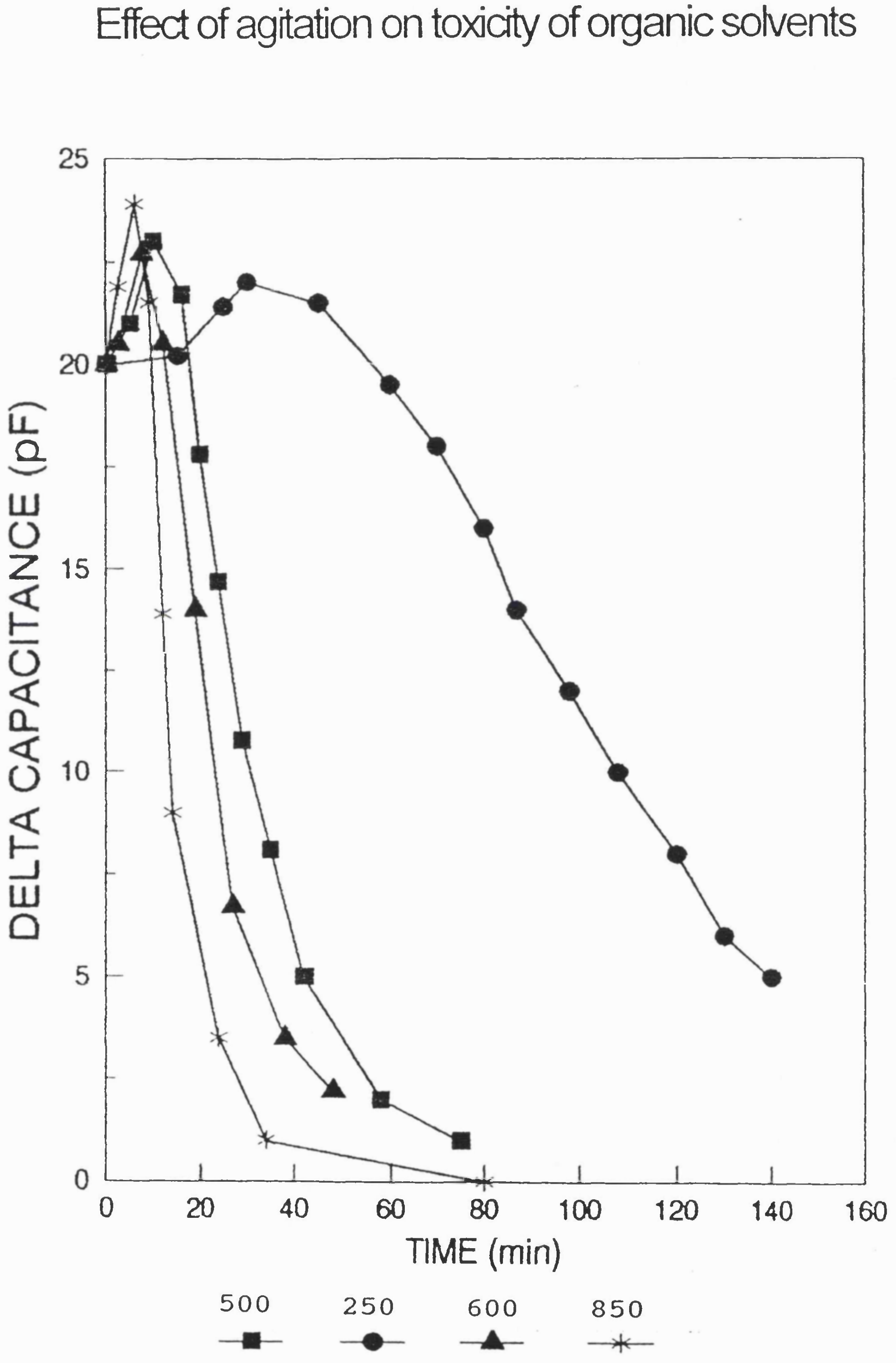
Agitation effect on solvent toxicity has been implied from work on *A.simplex* (Hocknull 1989). But Harrop (1990) described this parameter as unimportant with *P.putida*, for naphthalene hydroxylation.

From **figure** 3.24 a close correlation is established between stirrer speed and cellular toxicity of organic solvent. From this figure it is apparent that the correlation is not linear and that proportional increases in agitation do not produce proportional increases in toxic effects. The relationship between stirring rate and dispersion of droplets would also be expected to be nonlinear.

This correlates well with an expected increased impingement of cells onto the organic solvent interface or increase in aqueous/organic interfacial area, since the relationship between stirrer speed and droplet dispersion and therefore transfer of solvent into the aqueous would also be expected to be non-linear.

In **figure 3.24** a trend toward the increase in peak rise can be seen for the higher initial peak rise before the dramatic drop. In all cases the higher the initial rise, the faster the drop, indicating that once the solvent has partitioned into the cell, the damage is accomplished and that there is a maximum volume of solvent which can partition into the cell membrane before destabilising the membrane. The faster the agitation the quicker the transfer of the solvent into the cell membrane. The toxicity of the solvent may be limited by its mass transfer into the cell membrane.

FIGURE 3.24



Graph of capacitance against time for *S.cerevisiae* challenged with octanol at a range of stirrer speeds (3cm bar)

3.11 Effect of biomass in solvent saturated aqueous

From the previous experiments it is clear that transfer of organic solvent into the cell is of key importance. If organic solvents are absorbed into the cell, then the high biomass concentrations used in biotransformations may have a significant affects on the saturation levels of organic solvent in the aqueous phase. In the literature no attempts have been made to show any relationship between the biomass quantum and the level of solvent, despite the limitations of the solubility of the organic solvent in the aqueous phase.

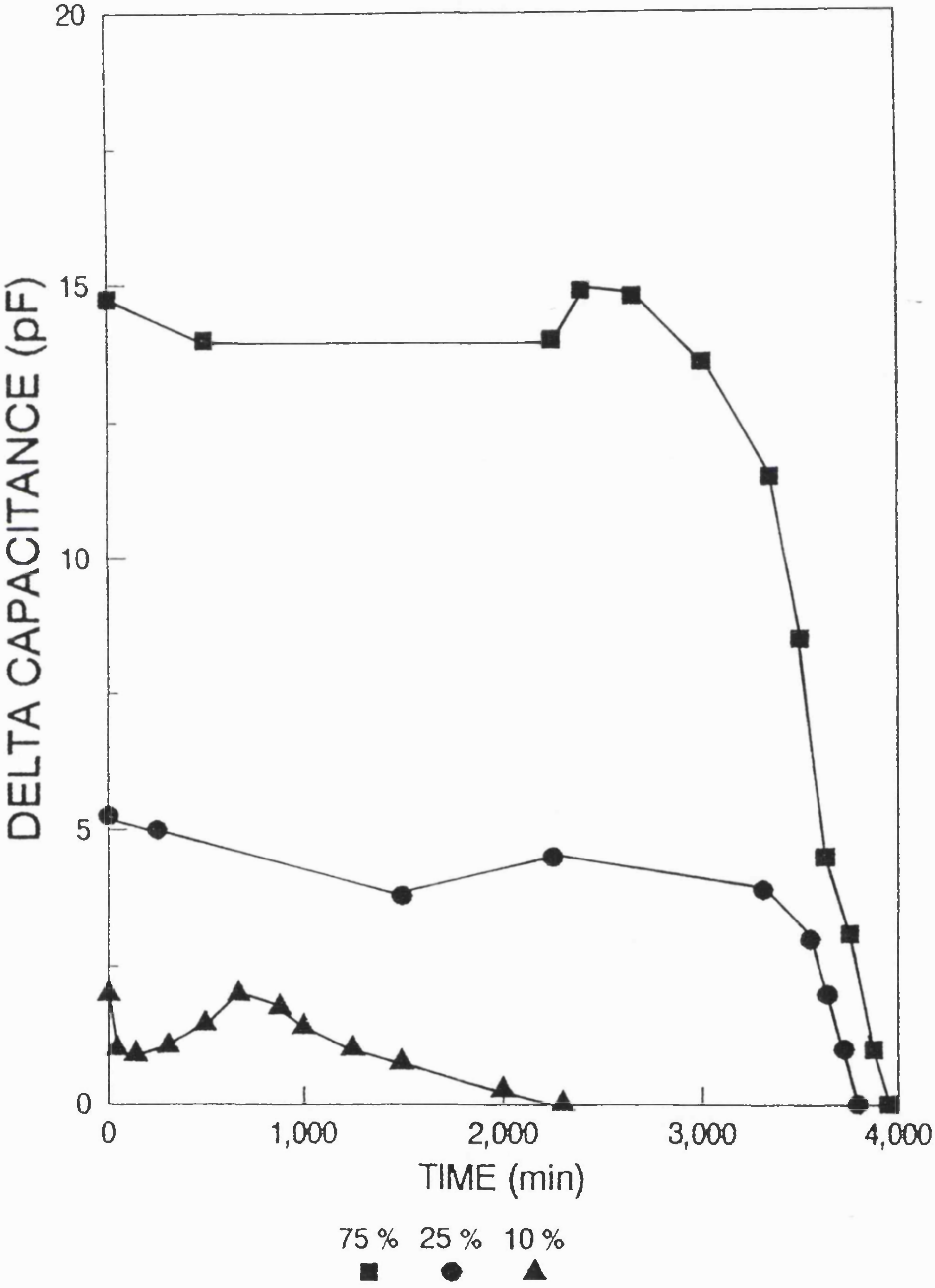
In this study, aqueous phase saturated (see chapter 2) with organic solvent was used to challenge *S.cerevisiae* at different concentration, always using the same volume addition of cells.

From figure 3.25 a correlation between biomass and solvent concentration is given. At the higher biomass concentration a slight agreement is achieved between the 75 and 25 percent cell additions. At 10 percent biomass concentration a significant difference can be seen. In both, the initial response of the cells to the solvent is an immediate drop, followed by the typical rise associated with solvent entry and increase in volume of the cell membrane. As a proportion of the cell biomass these are both significant (figure 3.25.1). The drop in the cell viability then proceeds at an increased rate , reaching zero viability at a considerably earlier stage than the higher biomass (see figure 3.25.2).

This clearly demonstrates the importance of aqueous saturation levels and the biomass concentration. To investigate the effect of organic solvent saturation of the aqueous in a two-liquid phase system, it is necessary to look at saturation of the bulk aqueous with a defined organic solvent interface. This was achieved by the use of a Lewis cell.

FIGURE 3.25

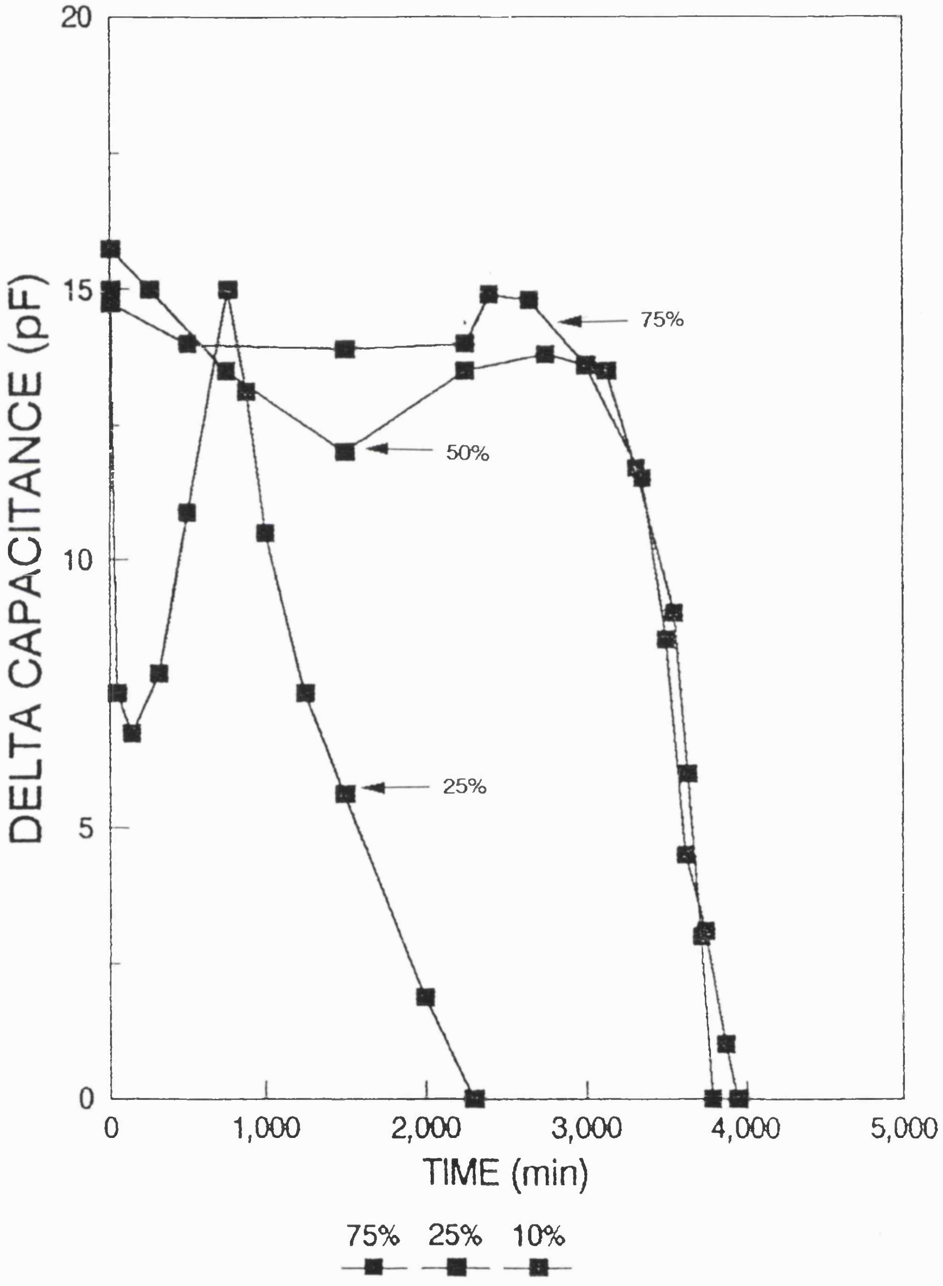
Toxic effect of organic saturated aqueous on biomass at different concentration



Graph of capacitance against time for additions of a range of biomass concentrations (all at the same volume), to aqueous saturated with octanol, stirred at 500 rpm (3cm bar)

FIGURE 3.25.1

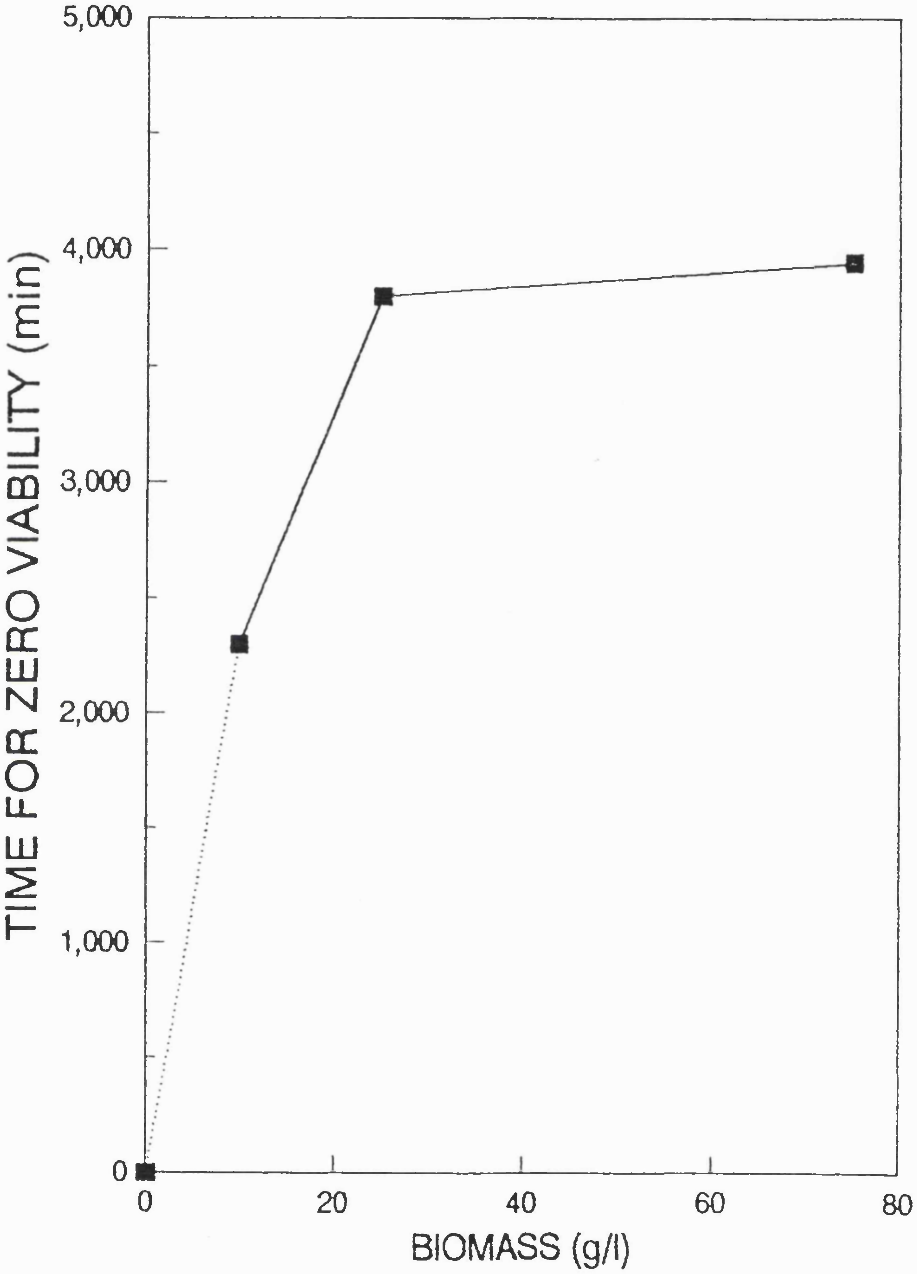
Toxic effect of organic saturated aqueous on biomass (biomass normalised)



Graph of capacitance against time for additions of a range of biomass concentrations (data from fig.3.25 normalised to 15pF starting concentration)

FIGURE 3.25.2

Relationship between biomass and toxicity of organic solvent



Graph of time taken to reach zero viability (data from fig.3.25)for a range of biomass concentrations. Dotted line is extrapolated data

3.12 Lewis cell type experiments

The previous experiments have been aimed at the addition of solvents, while allowing a variation in the organic aqueous phase ratio and interfacial area. To examine the affects of solvents when the interface is controlled, the challenge tank was modified. This required the use of a small stirrer bar that operated accurately at the low shear, necessary to prevent the interface being broken and within the rpm range of values which are necessary for accurate stirring. By the careful layering of the organic phase onto the aqueous phase a continuous interface was formed, without forming droplets. The aqueous phase was stirred by a magnetic bar, such that the organic/aqueous interface was not disrupted.

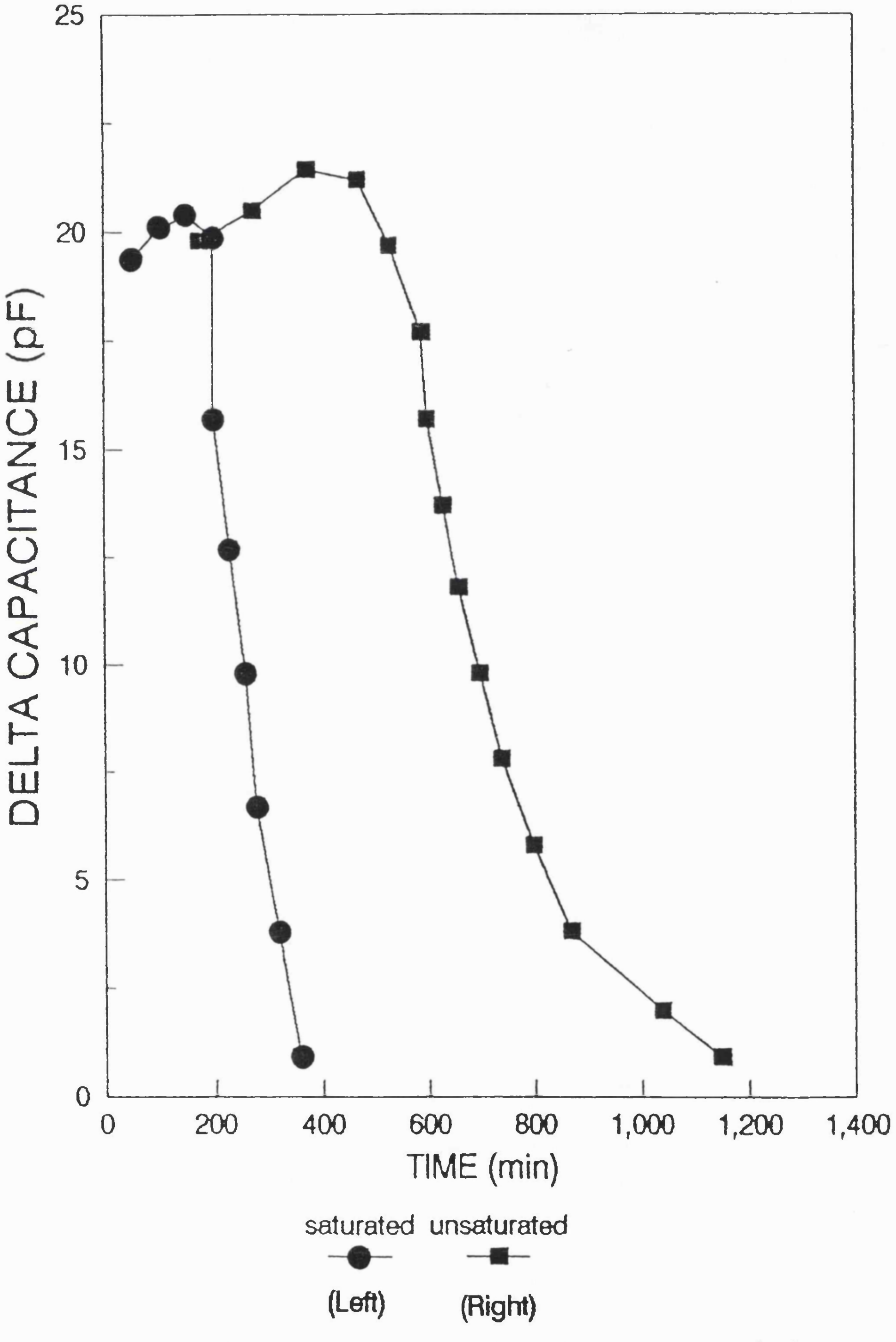
To overcome the dispersion effects of the organic into the aqueous, which are encountered with the different solvents, the aqueous phase can be presaturated using an organic solvent second-liquid phase, before the addition of the biomass solution.

To examine the effect of saturation of the aqueous phase, cells were challenged with a second-liquid phase (of defined surface area), where the aqueous phase was either presaturated or post saturated with respect to cell addition.

From figure 3.26, the presaturated aqueous is more toxic than allowing the aqueous to saturate up through the second-liquid phase. This demonstrates clearly the significance of saturating the aqueous phase and that the dispersion of the organic solvent into the aqueous is an important parameter.

FIGURE 3.26

Aqueous saturated and unsaturated with organic solvent prior to cell addition, challenged with a defined second-liquid phase interface



Graph of capacitance against time for *S.cerevisiae* challenged with a second-liquid phase of octanol with a defined interface. The aqueous is pre-saturated (left)and post-saturated (right)with octanol in respect to cell addition (stirred at 250rpm,using 3cm bar)

These graphs are not parallel and show a difference in the initial peak height reached. From these two pieces of information we can infer that the amount of solvent which partitions into the membrane from the aqueous phase is not the only determining factor that decides the toxicity of a solvent to the cell.

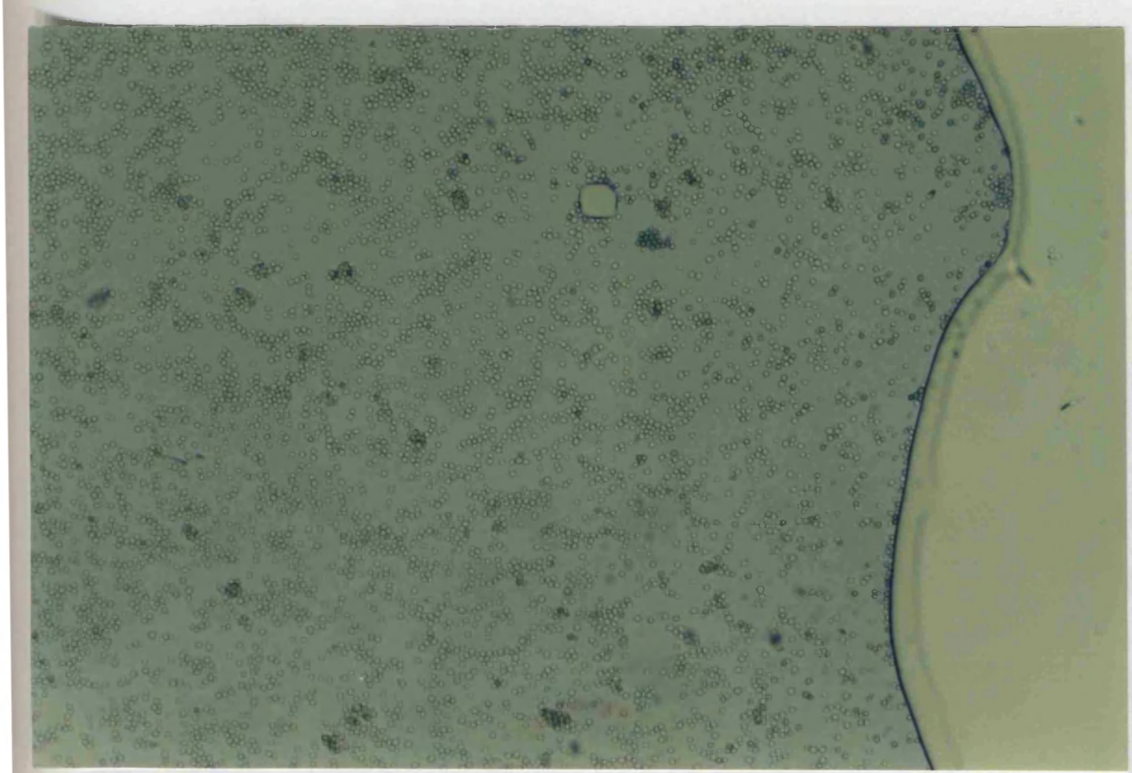
The dispersion of the solvent into the aqueous may be slower than the its partitioning into the cell (see also photographs chapter 3). This raises the question of the nature of the interaction of the interface and the cell (see section 3.16) and the effect of the organic solvent structure on the membrane (see section 3.12.1).

3.13 Interface proximity and viability

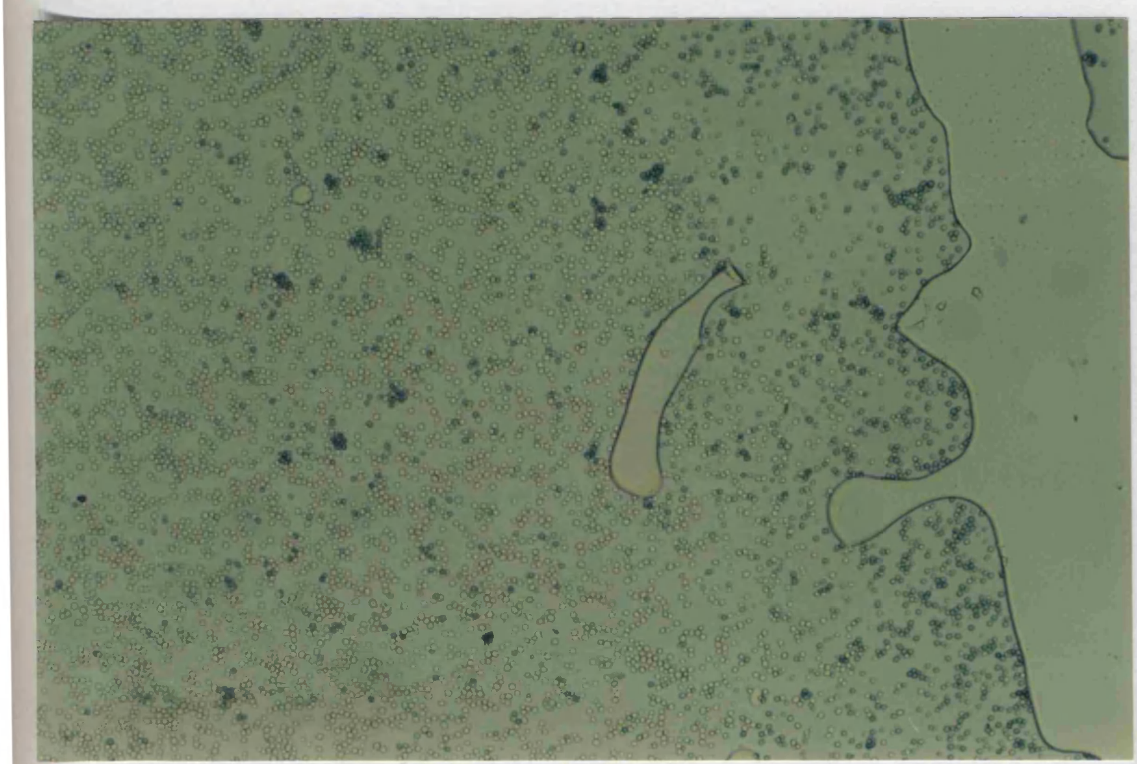
To illustrate the effect of the organic solvent in its different physical form (if a second-liquid phase is contacted) on the individual cells and on the population as a whole, cells were challenged with a well mixed second-liquid phase (figure 3.27.1), while other cells were challenged with an organic saturated aqueous (figure 3.27.2), saturated by a stable second-liquid phase interface, which was unable to come into contact with the cells.

This experiment was necessary to enable a distinct interpretation to be made between the individual cells and the data obtained from the whole population assays. No distinction has previously been noted between the individual cells and the whole population in the literature, when describing the effects of a solvent at sub and super saturation concentrations on the stability of an organism performing a biotransformation.

FIGURE 3.27.a
S.cerevisiae after challenging with 1-octanol present in a second liquid phase, stirred at 500 rpm with a 3cm bar.

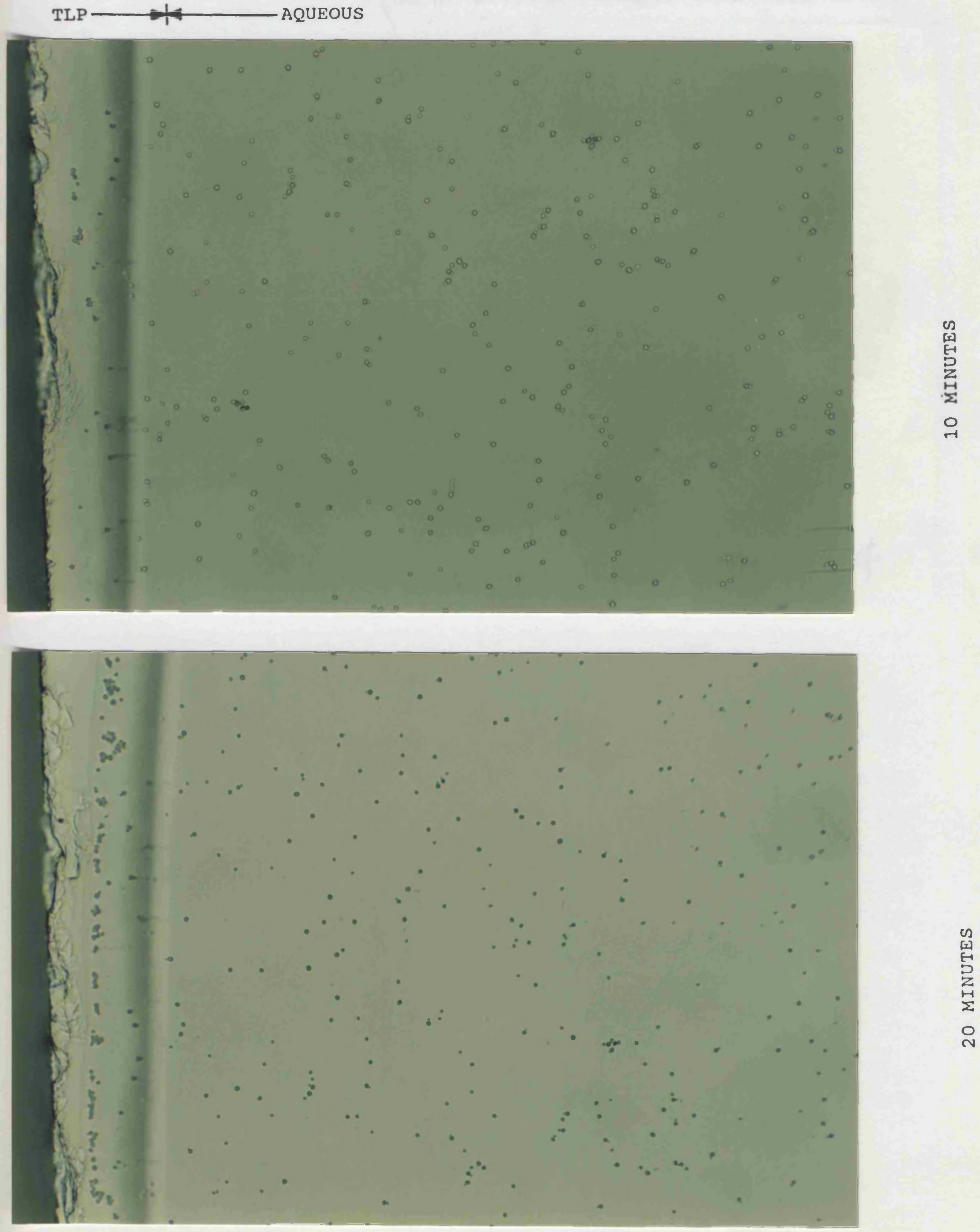


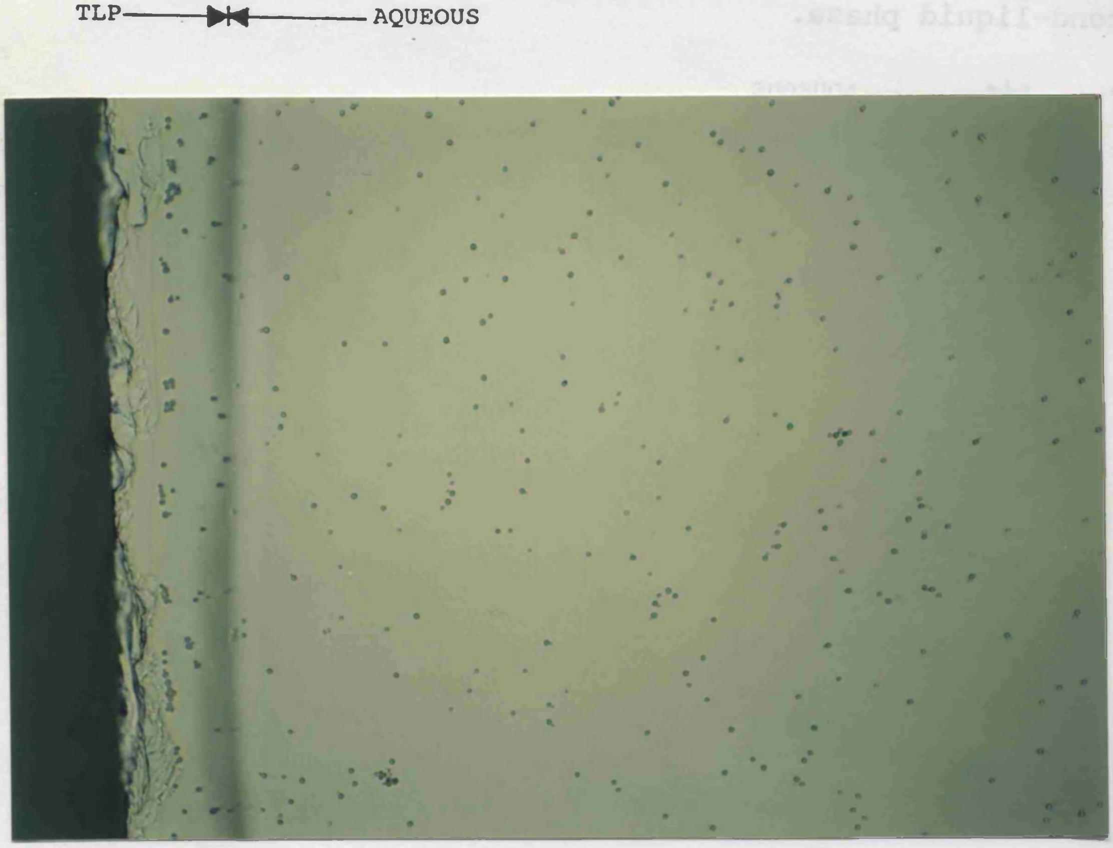
10 minutes after solvent addition



20 minutes after solvent addition

FIGURE 3.27.b
S.cerevisiae challenged with 1-octanol in a microslide challenge, where cells cannot come into contact with the second-liquid phase.


TLP
AQUEOUS
10 MINUTES
20 MINUTES


TLP
AQUEOUS

60 MINUTES

3.17 Budding cell versus single cell susceptibility to aqueous saturated solvent

From an average sample of *S.cerevisiae* the percentage of cells which are budding to single cells used was 14 percent. On examination of the effect of octanol saturated aqueous phase, using the methylene blue staining technique, it was observed that for samples taken between 10-30 minutes after addition of the cells, approximately equal numbers of 'non-viable' budding to single cells could be seen (staining blue). This indicated that cells which are budding are more susceptible to the aqueous dissolved solvent than the single cells.

3.15 Sub-terminally hydroxylated alcohols

The cell membrane is highly structured, unlike the octanol solutions which are used to derive correlations of log P for predicting solvent toxicity. This assumption implies that different organic solvent structures all have the same effect once in the cell membrane.

One of the aims of this project is to investigate the effect of solvent structure on the toxicity of organic solvents. The solvents studied in the literature have mainly been the linear aliphatic hydrocarbons with terminal substitutions. Osborne et al (1990) used Seemens (1972) correlation of partitioning to calculate the toxic membrane concentration of a cell. From the maximum aqueous solvent concentration and corresponding log P corrected for the cell membrane partitioning, the maximum concentration of the solvent which will partition into the membrane can be calculated. Estimation of the ability of a solvent to reach any critical toxic membrane concentration can then be calculated.

To show the clearest difference between the organic solvents with different maximum membrane concentrations, analogues of octanol were selected (1,2 and 3 octanol). As the octanol analogues have similar log P, the solvent with the highest membrane concentration should be the most toxic solvent (3-octanol), since the driving force for dynamic equilibrium is higher (see chapter 4). The reverse argument applies for calculated least toxic compound (1-octanol).

Figure 3.28 shows the effect of octanol analogue (1-octanol, 2-octanol and 3-octanol).

Figure 3.28.1 shows an enlarged version (0-50 min) of figure 3.28, to show the nature of the initial capacitance peak rise, which varies in accordance with the toxicity of the solvent as described by the drop in reading. Figure 3.29 shows the effect of aqueous saturated concentrations of organic solvents (1-octanol and 3-octanol). To confirm these finding analogues of heptanone were also tested (see figure 3.28.2).

Reliance on the log P term is not accurate when nonlinear organic solvents are used, to the extent that the reverse may be true. These findings were confirmed with both octanol and heptanone analogues.

3.16 Oxygen uptake rate (OUR)

Different solvents have different effects on the initial capacitance peak rise (see previous). If this rise is associated with an increase in the internal ion concentration or increased cell membrane fluidity of the cell, then it is possible that it may have a noticeable effect on the overall cell activity.

As already established, the capacitance has a strong correlation with viability as measured by (MB). But, no further understanding of the rise in capacitance was elucidated. The rise in capacitance is logically associated with the partitioning of the solvent into the membrane and increasing the beta-dispersion reading (see section 3.8). To further investigate this rise and its implications on the cellular activity, the endogenous oxygen uptake rate was monitored to give a measure of any changes in the overall cell activity.
The OUR was monitored at specific times over the organic solvent challenge time. It should be noted that no record could be found that these yeast oxidise octanol.

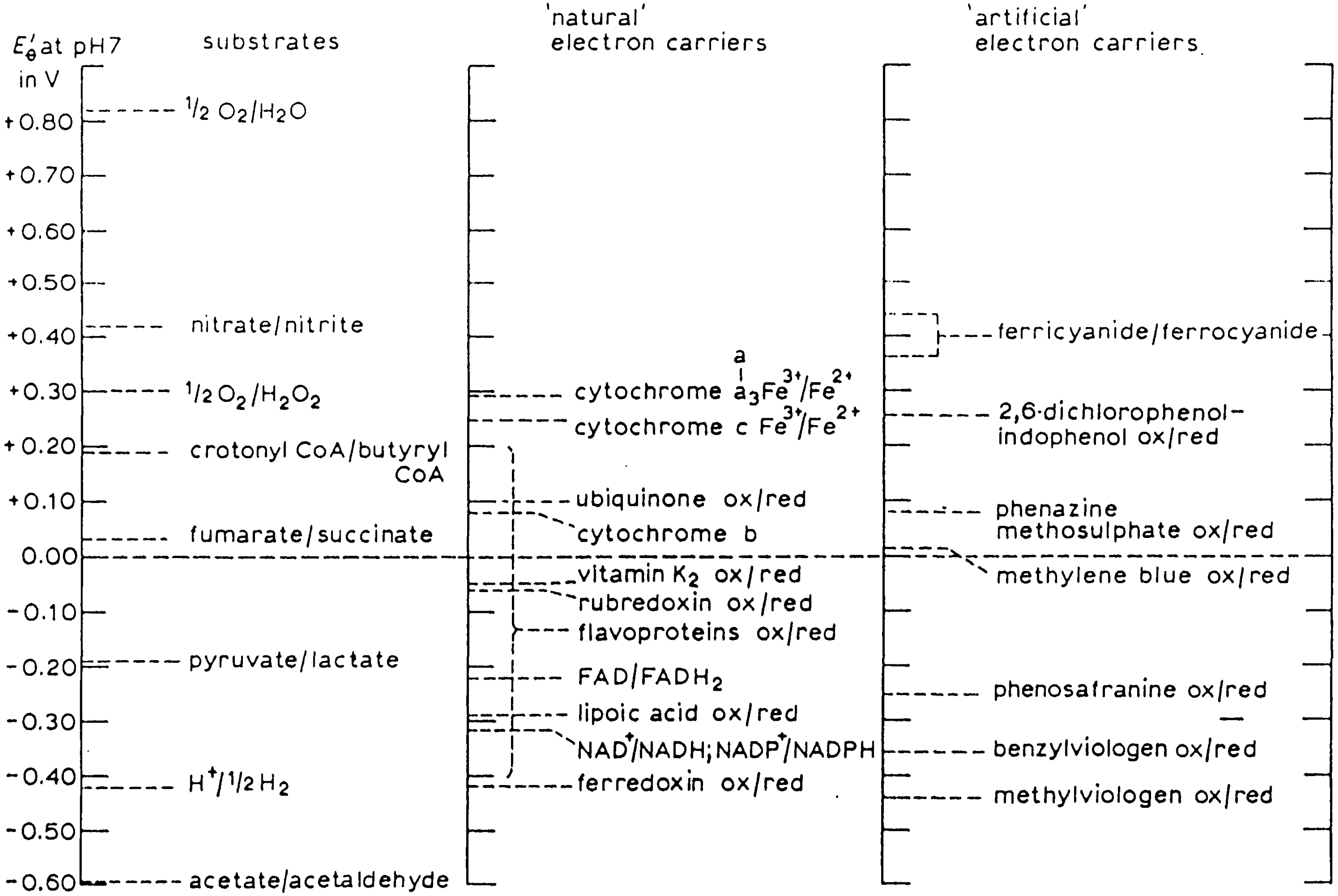
The first attempts to follow the OUR were successful (figure 3.30), enabling changes in OUR of the cells to be monitored over a 72 hour period.

The initial experiments with solvents were carried out in the challenge tank using the large impeller with a 1ml addition of octanol. The assay occurs over the maximum of a 100 minutes, during which time the OUR readings taken at zero hour may vary from a 100 percent to a value of 0. The first OUR results from challenged cells were not promising since they were all in the region of 10-20 percent of the unchallenged cell values. Whilst still of use they are difficult to analyses due to their similarity and give us no significant insight into the changes during the solvent challenge as reflected in the recorded capacitance (see figure 3.31.1 a).

One measure of viability has been described as whether the cells are respiring. Eukaryotic organisms have the components of the electron transport chain localised in subcellular organelles (mitochondria), while the prokaryotes have these processes located in the cytoplasmic membrane.

The highly exergonic oxidation of NADPH by O_2 is accomplished in a stepwise fashion through a series of electron carriers (Table 3.1). This makes a correlation of these techniques of particular importance (useful) in biocatalysts involving oxidative processes in organic solvents. Failure of these processes is of specific interest to the biological processes being considered here (outlined in chapter 1).

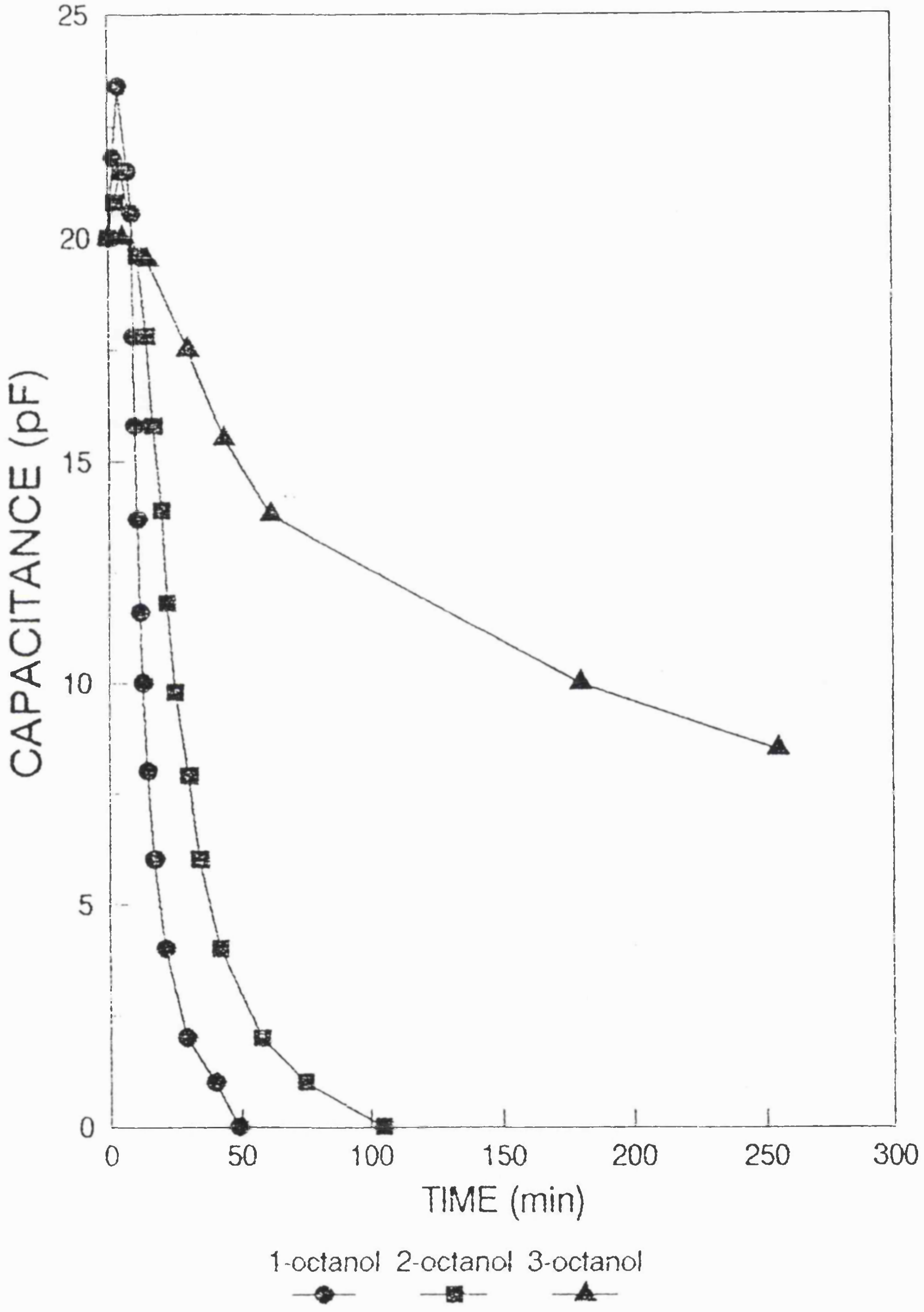
Table 3.1 Redox couples of interest to biologists



Values of $E'_{\ominus}$ at pH 7and 298 K (i.e. 25°C) for several redox couples of especial interest to biologists.

FIGURE 3.28

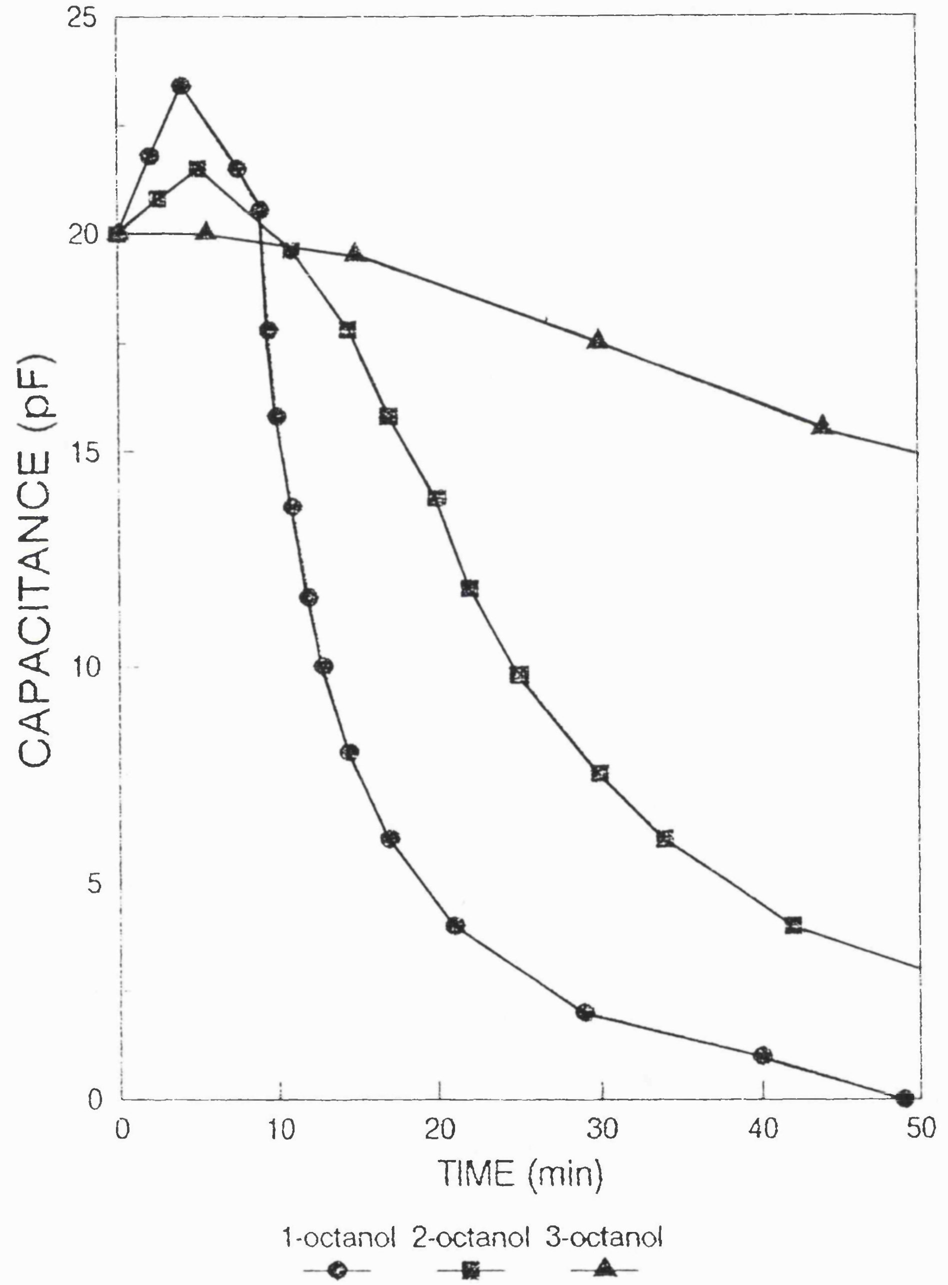
Addition of 1,2 and 3-octanols to *S.cerevisiae*



Addition of 1ml of 1-octanol and equimolar concentrations of 2-octanol and 3-octanol. Stirred with 3cm bar, at 500 rpm.

FIGURE 3.28.1

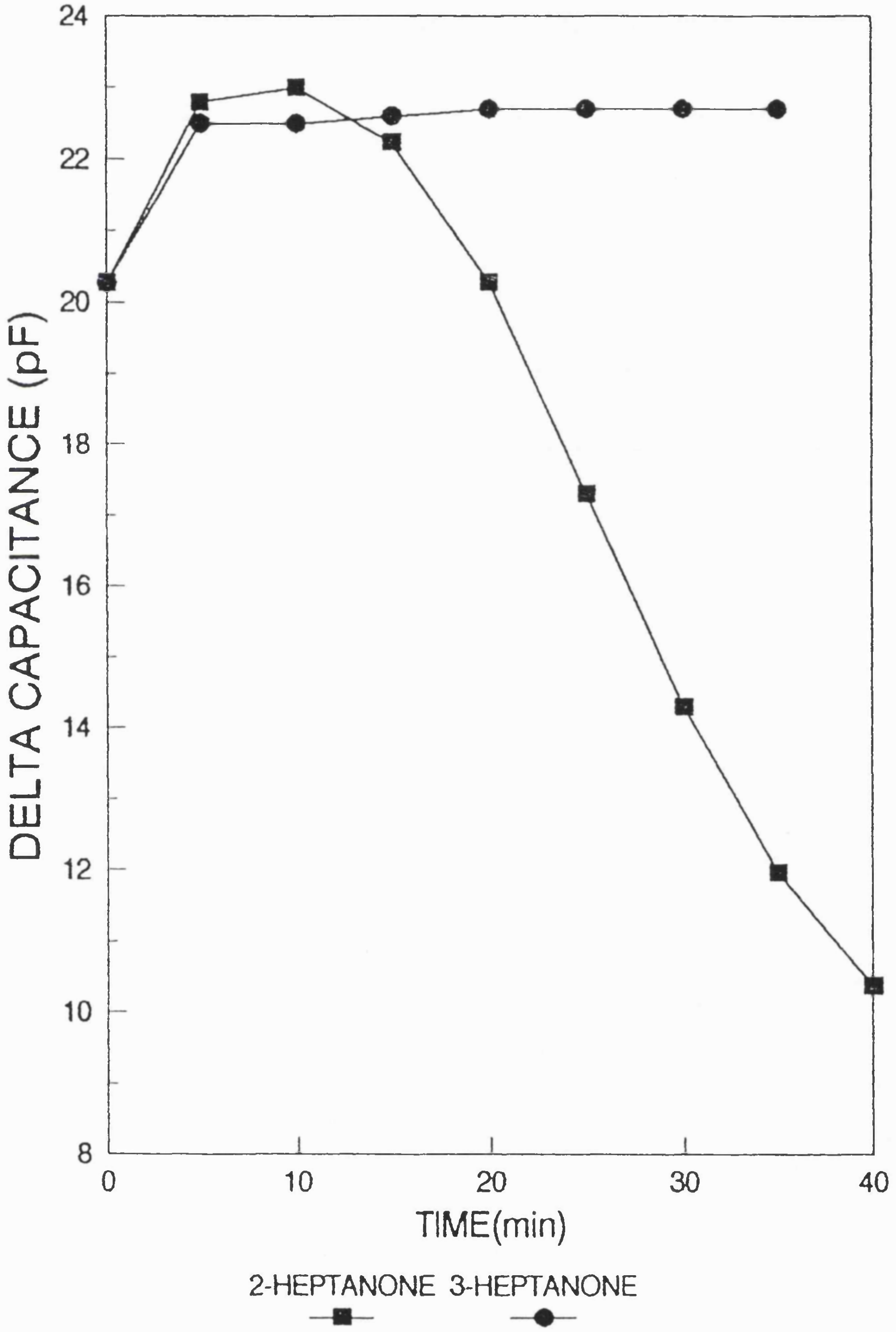
Expanded graph (0-50min) of addition of 1,2 and 3-octanol to *S.cerevisiae*



Addition of 1ml of 1-octanol and equimolar concentrations of 2-octanol and 3-octanol. Stirred with 3cm bar, at 500 rpm.

FIGURE 3.28.2

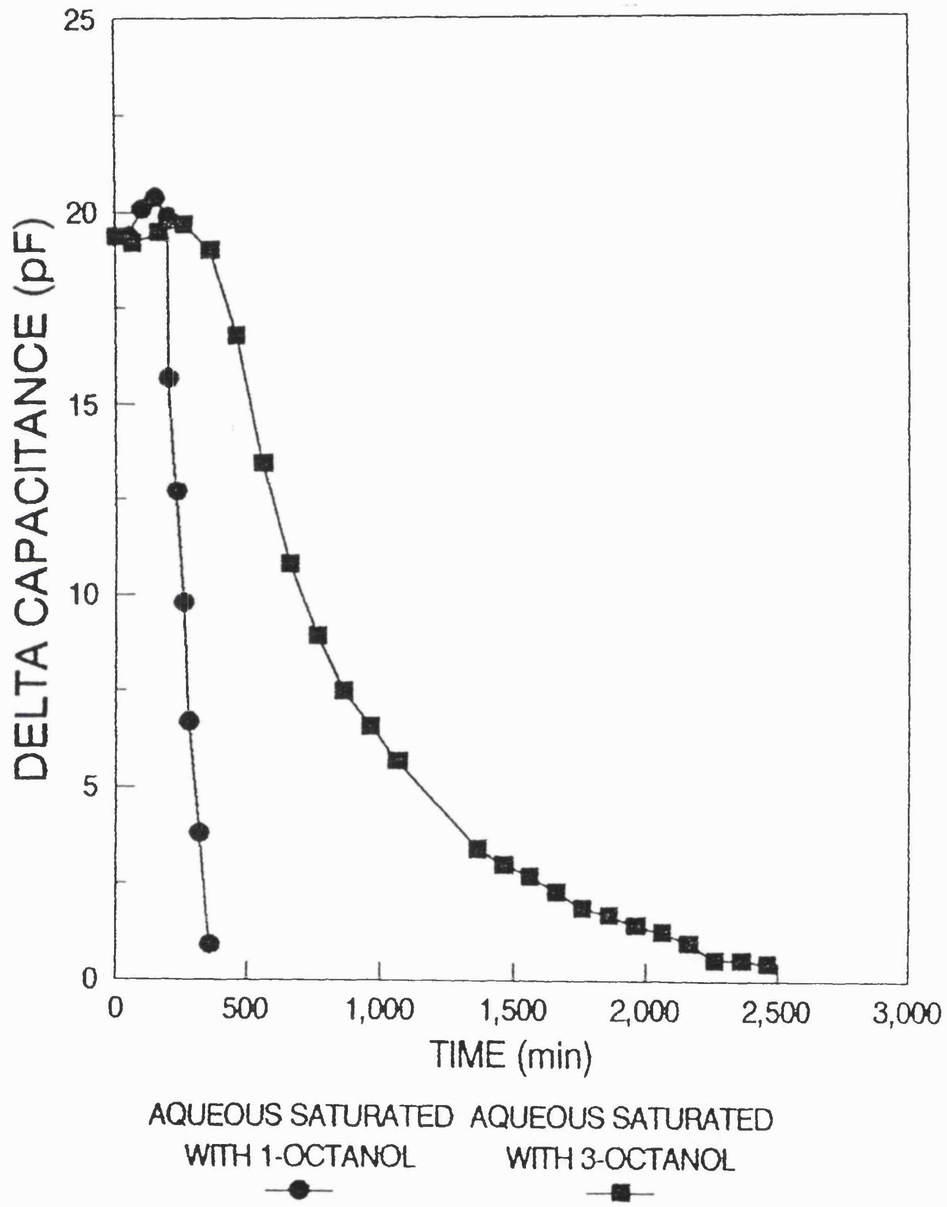
One volume addition of 2 and 3 heptanone to *S.cerevisiae*



Addition of 1ml of 2-heptanone and 3-heptanone, to *S.cerevisiae* in buffer, stirred with 3cm bar, at 500 rpm.

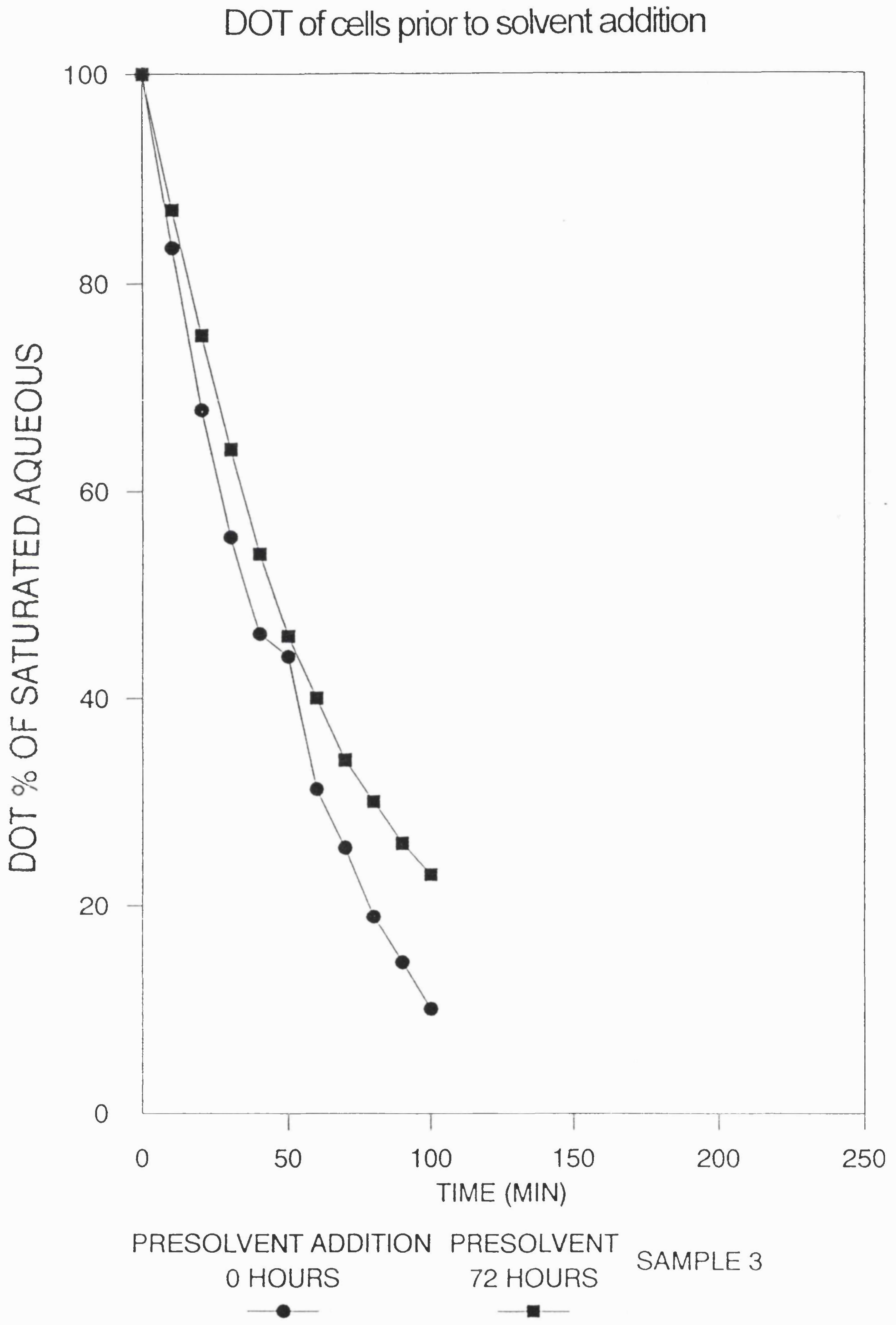
FIGURE 3.29

Aqueous saturated with 1 and 3-octanol ,challenged with *S.cerevisiae*



Graph of capacitance against time for *S.cerevisiae* challenged with aqueous pre-saturated with 1-octanol and 3-octanol, which are also present in a second-liuid phase

Figure 3.30



Graph of DOT against time for samples of *S.cerevisiae* removed from the challenge tank with no solvent addition

To increase the assay time differential between the two assay types (beta-dispersion and OUR), to produce a ratio where the rate of change of the beta-dispersion (see figure 3.31 for solvent toxicity) assay will be less significant compared to the time for the OUR assay (see figure 3.31.1, OUR). The effective toxicity of the solvent to the cells, as measured by the beta-dispersion is reduced by altering the challenge conditions (figure 3.31). The sensitivity of the OUR assay can then effectively be increased to a point where significant differences are apparent, allowing trends to be identified (simply the cell conditions are altered so that they are not undergoing rapid changes while being assayed for OUR).

These simple findings have serious repercussions when analysing results from the literature where log P curves from different experiments are compared, and then used to quantify the activity of a cell system subsequent to solvent addition. Each assay may produce different results according to its allowed conversion time.

These graphs also demonstrate that as the solvent partitions into the cytoplasmic membrane, the OUR is measurably increased when the time period for toxicity is relatively large (see figure 3.31.1 b).

3.17 Pentyl amine solvent addition

To demonstrate the affects of a solvent with the same general structure, but with a different solubility, pentyl amine was chosen (figure 3.32), since it has the same molecular shape as 1-pentanol, but is totally soluble in the aqueous phase.

Addition of this organic solvent

to the aqueous buffer increases the back ground reading (5%) without the presence of cells, this was due to an increase in conductance.

LEGEND FOR FIGURE 3.31

S.cerevisiae challenged with: 1-octanol (1ml), stirred at 500rpm, using 3cm bar; aqueous presaturated with respect to solvent addition, with a second-liquid phase present, stirred by a 1.5cm bar at 250rpm; aqueous saturated after cell addition by a second-liquid phase, aqueous stirred by 1.5cm bar at 250rpm.

LEGEND FOR FIGURE 3.31.1

OUR of *S.cerevisiae* from samples taken at various time intervals from the challenge tank, shown in figure 3.31

Figure 3.31

Selection of organic solvent challenges for *S.cerevisiae*

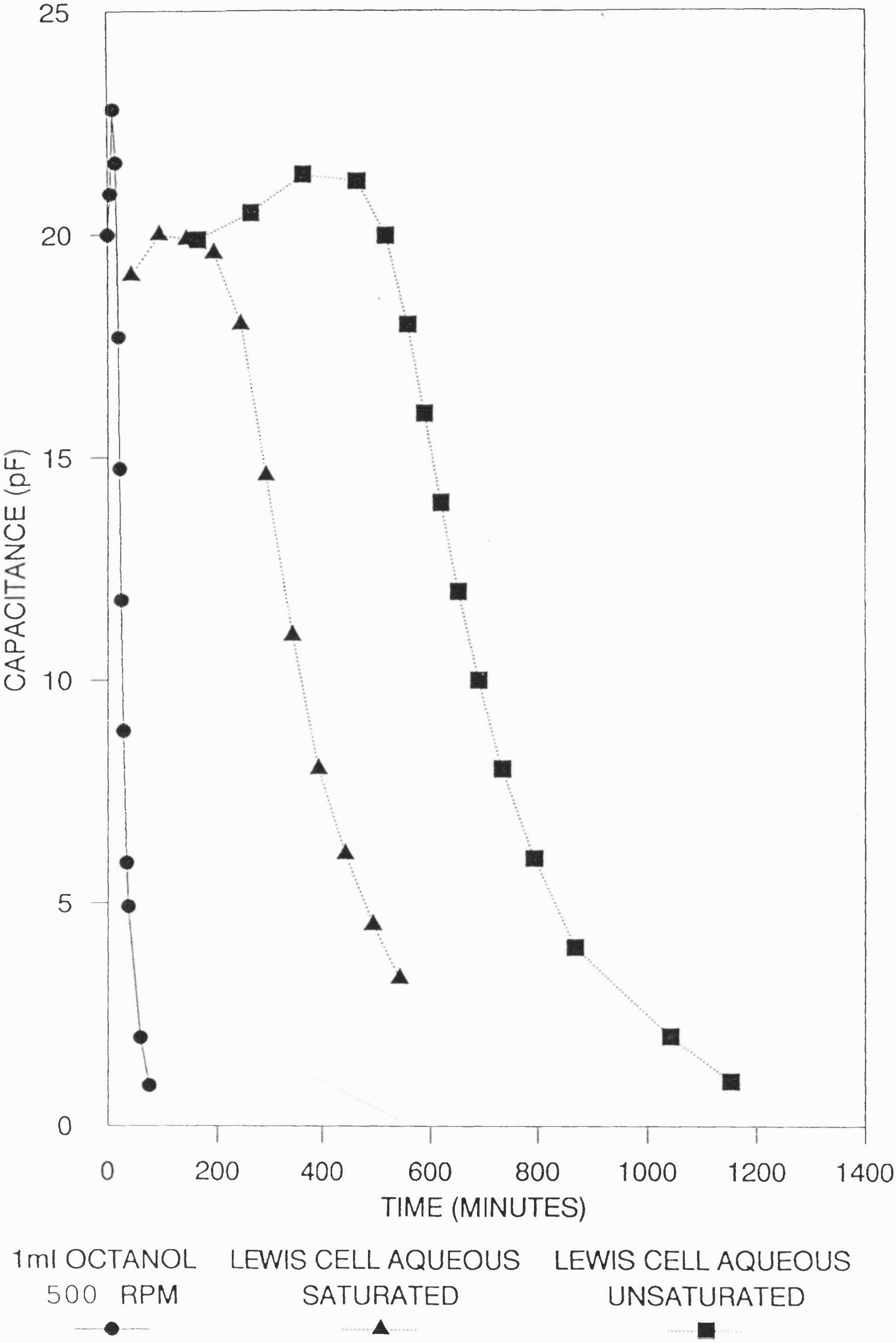
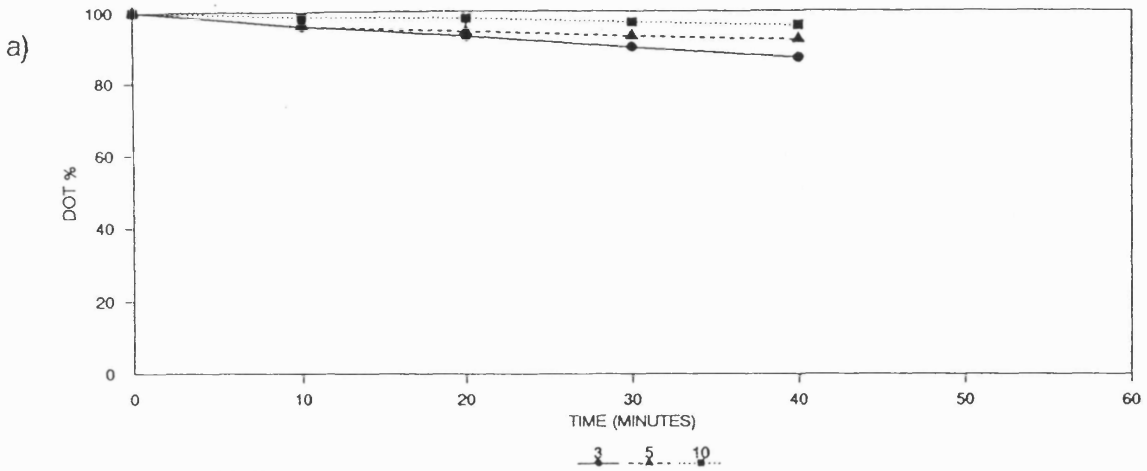
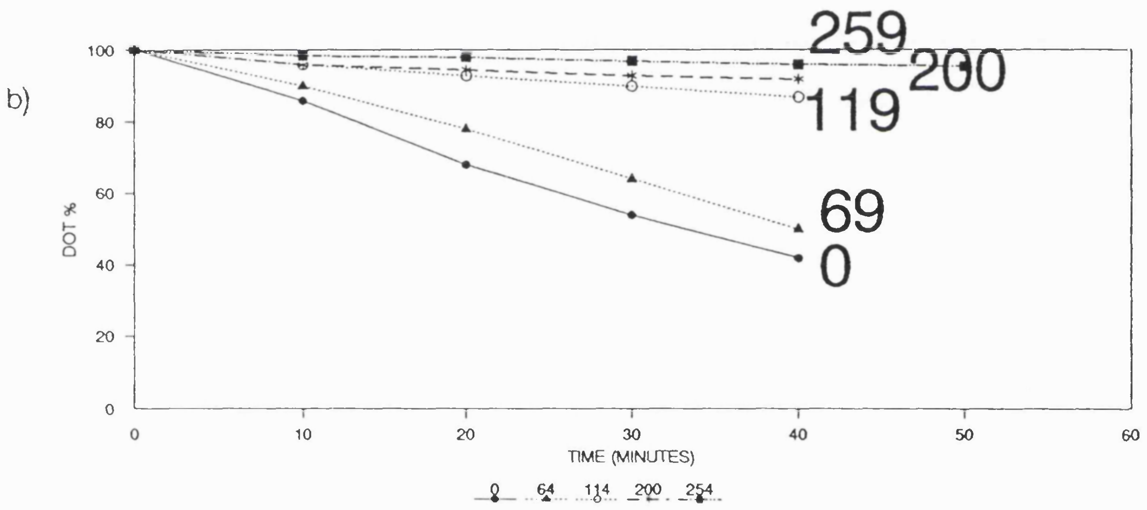


FIGURE 3.31.1

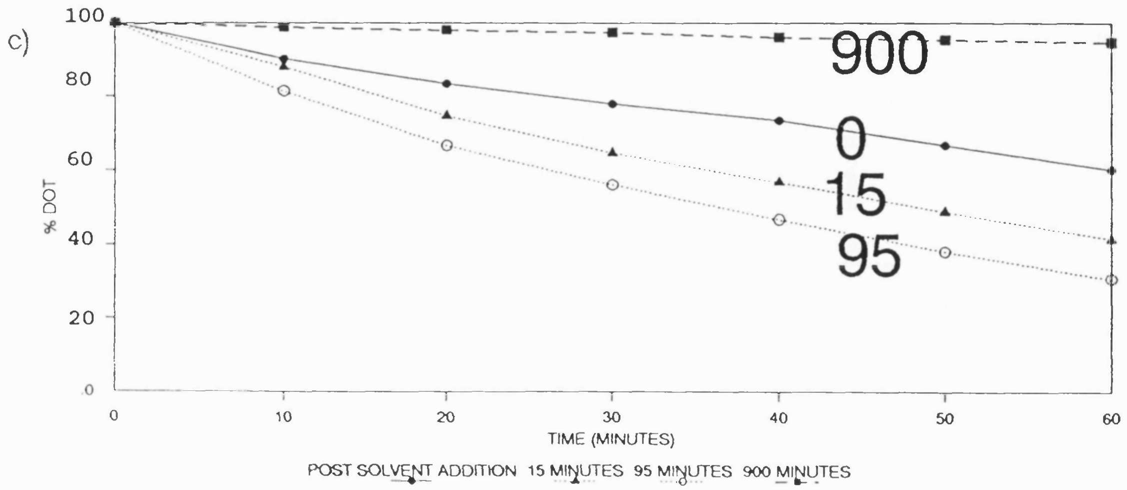
Oxygen uptake of cells challenged with organic solvent



1ml OCTANOL 500 RPM



LEWIS CELL WITH AQUEOUS SATURATED



LEWIS CELL WITH AQUEOUS UNSATURATED

From the graphs already discussed the increase in capacitance seems synonymous with an indication of solvent toxicity.

Pentyl amine is toxic at lower reactor concentrations than 1-pentanol. The trace characteristics are also different, the rise in capacitance occurs almost instantaneously, giving a linear rise followed by a gradual decease in reading. One ml of solvent driving the signal immediately down to zero. Smaller additions have correspondingly less toxic effects.

Figure 3.32.1 shows analysis points for OUR, given in figure 3.32.2 for yeast cells treated with 0.25ml of pentyl amine. Over a 5 minute period, a good correlation between the OUR and capacitance is obtained. The control OUR has the highest endogenous uptake rate, with each successive time point having a lower OUR.

The OUR is not an instantaneous measurement, unlike the beta-dispersion readings. It is the state of the cell over a time period. It is therefore effected by fluctuation in cell stability within that time period.

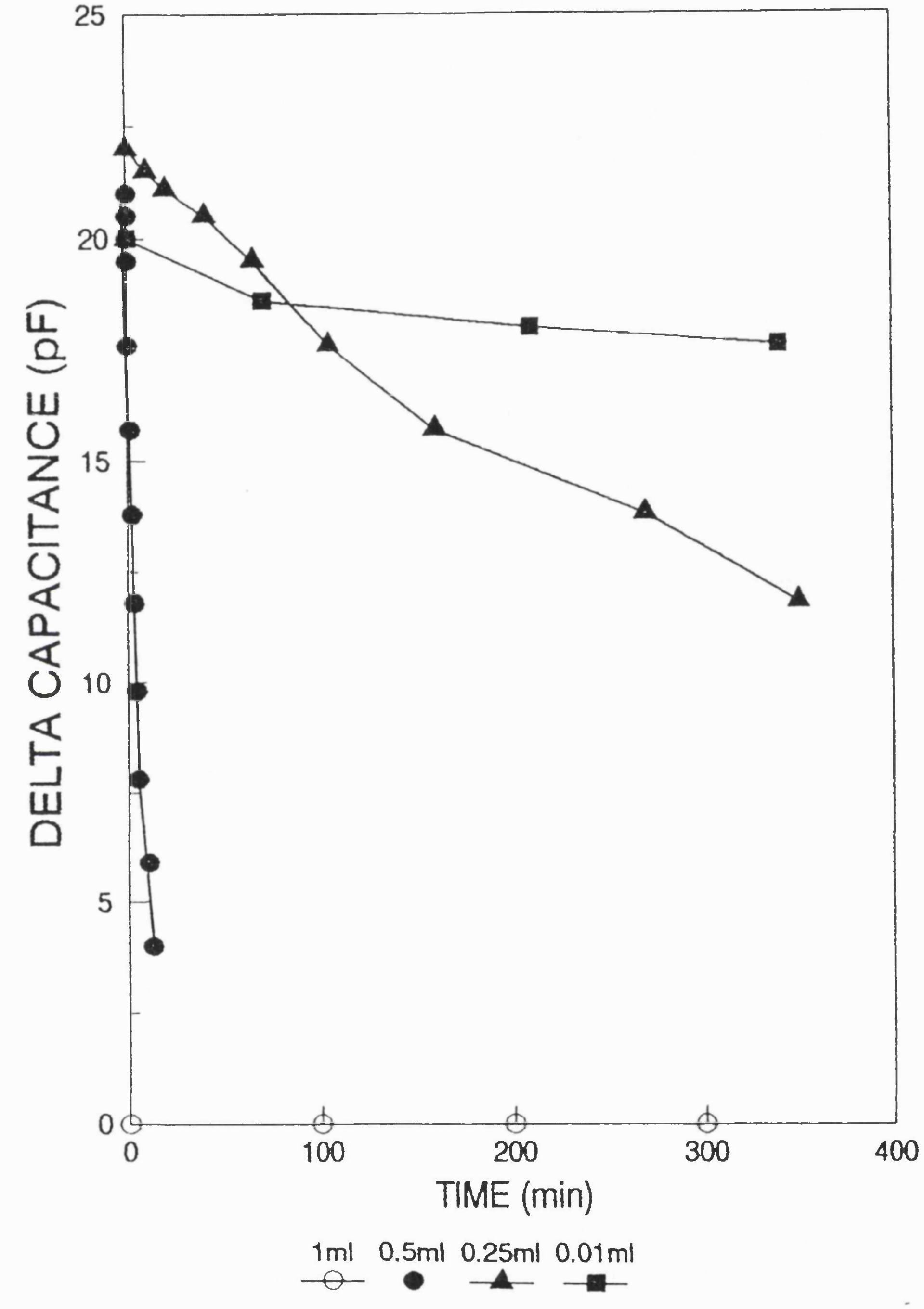
Figure 3.33 is an extended OUR assay after solvent challenge. Further examination seemed warranted when the trace of OUR indicated a recovery of the cells after challenging with solvent. This may be due to the dilution (1 into 60) of each cell sample when transferred into the OUR assay electrode chamber. In the control the OUR continues to drop, then begins to slow down as it progresses. The solvent treated cells have a very poor initial OUR, and then begin a steady increase of their OUR. The highest OUR being equal to the control (between 150-200 minutes). This may be a recovery of the cells after treatment with an organic solvent.

Even after the capacitance had reached a zero value the recorded OUR still showed a slight uptake ability (see figure 3.31.1), further discussed in chapter 4.

The drop in OUR is initially at a low ratio to that of the capacitance. When the capacitance drops below 75 percent of its original value, these OUR values drop in a much higher ratio to the capacitance, indicating the presence of a cut off point for the cell oxidation system.

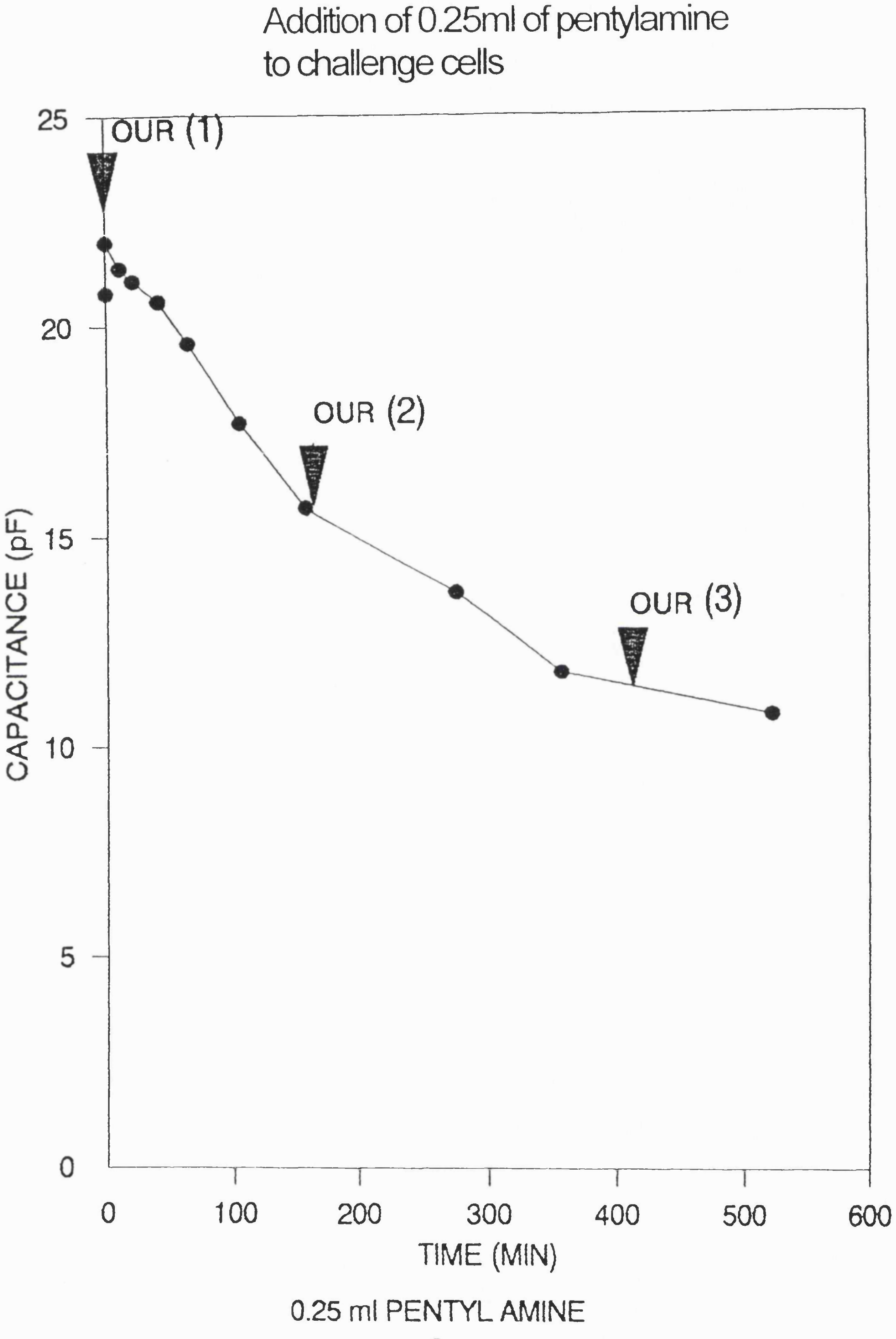
Figure 3.32

Addition of pentylamine to *S.cerevisiae*



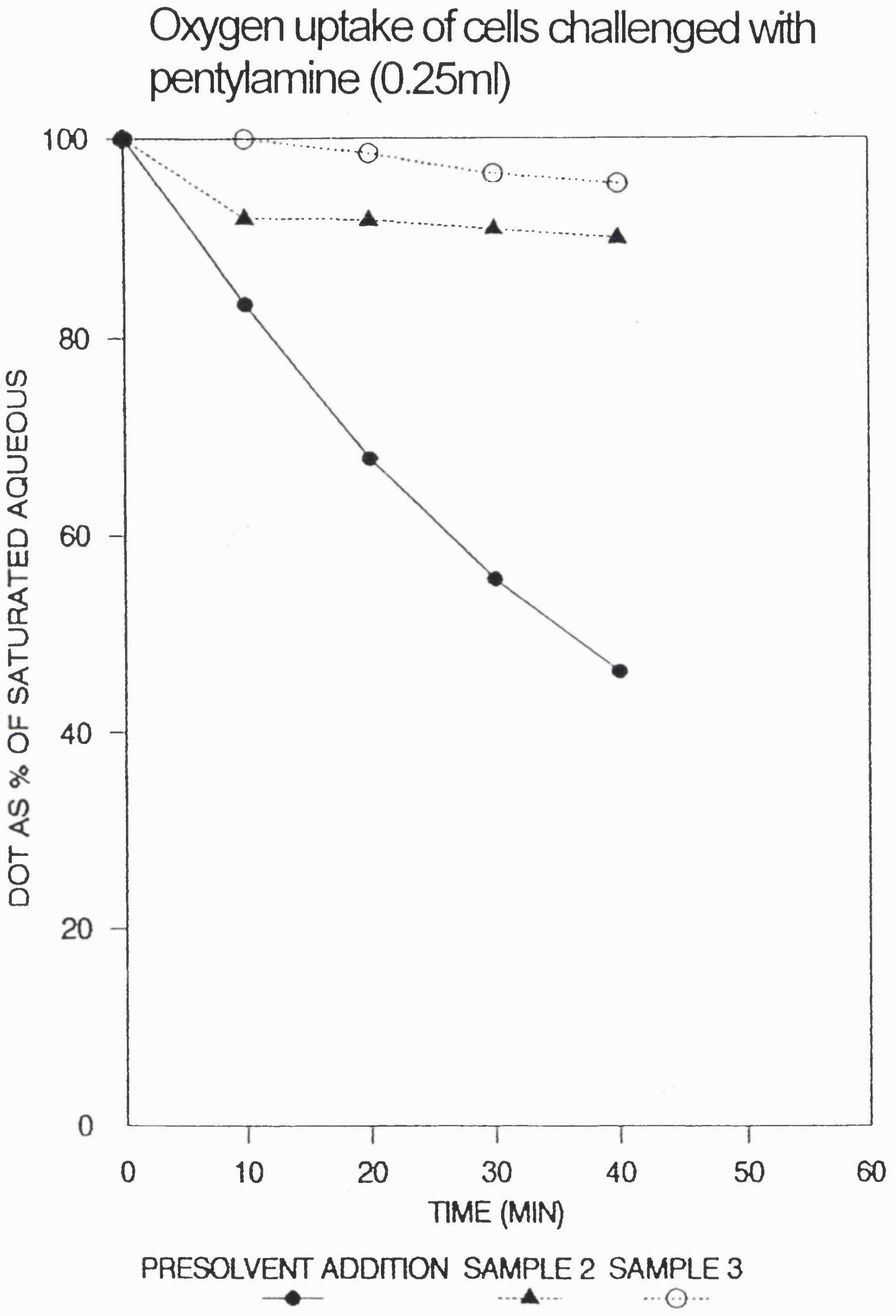
Graph of capacitance against time for *S.cerevisiae* challenged with pentylamine addition at various concentrations,stirred with 3cm bar at 500rpm

Figure 3.32.1



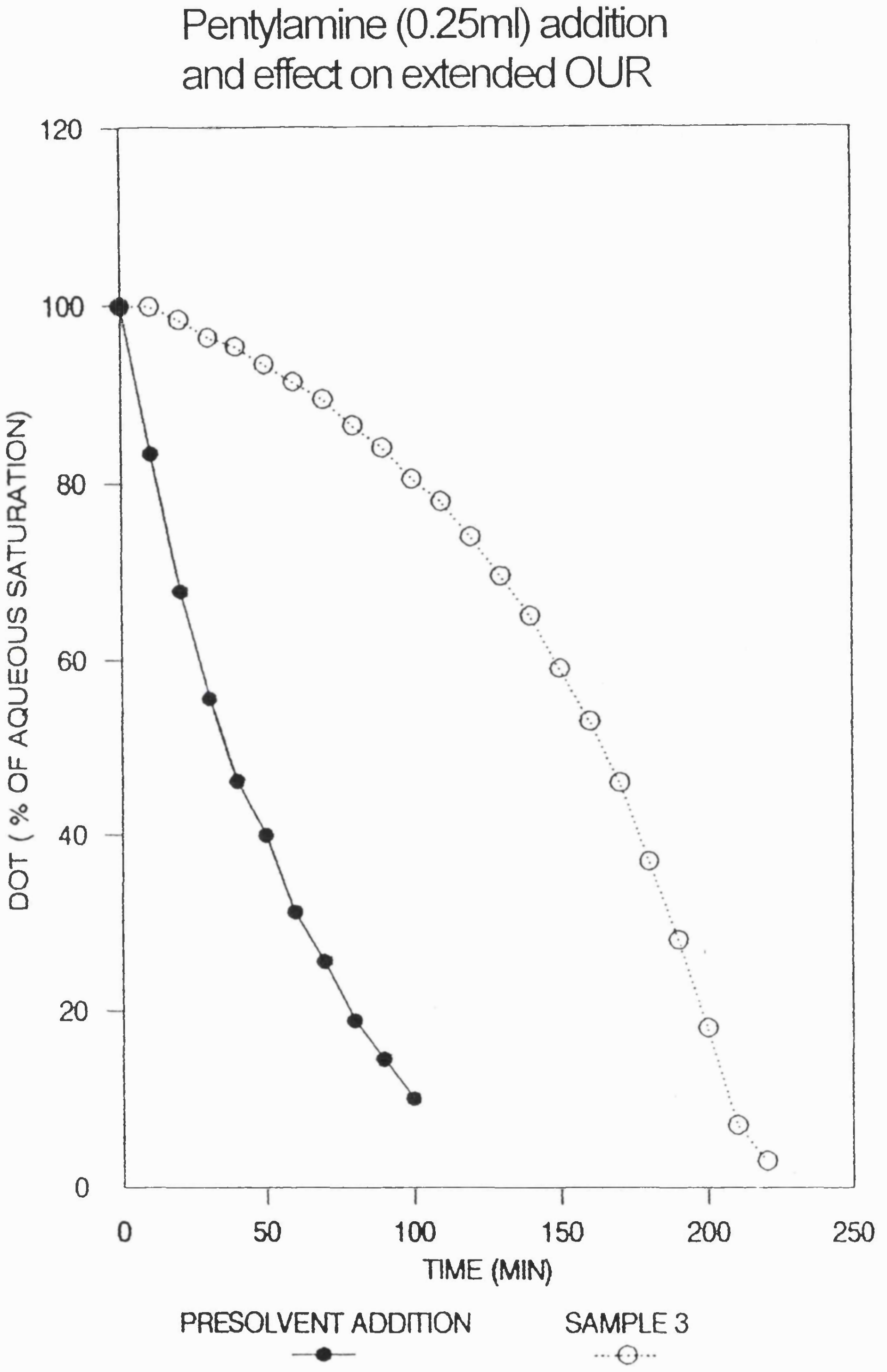
S.cerevisiae challenged with pentylamine addition (0.25 ml), stirred with 3cm bar at 500 rpm. Time points for OUR samples shown.

Figure 3.32.2



Graph of DOT against time to illustrate OUR of *S.cerevisiae* after challenge with pentylamine addition(0.25ml),time points shown in fig. 3.32.1

Figure 3.33



Graph of DOT against time, for extended OUR assay of *S.cerevisiae* after challenge with pentylamine(0.25 ml), from fig.3.32

3.18 Dihydroxylated aliphatic alcohols

By inspection of the earlier data, we can infer that the toxicity of a diol solvent, which is terminally dihydroxylated, may be less toxic (since it is very aqueous soluble and might not readily intercalate into or align with the phospholipid head groups in the cell membrane, unlike singly terminally hydroxylated alcohols (Hitzeman et al 1986)) than linear terminal hydroxylated alcohols. To establish an estimation of the solvent toxicity cells were challenged with aqueous concentrations of solvent. Figure 3.34 shows addition of 1,5-pentandiol (10 and 15 percent v/v) to suspended cells, no toxic effects were seen.

1,9-Nonanediol (20% v/v) was added to the OUR chamber with and without cells (figure 3.35). The solvent has no effect on the OUR electrode itself, but raised the OUR of cells above their normal rate.

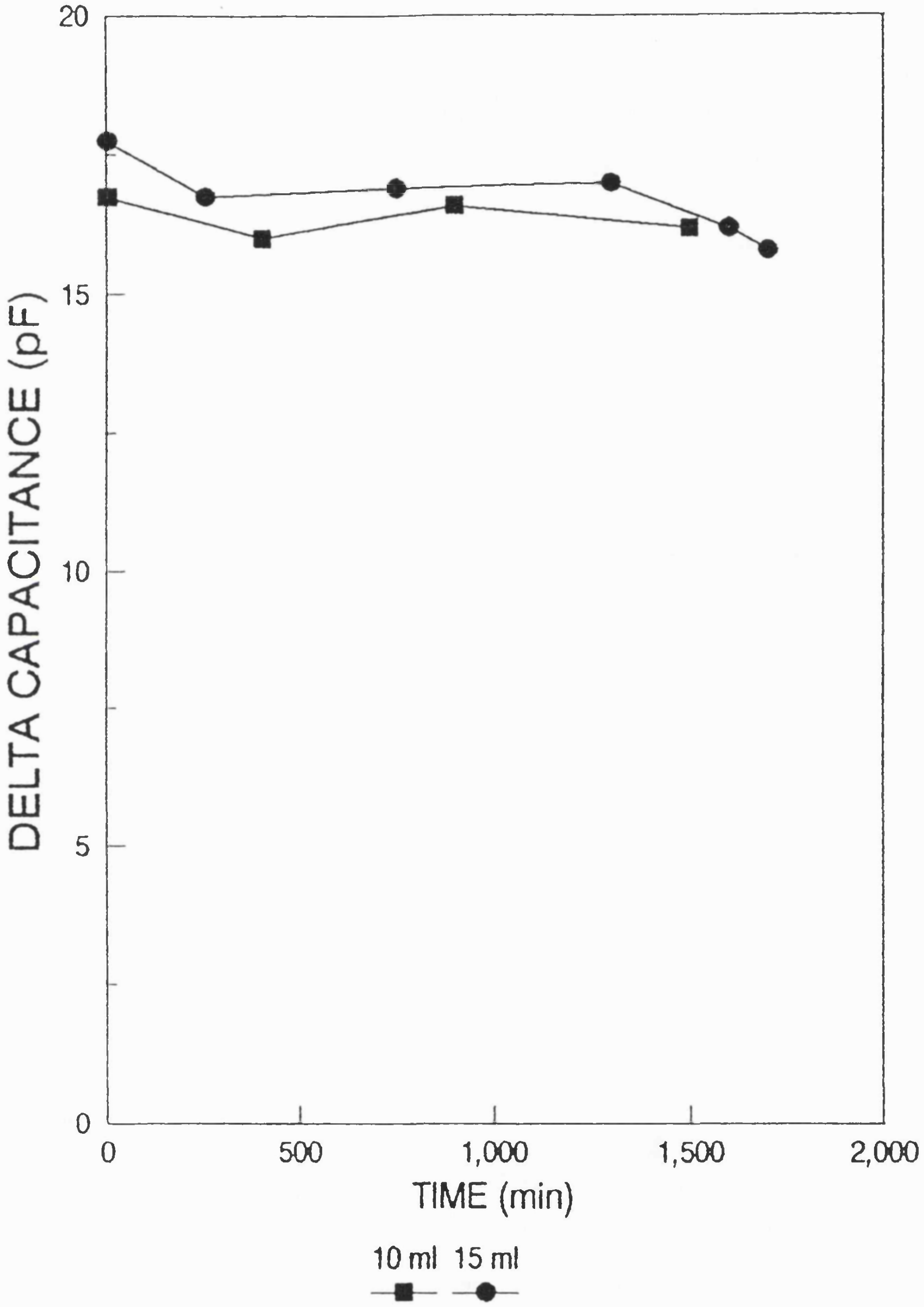
3.19 Hexadecane solvent challenge

If mass transfer is a limiting parameter to solvent toxicity, then it is likely that a very poorly-water soluble organic solvent, which does not have a hydrophilic group, would be almost non toxic. Hexadecane is a linear hydrocarbon chain with no hydrophilic group.

For hexadecane solvent challenges against time for *S.cerevisiae* in the challenge tank, over a 72 hour period showed a capacitance rise of 8 percent of the original value. If the data is extrapolated, we could infer that hexadecane might be toxic over a prolonged time period, but this is beyond the present scope of this project.

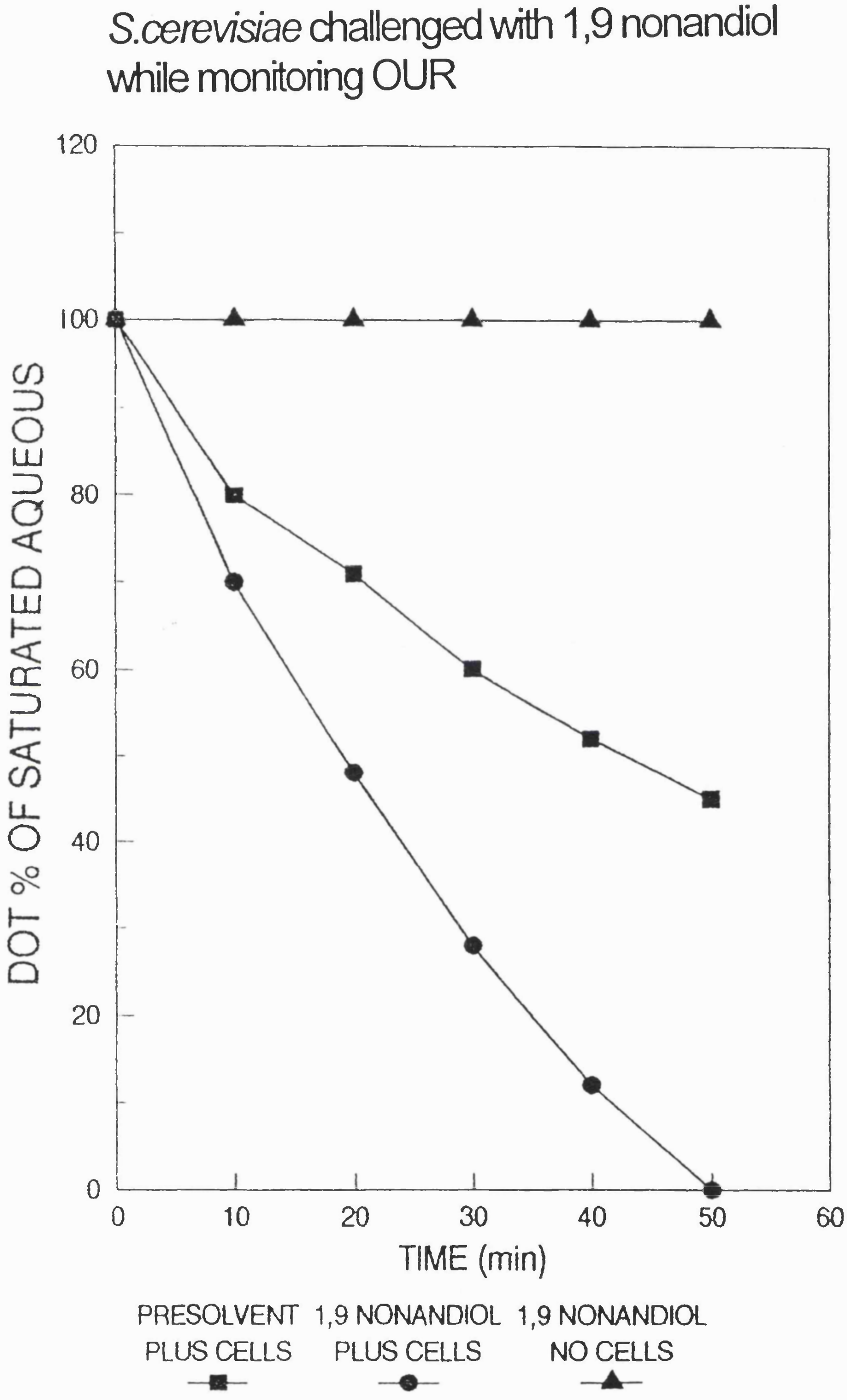
FIGURE 3.34

Pentandiol addition to *S.cerevisiae*



Graph of capacitance against time for pentandiol addition to *S.cerevisiae* in buffer (500rpm,3cm bar)

FIGURE 3.35



Graph of DOT against time for 1,9 nonandiol addition to OUR chamber, with and without *S.cerevisiae*, and addition of *S.cerevisiae* without solvent addition

3.20 Penicillin production and solvent addition

From earlier sections (3.14), a correlation between the rise in the beta-dispersion and the OUR for these cells was shown. This raises the question of whether a relationship between the addition of organic solvent at the correct concentration and the endogenous OUR exists. This phenomena may be limited to the oxidation of just a few compounds, which have a limited effect on the overall OUR and have no real application. The cell metabolism may remain unaffected or maybe peculiar to the yeast *S.cerevisiae*, which has an ability to metabolise ethanol and may be similarly stimulated by other alcohols. Although no reference to this occurs in the literature for *S.cerevisiae* or *Y.lipolytica*.

To test this hypothesis, another organism is needed which is unable to utilise alcohol (eg not a yeast). A common test organism is the eukaryotic organism *Penicillium chrysogenum*. This organism has been well documented and investigated by many workers and would make an ideal test case for the effects of organic solvents on the overall metabolism of a higher organism.

Description of the fermentation details

These experiments were carried out in shaken flasks (details see chapter 2). The precursor was phenyl acetic acid (which is nontoxic at this pH), added at the start of the experiment for penicillin V production.

A standard curve plot of peak area and

penicillin V is shown in figure 3.37, the graph is linear over the range investigated (0-1g/l).

The shake flasks minus the control were each inoculated with a volume of 3-octanol shown in figure 3.36. The 3-octanol was used since it has a high aqueous solubility but less toxic than the 1-octanol. From the previous experiments the solvent should raise OUR and possibly elevate metabolic activity.

A small volume addition of 3-octanol increases the biomass (dry weight) by 8 percent over the control without solvent addition. With greater volume additions the toxic effects of the solvent become apparent, decreasing the biomass obtained after 120 hours. The production of penicillin V also becomes reduced when larger volumes of solvent are added, showing the toxic effects of the solvent.

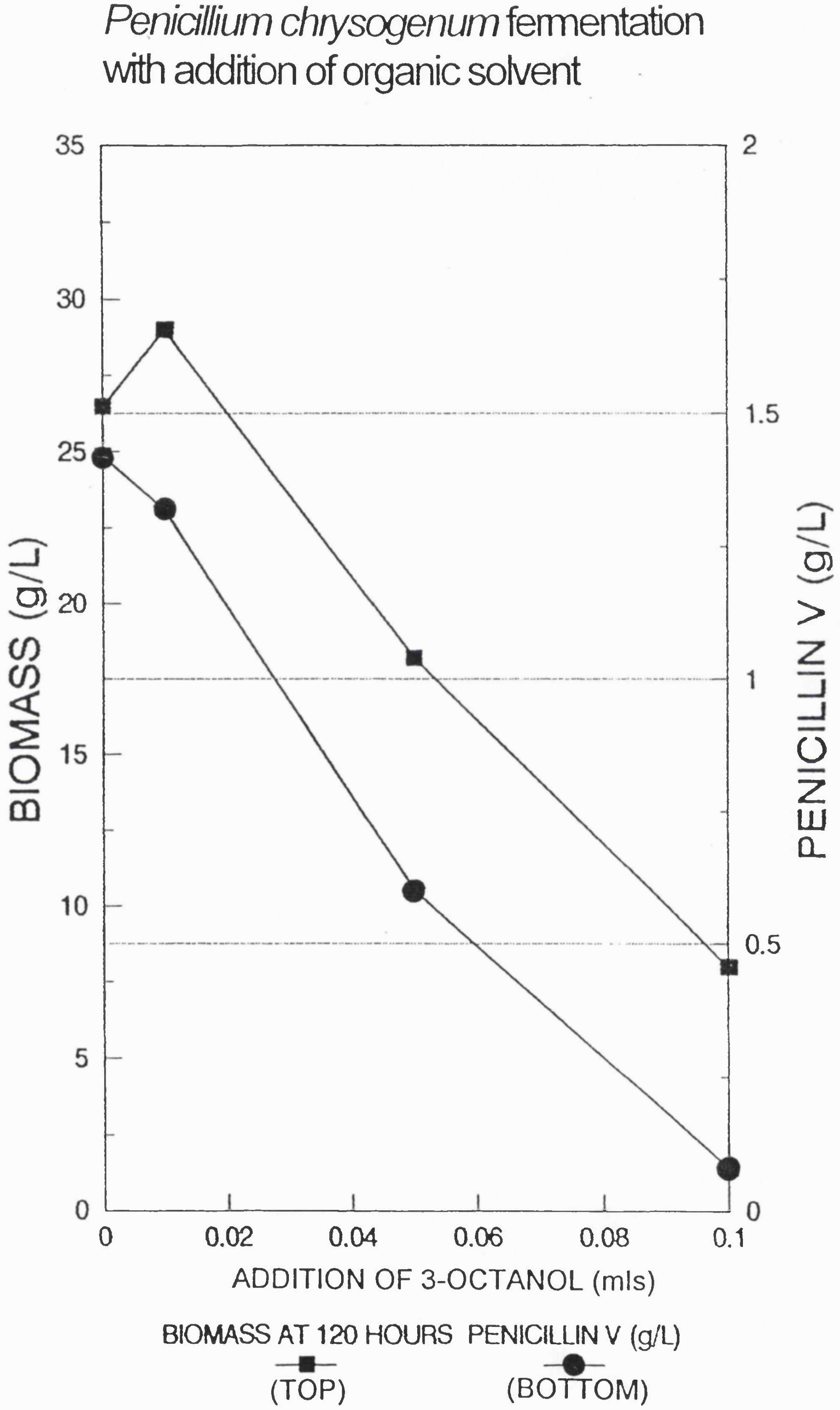
TABLE 3.2

Fermentation	Penicillin V (100 hours)
Control	1.50 g/l
0.01ml 3-octanol	1.50 g/l

Table 3.2 shows that earlier in the fermentation no difference in the penicillin production of the control and the 0.01ml addition of 3-octanol is apparent. Monitoring of the penicillin was not the aim of this experiment, but is included to show further evidence of the effects of solvent on these cells. From figure 3.36, in the 20 hours between these sample times the penicillin V has dropped in both cases, but drops faster in the fermentation with the addition of solvent (the penicillin V does degrade with time although it was selected for its stability). This shows a general trend associated with the

addition of this solvent and demonstrates the validity of using the capacitance as described and the meaning of OUR variations. The relationship of these trends and their cause is discussed further in chapter 4.

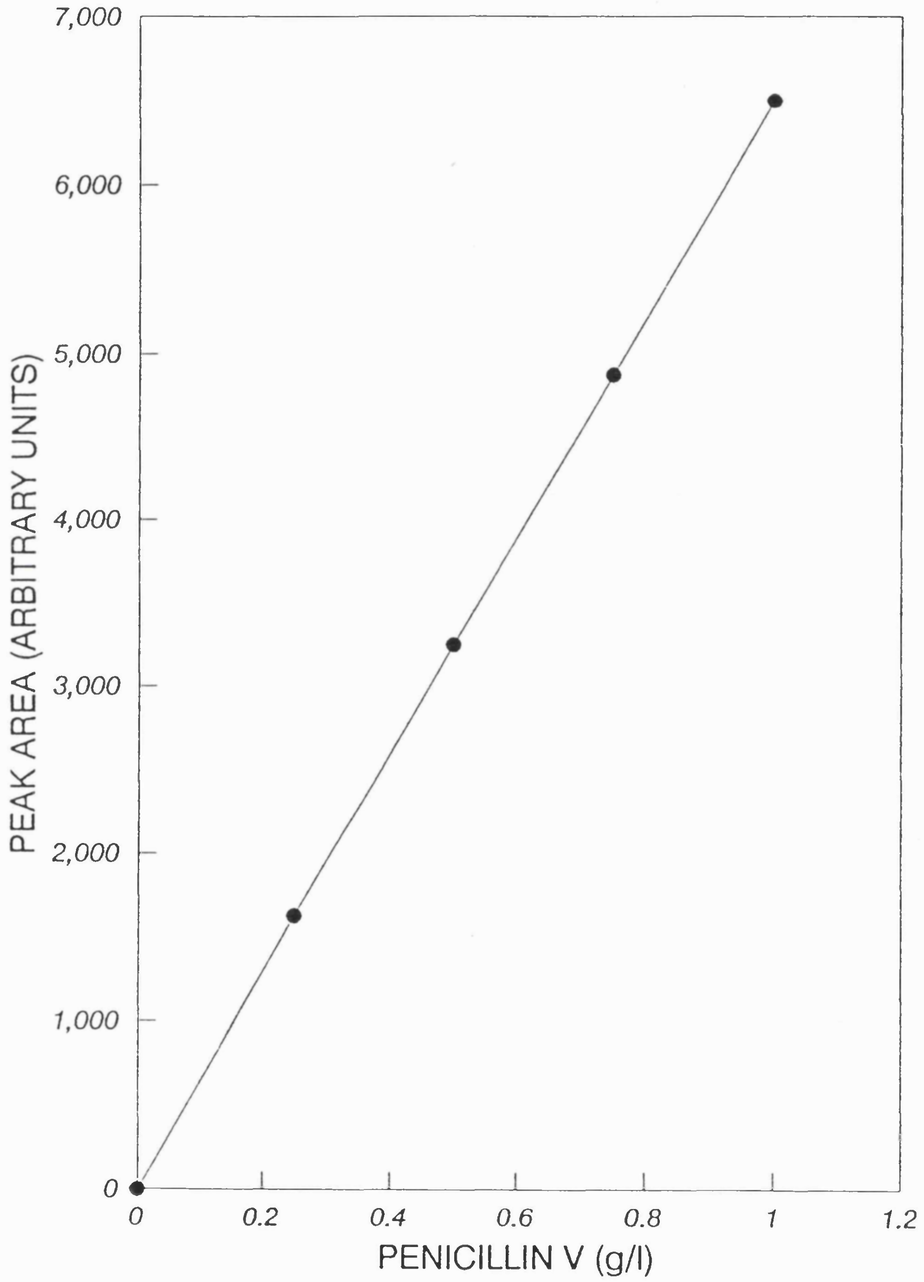
FIGURE 3.36



Graph of biomass against 3-octanol addition for *P.chrysogenum* fermentation

FIGURE 3.37

Standard curve for penicillin V against peak area



3.21 Effect of the cell wall and solvent structure

The solvents might well be selectively partitioning through the cell wall into the cytoplasmic membrane.

Two approaches to this problem are possible, the first is : To remove the cell wall from the yeast to form protoplasts. This is unsatisfactory in that it would be impossible to determine the effects of the protoplast process on the yeast cell membrane integrity ; The second approach to determine the effects of the cell wall is to challenge animal cells with organic solvents. Since they do not have a cell wall, are eukaryotic (as are yeast) and require no pretreatment, they were selected to test this general hypothesis.

The cells were grown in roller bottles and were then transferred into the challenge tank before solvent addition.

Treatment of animal cells

Special adaptations were made for the animal cells (lower shear, and careful handling as described in chapter 2). High shear (as used with the yeast cells) resulted in very fast lysis of the animal cells, which form stringy fibres of cells, leading to erratic results. Large changes in temperature were also noted to have a detrimental effect on the animal cells.

The growth medium used to culture the animal cells gave a conductance reading which was unacceptably high for accurate readings determination of biomass by the beta-dispersion technique (see chapter 1).

This required the use of a substitute solution for the monitoring of animal cells by beta-dispersion. To maintain the cell stability, the medium of choice was found to be a slightly higher concentration of phosphate buffer (see chapter 2).

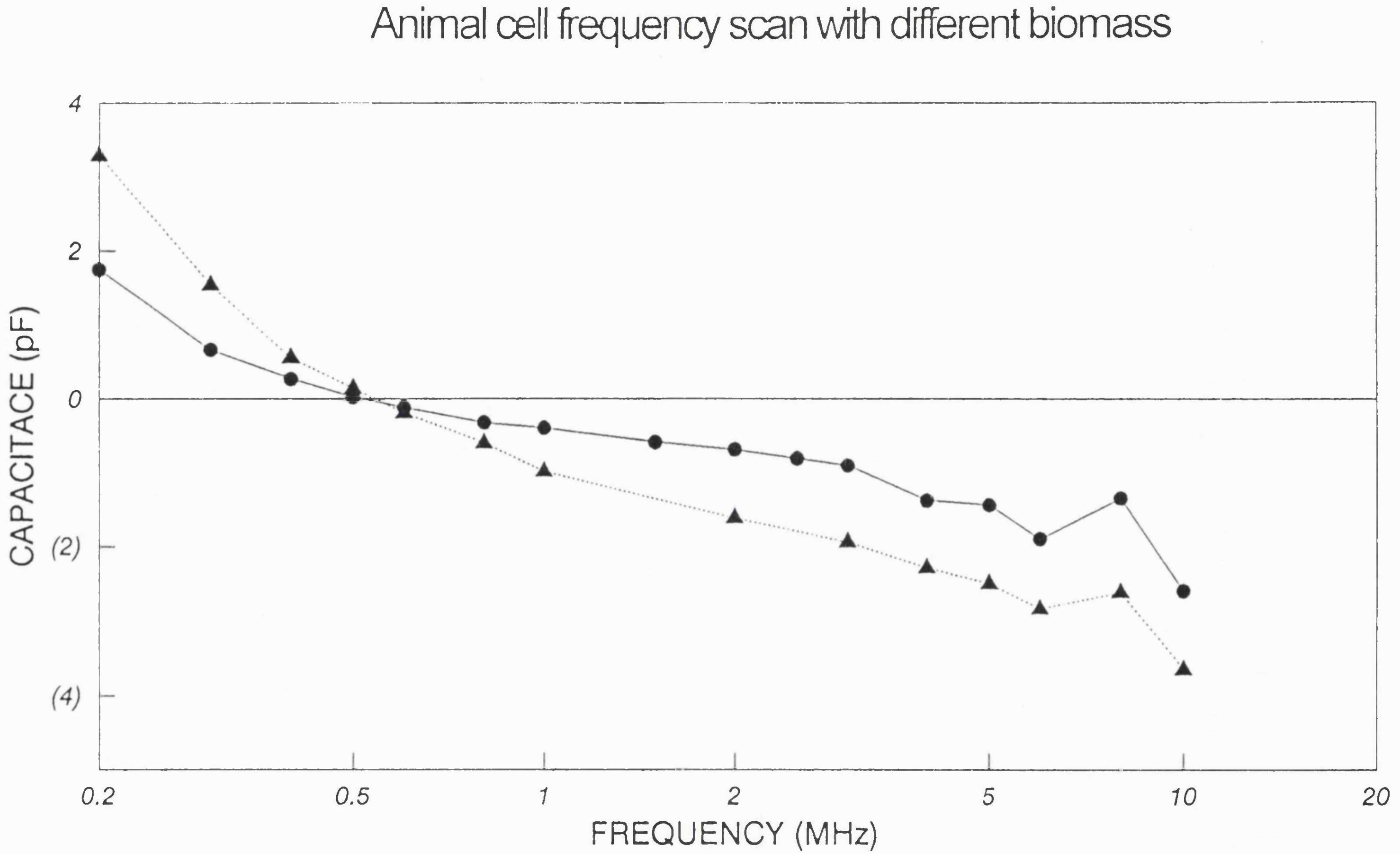
To establish a correlation between the total number of cells and those which were viable, the dye assay trypan blue was used (nonviable cells stain blue, note that dead cells may also stain light blue).

Figure 3.40 (p192) shows the characteristic frequency scan of the animal cells. The effect of solvent addition (1-octanol) to animal cells in a stirred reactor, monitored by the cells beta-dispersion is shown in figure 3.39.

At the sample times shown, cells were removed from the challenge tank and assayed by the trypan blue assay. In sample 1 the number of non-viable cells was 6 percent. At sample 2 all the cells observed were light blue, at sample 3 no clear cell structure could be defined. The last sample (4) had very few cells which could be identified, and all of those were stained light blue. The total number of cells used was $2.7*10^5$ cells per ml.

It is clear that for the animal cells a need exists to assess the intactness of the cell membrane, since it is difficult to establish a direct correlation of viable cells and the beta-dispersion recorded, although a general trend can easily be identified.

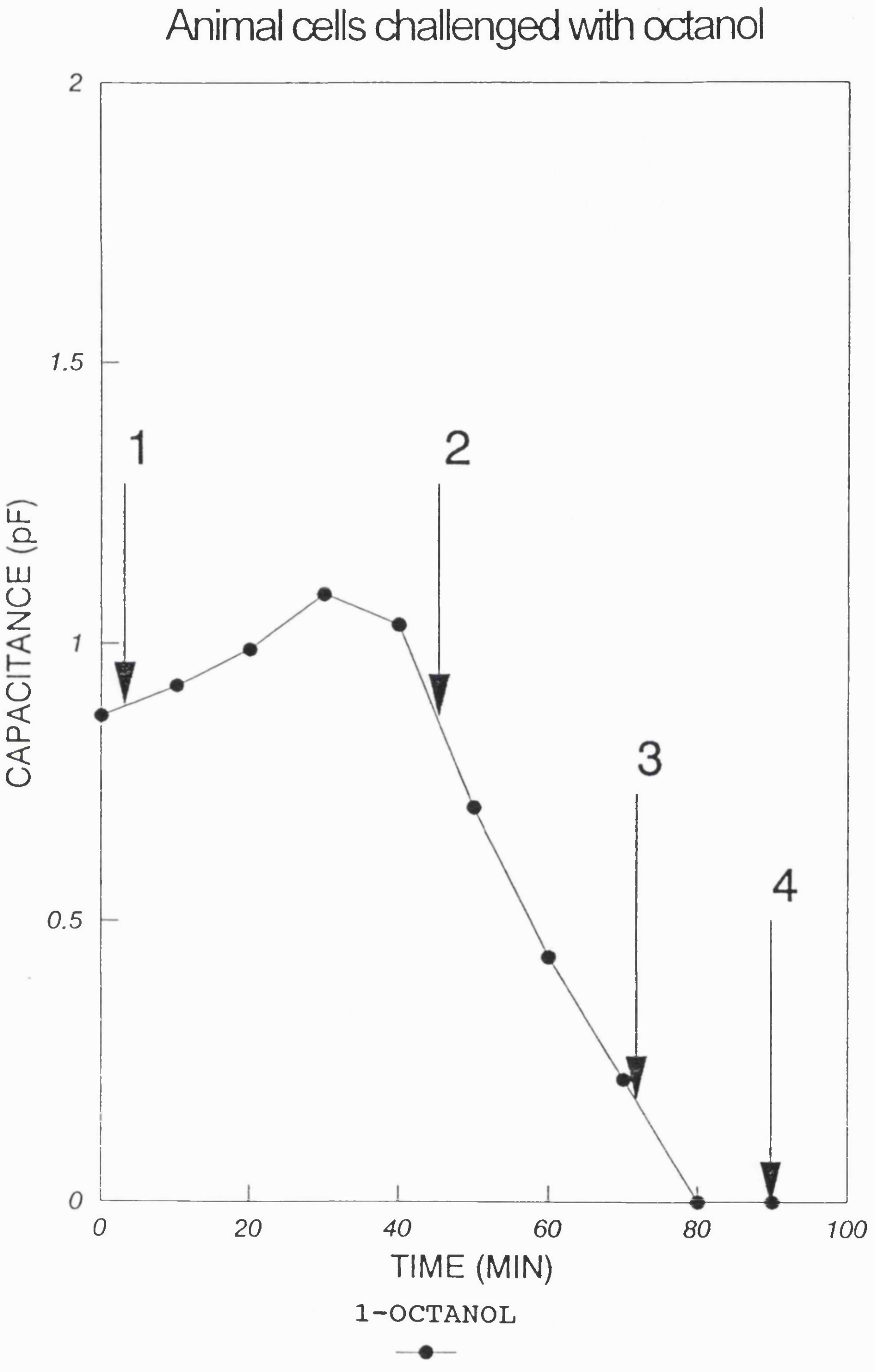
FIGURE 3.38



Animal cell frequency scan in challenge tank in buffer, stirred at 50rpm by a 1cm bar.

—●— 3.0g/l of cells
—▲— 1.5g/l of cells

FIGURE 3.39



Graph of capacitance against time for animal cells challenged with 1-octanol, samples removed for staining and microscopic examination

The total number of cells used with each solvent is the same within experimental errors throughout the experiment. The number of viable cells differ significantly between the solvents, the 1-octanol proving toxic after only two hours.

Viability in the high cell inoculum was 25 percent, while the 3-octanol sample was 95 percent. Less difference is noticed in the low inoculum challenge 80 percent to 97 percent for 1 and 3 octanol respectively.

3.22 *Y.lipolytica and P.putida* biotransformation and viability

The Gram-negative bacteria *P.putida* and the yeast *Y.lipolytica* adhere to the organic/aqueous interface and are therefore not suitable for the initial investigations by the beta-dispersion technique with a two-liquid phase system (see section 3.6). However the biotransformation involving naphthalene hydroxylation catalysed by *P.putida* and *Y.lipolytica* would be a suitable method for monitoring solvent effects in the presence of a second-liquid phase.

P.putida

At 10 and 20% (v/v) concentration 1,5-pentandiol had no effect on the biotransformation conversion (0.75 g/L) after 2 hours compared to the control (without solvent addition). It also had no effect on the viability of *P.putida* after two hours exposure with solvent (aliphatic alcohol) series at 20% (v/v), see figure 3.41.

Y.lipolytica

The *Y.lipolytica* biotransformation produced ca. 3.0 micrograms of naphthalene-1,2-dihydro-1,2-diol per litre, which was well below the level necessary to distinguish it from experimental variation (+/- 5 micrograms per litre by reverse phase chromatography), it also failed to hydroxylate indigo on agar plates, a strong indication of its lack of activity. This level of production is at the limit of detection and reproducibility.

3.23 Temperature pretreatment and solvent tolerance

Cells of *S.cerevisiae* were incubated at 15, 28 and 32oC in sterile conical baffled shake flasks, at 150 rpm, for 18 hours before assaying. The cells were then transferred into the challenge tank and allowed to stabilise for 15 minutes before addition of solvent (1-octanol 0.4% v/v, see figure 3.42). OUR measurements from the cells were taken immediately prior to the solvent challenge.

The plot of delta capacitance versus time shows a peak rise in capacitance after addition of solvent, which is inverse to the toxicity of the solvent. The 15oC trace gives the highest peak followed by the 28 and 32oC respectively. From previous observations the toxicity of the solvent can be indicated by the peak rise from the cells when exposed to solvents. This is not the case with the temperature challenged cells.

The ability of the cell cytoplasmic membrane to become charged appears to be enhanced by low storage temperature, while the converse is true of cells stored at a higher temperature. Ferris et al (1990) noted a positive temperature-dependence with the capacitance of biological membranes.

The OUR of the cells (figure 3.43) incubated at 32oC indicate that the cells were in a very poor state. The cells with the highest OUR were incubated at 28oC. From the presolvent addition traces of capacitance, where the cells are allowed to stabilise, the cells incubated at 32oC seem least stable (During the 15 minute stabilisation check the capacitance (viability) of the cells drop, see figure 3.44). In the case of the 28 and 15 oC preincubated cells, they did not show any fluctuations during this period.

When challenged with solvent the 15 and 28oC pretemperature incubated cells survived better than those cells preincubated at 32oC.

Storage temperature of cells prior to solvent challenge, alters the cell OUR (as measured at 28oC) and favours the cells stored at 28oC. However, when challenged with solvent the cells stored at 15oC are the most stable, despite the higher OUR of the 28oC cells. Hack (1991) also reported the increase in stability of cells stored for solvent challenge in biotransformation when kept at low temperature.

3.24 Time dependency of solvent toxicity

It is clear that transfer of solvent to the cell is a basic feature of solvent toxicity. Understanding this may explain many aspects of cells in a two-liquid phase system. Once a solvent has arrived in the cell membrane, the solvent structure and its interaction with the cell membrane then becomes of overriding importance. These two parameters form the basis of understanding solvent toxicity.

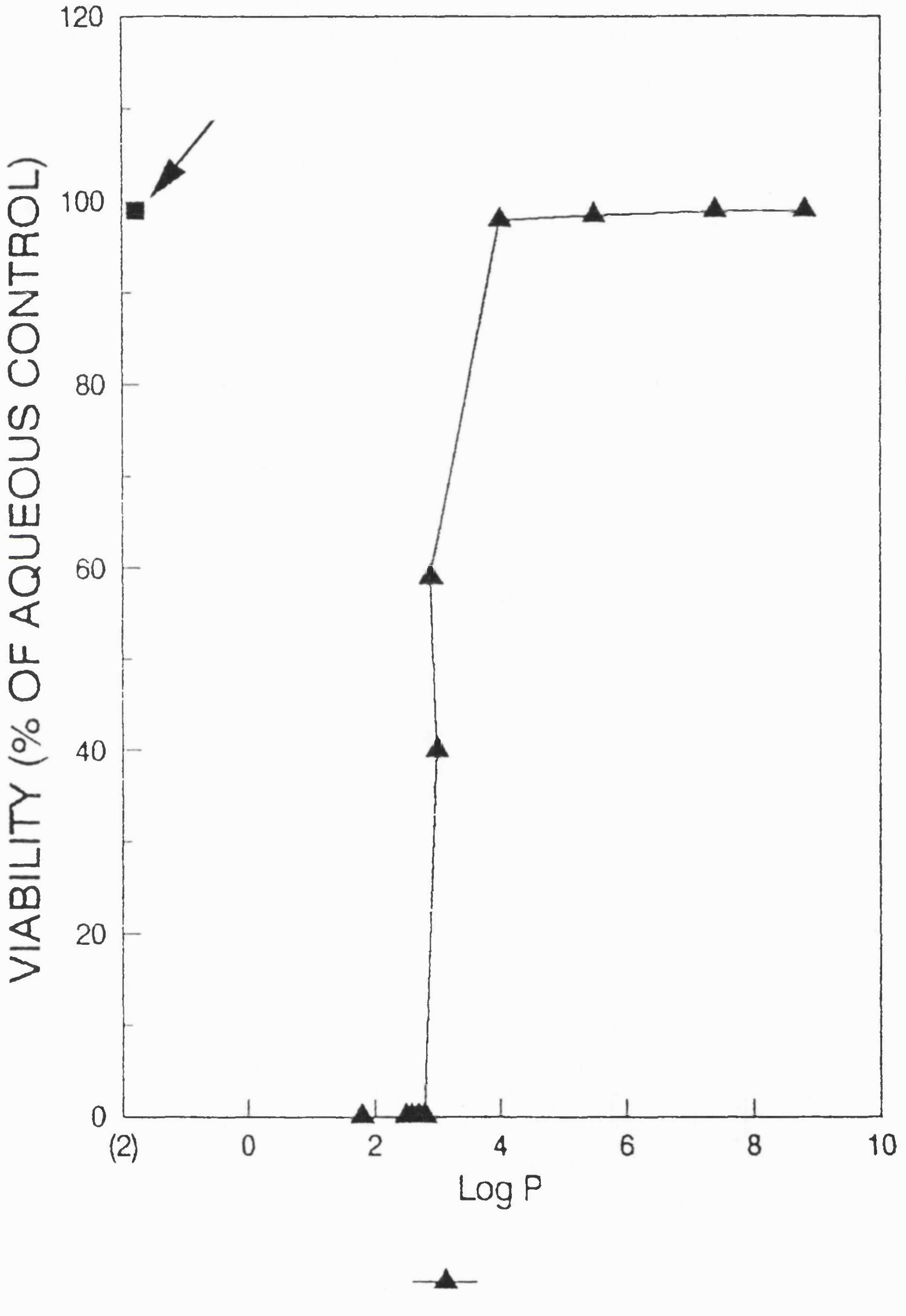
By looking at the transfer of solvents and the typical time dependency of transfer we can derive many of the reported results from the literature. From sections 3.9 and 3.10, the effect of solvent action over time can be demonstrated. To illustrate the affect this will have on the analysis time chosen, the viability profile obtained from a range of analysis time points are shown in figure 3.45 (data taken from the volume addition of 1-octanol, see figure 3.21) for different mixing characteristics.

The result of this time dependency of solvent toxicity and its effect on the interpretation of results is shown in figure 3.47, where the data in figure 3.46 is displayed in a simulation of a log P profile by the combination of volume addition and analysis time.

Thus the full effect of a solvent on a static culture of cells is not necessarily defined by analysis at one time point.

FIGURE 3.41

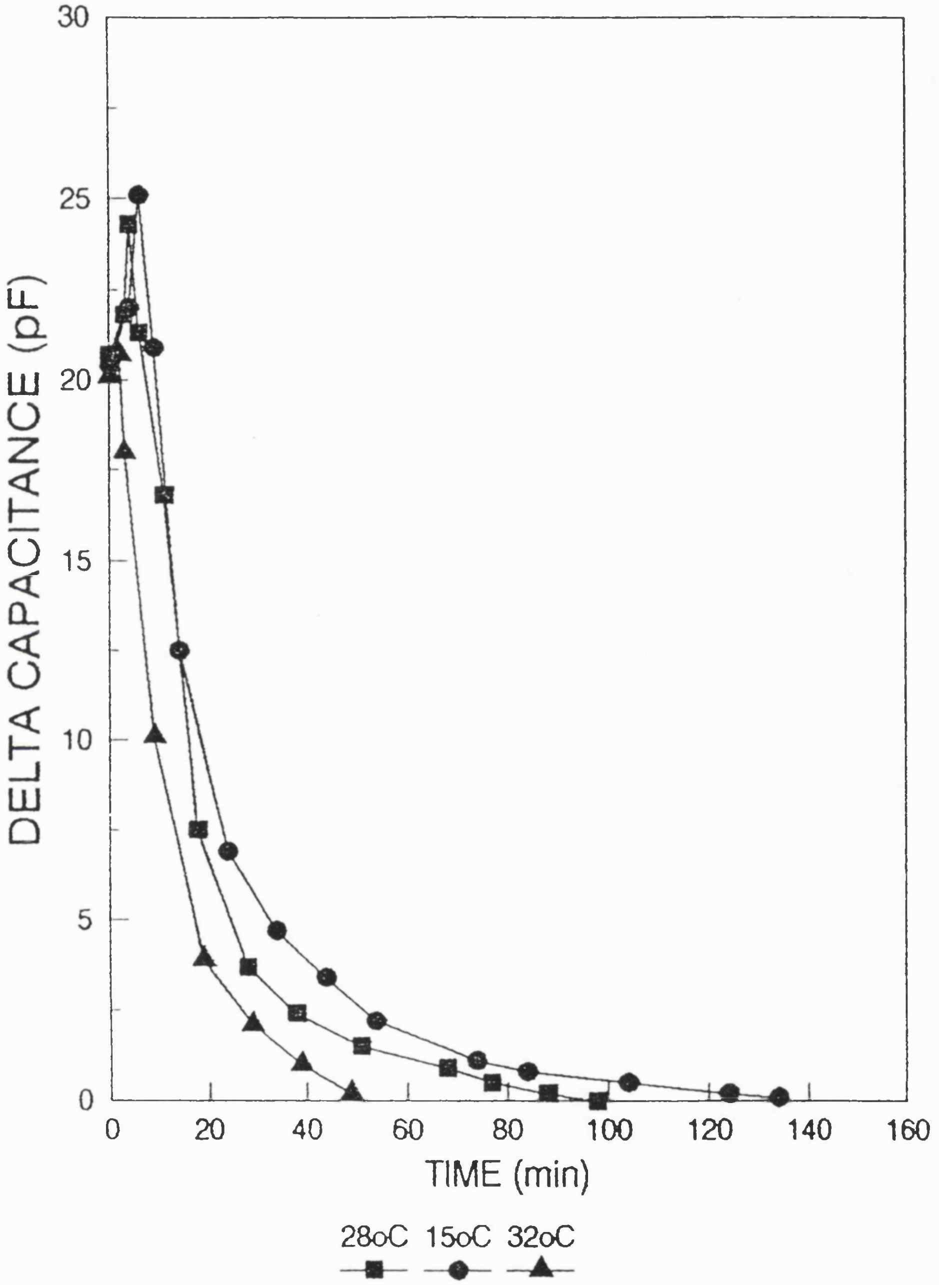
Cell viability of *P.putida* as a percentage of aqueous control after 2 hour solvent exposure



Graph of viability against solvent log P for *P.putida* viability as a percentage of aqueous control, two hours after solvent exposure

FIGURE 3.42

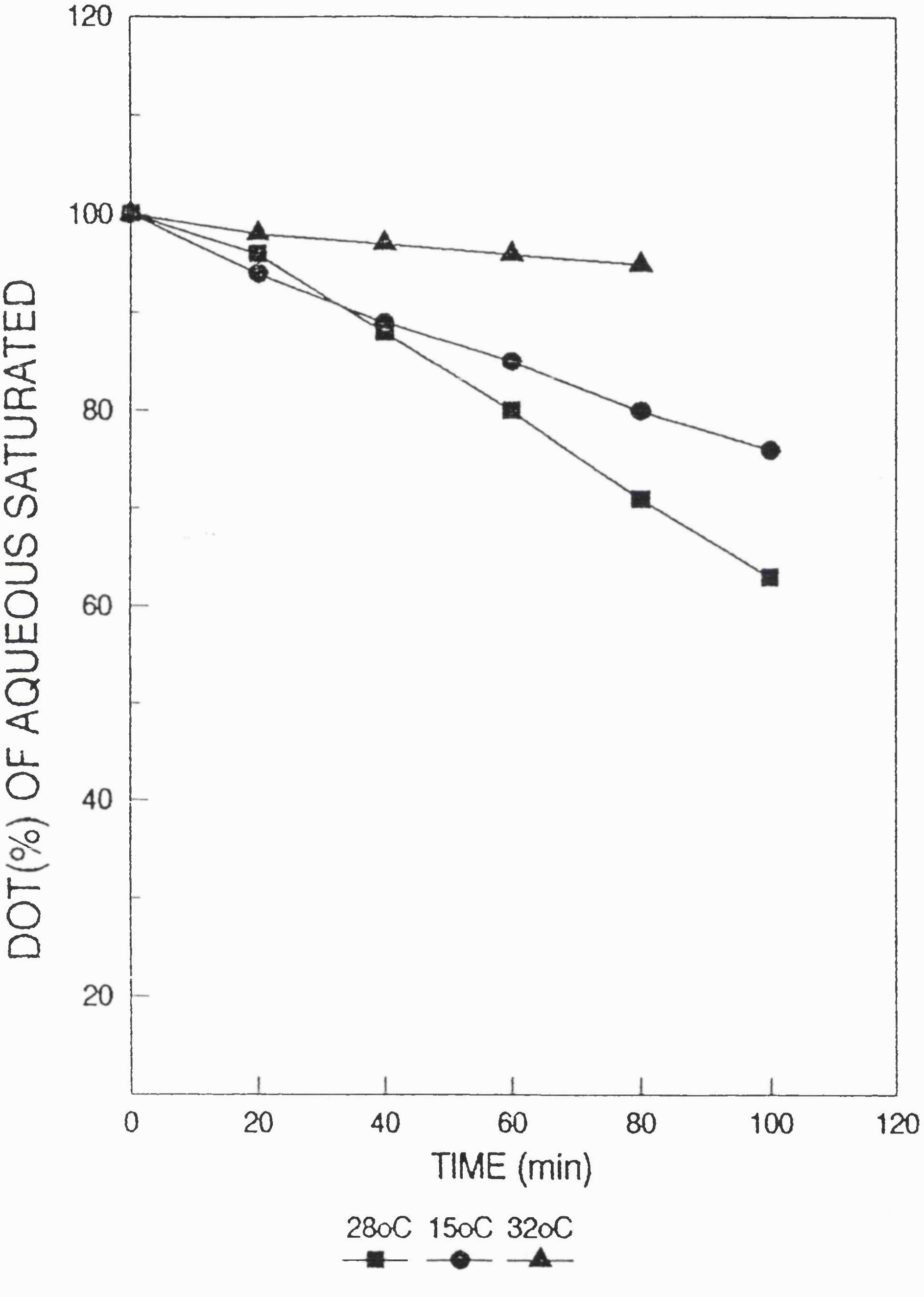
Solvent challenge after preincubating *S.cerevisiae* at different temperatures



Graph of capacitance against time for solvent (0.5ml of octanol)challenged after pre-incubating cells at different temperatures

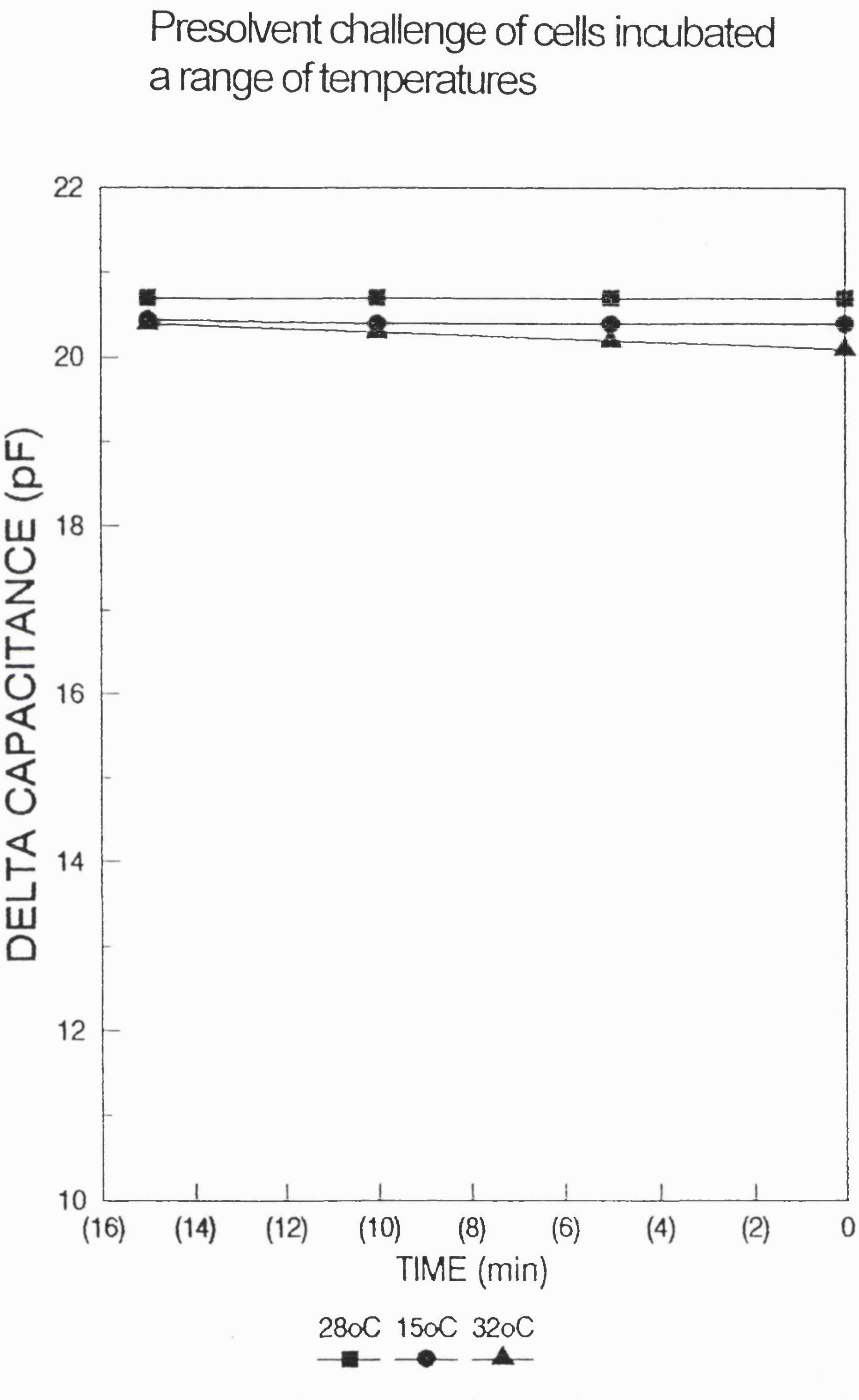
FIGURE 3.43

DOT of cells prior to solvent addition



Graph of DOT against time for cells after incubating at a range of temperatures, prior to solvent challenge (see fig.3.42)

FIGURE 3.44



Graph of capacitance against time for *S.cerevisiae* capacitance at 28oC, after incubating at a range of temperatures, prior to solvent addition

FIGURE 3.40

Figure 3.40 shows cells treated with both 1 and 3 octanol at similar concentrations. The right hand graphs of 1 and 3 octanol show the total number of viable cells, while the left hand set show the number of viable cells. The time of samples increases downwards (0 hour, 2 hours and 25 hours).

Animal cells incubated in growth medium at 37oC and gently rotated in flat bottles in the presence of 1 and 3 octanol. Samples removed for counting total cell numbers and viability by trypan blue microscopic examination.

FIGURE 3.40

Histogram of animal cell viability and cell numbers after treatment with solvent

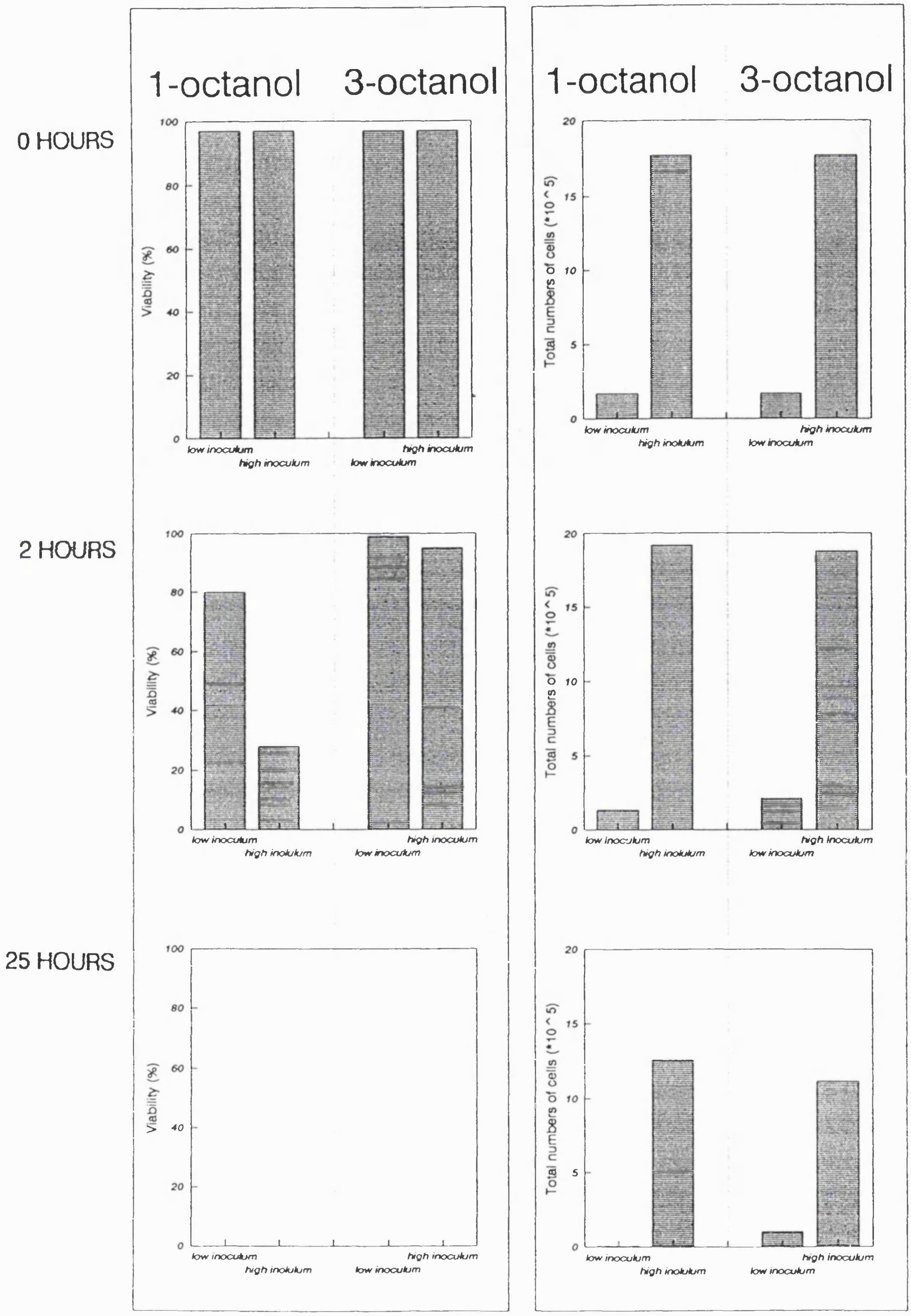


FIGURE 3.45

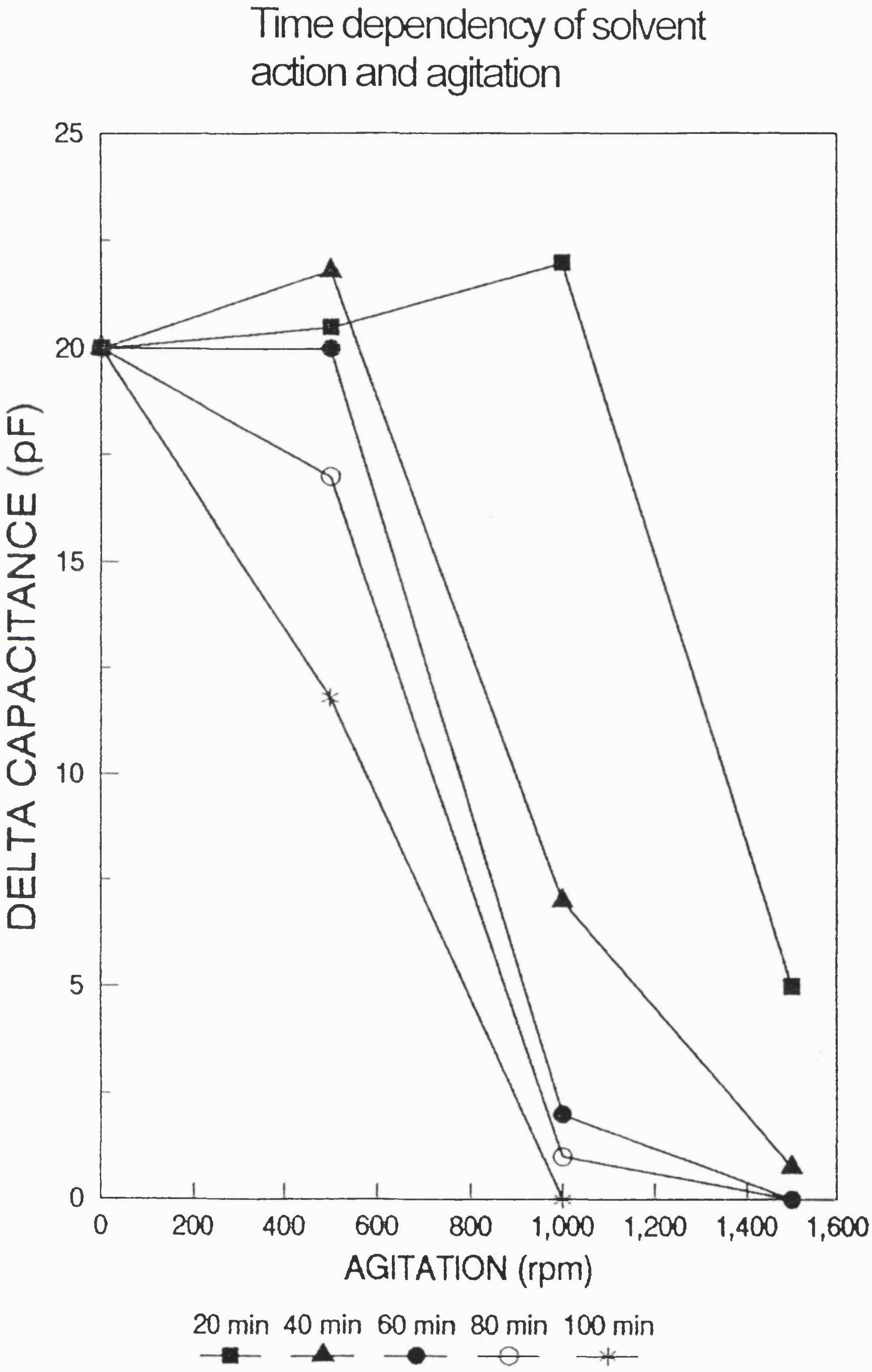
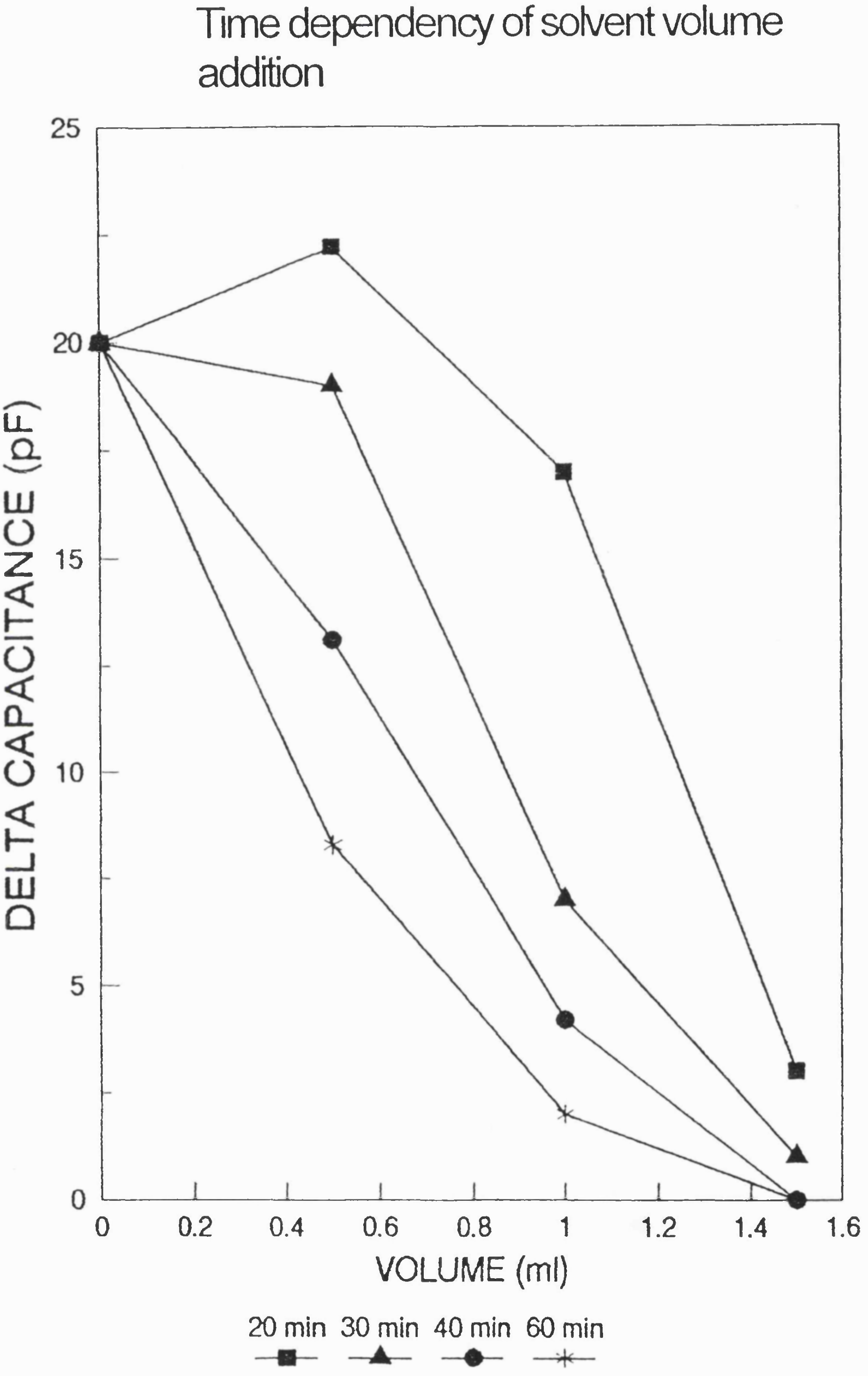


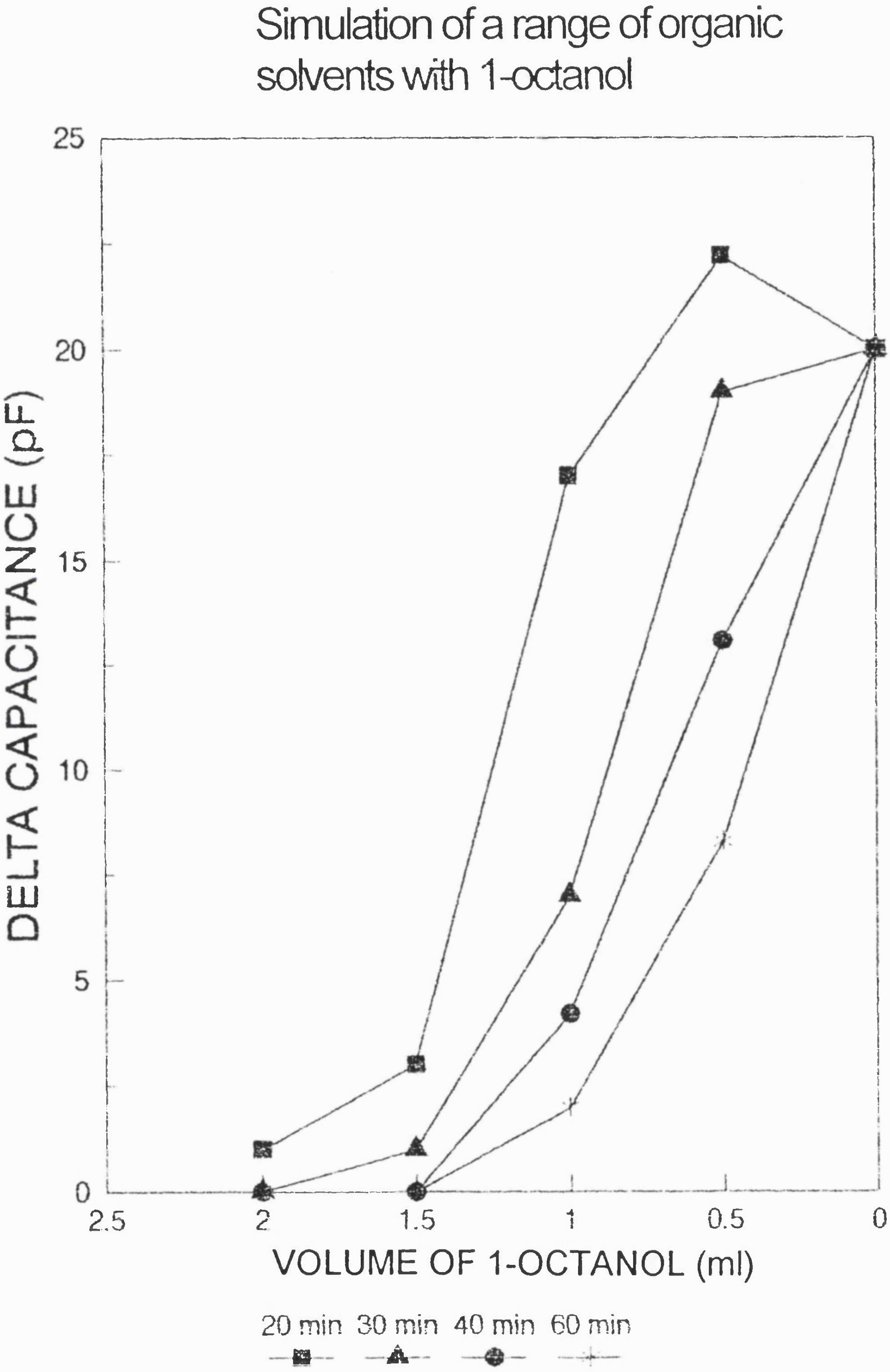
Illustration of time dependency of solvent action and the result of analysis at particular time points. Data from figure 3.24

FIGURE 3.46



Graph of capacitance against solvent addition to illustrate time dependency of solvent action and the result of analysis at particular time points. Data from fig.3.22

FIGURE 3.47



Graph of capacitance against volume addition to illustrate the effect of the time dependency of solvent challenge and the alterations to the log P curve, all points derived from 1-octanol

CHAPTER 4

CHAPTER 4
DISCUSSION

4.1 Rationale and justification for development of the approach adopted

Previous workers investigating biotransformations in the organic media have used single enzyme systems. A number of problems arise with this approach, including:

1) Finding a suitable biotransformation where the whole system is well characterised, so that any breakdown in the system can be pin pointed.

2) Development of the respective assay system for both substrate and product in the aqueous and or the organic phase.

3) Finding a blocked cell system, ensuring it is not leaky (further assay development if this is not satisfied).

4) The ability of the host to express this foreign gene, for 'normal or expected' function.

By use of a multi-host vector it can then be moved easily between a number of organisms and their organic solvent tolerance examined. This approach is likely to yield much information on the genetic expression and enzyme activity *per se*, but in many respects will prove of limited use in elucidating the effects of organic solvents on the cell system. This approach will also require extensive and time consuming work.

The aim of this project is to examine the underlying trends of organic solvent toxicity on cell stability. The approach selected was to use dielectric spectroscopy, which obviates many of the problems associated with the above, while still meeting the initial criteria selected (chapter 3). It can also be used on a broad range of cells, giving maximum information from a number of hosts, without compromising the quality of information obtained or being excessively time consuming in background work before the start of the project. This choice allows a flexible experimental approach, but limitations were encountered in certain experimental configurations (chapter 3). However several interesting avenues were highlighted during this study.

Monitoring the cell beta-dispersion by this technique gives an on-line, noninvasive technique for measuring cell viability of microorganisms.

Frequency scans were obtained for several organisms and the reproducibility of the technique confirmed. Further preliminary work demonstrated that the technique was suitable for use in the presence of an organic solvent.

To create a bridge with other techniques, a correlation with viability (by methylene blue assay) was performed. This clearly demonstrated a link between beta-dispersion and cell viability.

When observing the viability of the cells by methylene blue, it was evident that the viability of a cell population, exposed to an organic solvent in a second-liquid phase, was in a nonuniform state ie the viability of the cell population is heterogeneous. However cells challenged with a solvent, not in a second-liquid phase, but with aqueous saturated with organic solvent, appear to loose viability in a more uniform homogeneous manner. This indicated that the cells in a second-liquid phase environment are not exposed to a uniform environment. It is therefore apparent that the "phase effect" noted in the literature, associated with the presence of a second-liquid phase, is the result of a population with heterogeneous viability.

The literature contains numerous reports that the cell membrane is affected by slight structural alterations in its unit components (eg changes in length or degree of saturation of phospholipids). Since the cell membrane is a highly structured organelle, it is quite likely that the particular structure of an organic solvent once in a membrane may have a strong effect on the stability of the membrane and hence the cell.

Since the phospholipids themselves are highly polar molecules, other highly polar molecules (eg organic solvents) might be expected to adopt a similar alignment. Structure is important in a membrane, and the consequence of adding analogues of a solvent (ie with the same components but with different structural arrangement especially with regards to the hydrophilic group) to a membrane could be hypothesised to have different effects on the cell. If this working hypothesis is correct then analogues of organic solvents with the same log P should have a different effect on the cells if they are challenged with the organic solvent saturating the aqueous (since the driving force should be the same). This work confirmed this for 1,2 and 3-octanol and analogues of heptanone.

It was interesting to note that log P in its various forms, is the standard for determining solvent toxicity, but does not predict this outcome.

Further, these findings are in contradiction to those log P predictions. This is presumably due to the highly structured nature of the cell membrane compared to the structureless nature of octanol.

Further confirmation of this hypothesis was sought by using animal cells, which do not have a cell wall, the presence of which might influence the findings. The findings were not contradictory.

When cells were challenged by an organic solvent an initial rise in beta-dispersion was measured by capacitance, followed by a drop in capacitance, concomitant with a drop in cell viability. The rise in beta-dispersion, which is due solely to solvent/cell interaction, was hypothesised to be caused by an increase in ion concentration within the cell and due to an alteration in the cell membrane permeability. An increased ion concentration in the cell may well have a positive knock on effect within the cell during this period. To investigate if a detectable positive effect occurs during this "rise", the "rise" period needs to be extended so that a measurable effect can be assessed.

Low concentrations of solvent raise the beta-dispersion over a period of time. A correlation exists between the concentration of solvent added and its effect on cell stability. If a sufficiently low concentration of solvent is added, the "rise" can be extended over a time period sufficient to perform an OUR assay to assess cell changes during this period. In chapter 3 certain organic solvents were shown to raise the endogenous cell OUR. It was also noted that cells challenged with totally aqueous soluble organic solvent appeared to undergo reversal of toxic effects of the solvent when assayed for OUR (this assay involves a dilution (1:60) of the cells into an aqueous solution). To further demonstrate this effect, low concentration of organic solvent were added to *P.chrysogenum* penicillin V fermentations. Addition of solvent gave a significant increase in *P.chrysogenum* biomass during the fermentation.

To quantify the effect of solvent addition at low solvent concentration, it is important to consider the adsorption of solvent into the cell was investigated. Although this is an important parameter it has not previously been investigated in the area of two-liquid phase. This prompted experiments to develop a correlation of biomass and solvent adsorption into the cell.

This is a brief bullet point overview of the core experiments and the logic which links them.

4.2 Capacitance measurements at fm

From chapter 3 a correlation between biomass and capacitance by the beta-dispersion, as measured at fm, was shown. The correlation is repeated in a cell suspension with a range of different aqueous conductivities. These results illustrate the decision to operate at the low frequency for fm of 0.4MHz, where a plateau occurs over a range of conductivities. This technique gives reproducible results, and can be successfully applied to cell stability in the presence of organic solvents.

4.3 Cell stability and the membrane

The primary site of attack of organic solvents on cells is at the membrane. This study provides further insight into the action of solvents on the cell and

4.5 Type of biocatalyst

Clearly the transfer of solvent into, and to the cell is of paramount importance when selecting a biocatalyst and a reactor design.

A crucial factor in reactor design is mixing, this promotes transfer of solvent from the bulk organic into the aqueous and into the cell. The choice of a biocatalyst cell can also affect the transfer of solvent into the cell. Some important features which must be considered are listed below:

1) Permeability of the solvent through any outer membrane or cell wall. This may alter the range of solvent which can effectively be used. This is also applicable to the range of reactants which may be similarly affected by permeability (as inadvertently demonstrated by Inoue 1991 and Horikoshi 1991).

2) The cell itself may affect the transfer of the solvent from the bulk organic into the cell by the nature of the cell outer surface. A cell with a hydrophobic surface may become located at the organic/aqueous interface. This may greatly enhance the transfer of solvent from the adjacent bulk organic into the cell, and/or slow the transfer of the solvent into the bulk aqueous. This may also alter the transfer of poorly-water soluble compounds between the two-liquid phases and may therefore also alter the cells ability to act as a biocatalyst.

When cells adhere to an organic/aqueous interface, it stabilises the interface, which enhances the formation of an emulsion. The location of the entire cell population, within an agitated two-liquid phase reactor, may thus be determined by the cell wall hydrophobicity.

The hydrophobic nature of a biocatalyst cell wall will determine the cells affinity for a range of log P organic solvents. In a two liquid phase system, it will also determine the solvents ability to remove the cells from the bulk aqueous.

Because of the hydrophobic nature of the cell wall of *Y.lipolytica* and *P.putida,* they were unsuitable for many of the initial experiments as their inclusion would cause unnecessary complications. The formation of emulsions could be partially overcome by immobilisation.

4.6 Volume addition of organic solvent and agitation

The toxicity of the organic solvents tested increases with the volume added, whether present as a second-liquid phase or not. When a second-liquid phase is not present, then the ratio of biomass to dissolved solvent is important. This is illustrated in Chapter 3, by organic solvent partitioning into the cells, which effectively reduces the aqueous concentration. Although this is perhaps an obvious fact which could have been predicted it has been overlooked, but is, never the less, an important parameter for the use of cells in organic media and has been neglected in the literature.

Cell stability is decreased by increased agitation when in the presence of a second-liquid phase. While other workers have inferred this correlation (Hocknull 1988, Cho and Shuler 1986), it is unclear if the phase effect exists in its own right or as a result of a faster transfer of solvent from the bulk organic phase through the aqueous phase and/or by organic phase transfer into the cell membrane. It is important to note that no difference is observed between cell death at sub or super saturated concentrations (also see figure 3.22 and section 4.11), which gives no evidence of a separate effect, except to emphasise that a faster transfer of solvent into the cell has occurred in the presence of a second liquid phase.

4.7 Log P and selection of an organic compound

The determination of cell stability is not fully understood, although some general rules have been proposed. The Laane log P versus activity plot indicated the influence of log P, but it has a limited use, as does the critical membrane concentration concept (Osborne et al 1990). The toxicity of the molecules appears to vary with their structure, as demonstrated by challenging cells with solvents with subterminal hydrophilic groups (see chapter 3). The further the hydrophilic region is from a terminal end of the aliphatic hydrocarbon chain, the less toxic the organic solvent appears to be. Due to the structured nature of the cell membrane, a compound once within the membrane may have a range of effects depending in it structure. As previously stated (chapter 4) this observation is a clear deviation from the current adherence to log P. Animal cells were also used in this study to show the general application of these findings and that they are not the effect of cell wall. These findings point to two separate effects, the first is that all solvent cell interaction is limited by transfer of solvent to the cell, and secondly by the intrinsic toxicity of a molecule once in the membrane (see latter).

A noticeable feature of solvent toxicity is the time within which the solvents exert their effects. The time dependency relates to the parameters governing transfer of solvent from the bulk organic into the aqueous and the cell.

4.8 OUR

The main criteria when selecting an organic solvent for use with a whole cell biocatalyst is often that it does not impair the oxidation system, since it is often integral to the stability of that organism.

The effects of the solvents have been shown to raise cell cytoplasmic membrane fluidity (Gordon 1980). They also effect the cells endogenous OUR. In chapter 3 a correlation between capacitance and the OUR in the presence of solvents was demonstrated. This is presumably via the alteration in the internal ion concentration within the cell.

An increase in the OUR was demonstrated (see chapter 3) to occur under certain conditions of solvent addition to cells. This finding has broad application as shown by its use in raising the cell biomass (Eglin 1992) obtained in a Penicillium fermentation (see chapter 3). This is thought to be due to a higher state of activity of membrane associated enzymes (Gordon 1980) and on the ions within the cell.

The addition of an organic solvent second-liquid phase can enhance oxygen transfer in Penicillium fermentations, noted in the literature (Chester et al 1990), where it acts as a oxygen reservoir. But, the results from this work showed that a very small volume addition (0.005 percent organic solvent v/v), a factor of some 400 times less than that added by Chester et al (1990) and not present as a second-liquid phase, can be used to achieve an increased biomass production. This has significant implications for many cell processes, which might be enhanced by the addition of an organic solvent.

4.9 Limitation of transfer of ethanol and oxygen across The cell membrane

From initial calculations it appears that the alteration in permeability of the cell cytoplasmic membrane has little effect on the permeability of hydrophobic compounds or of oxygen (see below) which might otherwise raise the cells endogenous metabolic rate, as measured by the uptake of oxygen.

From Jobses et al (1986) calculations, using Wilkes empirical formula, ethanol diffusion into the cell is not limited by the cell membrane. This was determined by the relative diffusion rates of ethanol in an aqueous phase and in a hydrophobic phase. The basis of this conclusion rests on the solubility of a hydrophobic compound in a hydrophilic or hydrophobic environment. The resultant diffusion in the hydrophobic environment is an order of magnitude higher than in the hydrophilic environment for ethanol. Thus any increase in the diffusion rate in the hydrophobic environment will have little if any effect on the diffusion of ethanol through the cell membrane, since the diffusion through the aqueous is the rate limiting step.

Oxygen transfer into the cell and its diffusion rate through the membrane are an order of magnitude different. This indicates that as in the case of ethanol, the rate limiting step in diffusion of oxygen transfer is in the aqueous phase. Thus the effect of organic solvents on the OUR is unlikely to be by its effect on diffusion of oxygen through the membrane. Thus it is likely that it is the result of increased enzyme activity and or the increase in ion concentration with the cell.

4.10 Recovery of cells after challenging with organic solvents

Pentyl amine appears to have a reversible toxic effect on yeast cells (*S.cerevisiae*), as discerned from the OUR results, with the implication that metabolic activity is also revived. This has far reaching implications for work in two-liquid phase and implies that recovery of cells after treating with organic solvents is possible.

Pentyl amine is totally soluble in the aqueous phase. If cells previously challenged with organic solvent (so that the solvent partitions into the cell membrane), are then transferred into solvent free aqueous, the solvent may then partition out of the membrane and into the aqueous, allowing recovery of cells.

Cells which had been challenged with octanol or pentyl amine that had reached a zero capacitance value, failed to show any increase in the OUR, even after 2 hours in the OUR chamber, indicating that the initial effects of the solvent may be reversible, but once the cells are 'non-viable' the effect of the solvent damage is irreversible.

Cells showed a slight ability to take up oxygen even when the cells were predicted by capacitance as having died (Other workers have noticed a similar trend Osborne (1990), Hocknull (1988)). This implies that the oxidation system itself is very robust and not destroyed by the solvent challenge, although its ability to function becomes drastically reduced. This then indicates that cells can recover, but there exists a point from which the cells cannot recover.

4.11 Phase effect

No distinction is apparent between the effects noted when cells are challenged with sub or super saturated aqueous concentrations of organic solvents using gross measurements of the cell population.

From the capacitance peak rise data after solvent addition it is clear that the better the mixing the greater the peak rise. This implies that during better mixing the cells are in a more homogeneous state, ie more cells are giving a high peak reading at that time.

The photographs shown in chapter 3 illustrate the nature of the solvent toxicity on the individual cells of the population challenged with organic solvent. When the aqueous is saturated with an organic solvent and no second-liquid phase contact with cells occurs, the toxic effect on individual cells is uniform. When an organic phase contacts with cells, the toxicity on individual cells is heterogeneous. That is, the whole population is effected in defined ways, depending on the nature and concentration of the organic solvent.

The second-liquid phase is toxic in a non-uniform manner. We can infer that an organic solvent present as a second liquid phase increases toxicity to cells. This may be by "massive" transfer of solvent to the cells or due to high shear at the interface which disrupts the cell. We know that saturation of the aqueous is not instantaneous (figure 3.26), also that the cells themselves take up solvent (figure 3.25). Therefore the presence of a second-liquid phase will allow continuous resaturation of the aqueous, which will effectively raise the aqueous concentration which the cells "see" above that experienced by cells at sub-saturated solvent levels in the aqueous (with no second-liquid phase).

If the second-liquid phase does allow a "massive" transfer of solvent into the cells then this again is a function of solvent transfer and is not a different effect, but a direct transfer of solvent where the transfer through the aqueous phase is obviated.

Shear has little effect on the Gram-negative bacteria *P.putida* or *S.cerevisiae* in an aqueous stirred tank reactor. However, when an organic solvent second liquid phase is added, it is hypothesised that the yeast and Gram-positive bacteria cells become shear sensitive while the Gram-negative bacteria do not. Since we know mass transfer of organic solvents into the cell is important, and that the Gram-negative bacteria have restricted transfer of organic solvent into the cell, we can infer that the "phase" effect in this respect is the result of increased transfer of solvent into the cell.

Attempts to define the interface in the literature have so far only considered the second-liquid phase as a ratio of the aqueous phase (phase ratio). This is a useful concept for the calculation of the distribution of the reactants between the phases, but is of little use when trying to establish a relationship for any 'phase effect'(See chapter 3). A more useful model would involve the effect of the ratio of solvent interfacial area to the aqueous volume. This may still be incomplete, except for a comparative study, since it omits the effects of mixing in the aqueous phase. However it is clear that the cell contact with solvent interface is important.

4.12 Systematic approach to solvent toxicity

Driving force of the solvent and log P

This work has highlighted several important areas, most aspects discussed so far are all components of mass transfer. The transfer of solvent into the cell is related to the organic solvent interfacial area, the number of cells, the volume of the aqueous phase and the rate of agitation of the aqueous phase.

Mass transfer equation and relation to the log P :

$$\text{Mass transfer} = Kl_a \ (C_0 - C_1 P) \qquad -(4.1)$$

The driving force is the difference in concentrations of an organic solvent between two points (eg aqueous and the membrane). This will give a rate of transfer for a solvent as stated in Ficks first law (V=P*A*delta c), but cannot be considered as its driving force where partitioning occurs, since the dynamic equilibrium of the solvent must be reflected. This can be compensated for by Equation 4.1, which describes the driving force of the solvent between two phases where partitioning occurs. Restrictions of the solvent into the cell may occur as in the case of the Gram-negative bacteria (Nikaido and Vaara 1985), but equation 4.1 should still hold true.

Thus the mass transfer can be used to describe part of the solvent toxicity, since it describes the transfer of the solvent between the bulk organic and the aqueous phases and also the aqueous phase and the membrane.

The mass transfer of a solvent can describe the permeation of the organic solvent into the membrane but does not elucidate the intrinsic molecular toxic effects of the solvent once inside the membrane.

Intrinsic molecular toxicity

Well established reports (see chapter 1) indicate that the nature of a new compound introduced into a membrane has an impact on the nature of the bilayer and its ability to function 'normally'. From these findings alone we can conclude that the structure of a compound inserted into the bilayer should determine its effect in the bilayer. The work in chapter 3 has confirmed this hypothesis.

The molecular toxicity of the organic solvent molecule will be described by the concentration and structure of the molecule: its length, number of hydrophilic groups and their relative position. This hypothesis is extended from the data to include the number of branches and a number of other parameters to be defined.

From chapter 3, the organic solvent toxicity may be described;

$$\text{Organic solvent toxicity} = (\text{Intrinsic molecular toxicity}) * (\text{Mass transfer of the solvent into the membrane})$$

An analogous argument might be developed for the calculation of the cell recovery, after solvent challenge, where the cells are transferred into a solvent free environment.

Implications for engineers

The concepts outlined in this project may have a number of implications within the design and operation of reactors involving a two-liquid phase systems with organic solvents. It is impossible to outline all the ramifications of any one piece of work, with the infinite combinations of unforseen circumstances which are unique to any new process. However some implications warrant further emphasis.

A new reactor design can be developed with many pressures governing the direction of its evolution, eg having to use a specific organism, or a specific organic solvent which must be used since it is one of the reactants. Ideally, the start point for the design of a new reactor or system should be from the most complex basic interactive unit which is necessarily involved in the design or system. In many cases this will simplify the design process, eg if a specific organism is necessarily involved in a design, then almost certainly it should be used as the starting point for the design, from which all permutations can be developed eg solvent-organism interaction, or operating temperature. If the organism is robust and can operate in a wide range of temperatures and maintain a high conversion rate then perhaps it would not be considered as a complex interactive unit with regards to reactor parameters.

A more pragmatic approach can be taken to reactor and process engineering: When designing a two-liquid phase system, the nature of the cell wall of the biocatalyst must be considered, as to whether it will cause the formation of emulsions; Agitation is another important parameter, which will define transfer of solvent from the bulk organic to the biocatalyst; An increase in defined organic solvent surface area may lead to an increase in toxicity of the solvent; Aqueous concentration of solvent; Ratio of biomass to solvent; Temperature pretreatment of cells prior to solvent addition; Permeability of the cell to the solvent. All these parameters are involved in governing solvent mass transfer, which influences cell stability.

Another important parameter is the organic solvent structure, which defines its "intrinsic molecular toxicity". Solvents may however, if carefully selected and monitored, be used to increase general cell activity, which may be of great commercial importance.

Further aspects of this work involve the reversal of solvent action, which might be accomplished by simple dilution into aqueous solution or ironically by adding a suitable organic solvent.

A further possibility would be to use the current data to design a non-toxic solvent. From the observations outlined in this project, it might be anticipated that a non-toxic solvent would be one which has no "intrinsic toxicity" and is able to align with the cell membrane without disrupting it. This might be accomplished by using lipids with a similar structure to the membrane components (ie phospholipids). Other designs would involve branched hydrocarbons chains with a hydrophilic group eg $(CH_3)_2CHCHOHCH(CH_3)_2$, which would be expected to be considerably less toxic than heptanol. It must be emphasised that this outlines the direction in which further research might proceed and is not intended as an exhaustive treatise on the possible structures which might prove fruitful for use in two-liquid phase, but to illustrate the factors to be considered when looking for other biocompatible factors of solvents (eg melting point).

In summary, two important concepts are

outlined in chapter 3, with respect to solvent toxicity. The first is the transfer of solvent from bulk organic and bulk aqueous to the toxic site within the cell. Secondly the structure of the organic solvent, described as the "intrinsic molecular toxicity", and the effect it will have once it is in the membrane. These two parameters are the governing factors for engineers to consider for biocatalysts stability in reactors involving organic solvent.

CHAPTER 5

CHAPTER 5
CONCLUSION

5.0 Conclusion

Predictable and reliable manipulation of microorganisms in organic solvents, is only possible when the parameters which effect cell stability are known and defined.

This study utilises the Beta-dispersion present in all live cells, which have a cell membrane. It was found to be a useful, non-invasive technique, which allows constant monitoring of cell viability. This work demonstrates the importance of free ions, for the normal internal functioning of the cell, and also their concomitant loss after organic solvents addition.

From chapter 3 it is evident that challenge time with an organic solvent is also significant factor, illustrating that analysis at different times will alter the cell stability profile obtained. Therefore, one of the most important parameters to ascertain when looking at cell stability, is at what time point to analyse for cell stability. This appears to have been largely ignored in the literature.

One of the most obvious forms of engineering application with microorganisms, is reactor design, where mixing is of primary importance, affecting most of the reactor operations. In chapter 3, a correlation was noted between cell stability and stirrer speed in a two-liquid phase system. In this yeast system it is clear that avoiding a high degree of agitation is important to maintaining cell stability. Increased agitation enhances solvent transfer across the cell wall and into the membrane. In Gram-negative bacteria the mixing is not the rate limiting step (Nikaido and Vaara 1985) for solvent transfer, which may explain data of Harrop et al (1989).

This work also demonstrates that the type of organic solvent system chosen, will effect the cell population in a hitherto unexpected way: When the individual cells are observed for viability it is apparent that in a two-liquid phase system the cell population is heterogeneous with respect to cell stability, while in the absence of a second-liquid phase the population is more homogeneous, eg a 50 percent viable cell system with a second-liquid phase, may consist of a population of cells, 50 percent of which are nonviable, while the rest remain viable. Whereas without a two-liquid phase system the whole population would be in a homogeneous transition state, somewhere between viable and nonviable. This is a significant factor to consider for developing the optimal state and activity for biocatalytic operation and for any cell regeneration by reversal of solvent partitioning. Immobilisation would allow the cell state to be monitored accurately to give predictable results in a two-liquid phase system, since the cells would be in a homogeneous state.

Whilst stirrer speed is an easily measured parameter, it is indicated from this work that a more basic understanding is needed. Useful information could be obtained by measuring cell stability against the ratio of aqueous phase volume to organic solvent surface area. As yet this has not been identified as an important parameter in the literature.

Other important conclusions include the apparent susceptibility of budding cells to solvent toxicity and the positive effect of low storage temperature on the cell population, prior to solvent challenge.

In the absence of a second-liquid phase the effect of organic solvent in the aqueous partitioning into the cells can be demonstrated and may significantly reduce the aqueous concentration of the organic solvent and its affect on cell stability. This is especially important for organic solvents where the aqueous solubility is low. For such systems it becomes important to carefully monitor the aqueous solvent levels.

In view of all of these considerations, to expect one term to encompass all of these parameters would lead to a very rough and generalised correlation (ie log P on its own). The use of maximum membrane concentration (which also uses log P) is a step forward, but is limited to certain solvents (outlined in chapter 3) and as indicated in this work it is not universally applicable.

Using this information, a systematic approach to cell stability in organic media can be outlined. Three important considerations are highlighted: The first consideration is the transfer of the organic solvent from the second-liquid phase into the aqueous phase. For many solvents (eg the low log P solvents) this will be almost instantaneous, but for very poorly-water soluble compounds used in two-liquid phase this may be a rate limiting parameter.

The second consideration is the transfer of the solvent from the aqueous phase into the cell and its membrane. Both the first and second consideration can be described by equation 4, where the driving force, is the concentration gradient, modified by the partitioning coefficient as defined by P. The second consideration may be obviated by direct transfer of the solvent from the bulk organic, to the cell, by what has been described as the phase effect.

The third consideration is the intrinsic toxicity of the organic solvent molecule itself. It is evident from chapter 1 and 3, that each molecule has an effect on the cell membrane, dependent on its structure. Log P is itself a reflection of organic solvent distribution by partitioning, but also reflects a progressive change in structure for a series of organic solvent. The importance of structure arises due to the highly complex structure of the cell membrane, not present in n-octanol or therefore represented by partitioning.

In summary this work defines several new parameters which must be considered when using microorganisms in organic media and also brings into focus other relationships between interacting parameters.

5.1 Further work

Many parameters have been examined with respect to cell stability in the presence of organic solvents. Several facets of each of these parameters need further investigation. To further understand cell stability in the presence of organic solvents, the solvent mass transfer into the cell and the intrinsic toxicity once in the membrane seem like fruitful avenues for future research.

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APPENDIX 1

Equipment

Aber instruments Ltd.
Unit 4,
Aberystwyth Science park,
Cefn Llan
Aberystwyth, Dyfed SY22 3AH.

Aldrich Chemical Co.
The old brickyard
New Rd.,
Gillingham Dorset,
SP8 4JL
UK

ATCC Rockville
Maryland
USA

BDH Po Box 15,
Freshwater Rd.,
Dagenham,
Essex RM8 IRF

Charles austen air pump
Ordnance street
Blackburn
Lancs.

Dextrostix,
Reagent Strips

Ames Division,
Miles Lab. Ltd.,
Stoke Poges,
Slough SL2 4LY
UK

Difco Laboratory
Detroit,
Michigan,
USA.

Corning glass works,
Corning,
New York 1483.

FSA (Fison's) Laboratory supplies,
Bishop Meadow Rd.,

Lestershire LE11 0RG

Grant instruments (Cambridge) Ltd.
Barrington,
Cambridge CB2 5QZ

HPLC Technology
Wellington House
Waterloo street
West Macclesfield
Cheshire

ICN Eagle house,
Perigrin business Park,
Gomm Rd.,
High Wicombe,
Bucks. HP13 7DL

Ikamag reo
Drehzahl electronics
From BDH Ltd.

Lloyd JJ instruments Ltd.
Brook Ave.
Warsash,
Southampton SO3 6HP

Millipore (UK) Ltd
The boulevard,
Black lane,
Wastford,
Herts, WD1 8YN

Philips
Pye unicam Ltd.
Cambridge
England

Sigma Chemical Co. Ltd.
Fancy Rd.
Poole,
Dorset BH17 7NH
UK

APPENDIX 2

ABBREVIATIONS

OUR	Oxygen uptake rate
TLP	Two liquid phases
Log P	Log of the partition coefficient of solvent between n-octanol and water
fc	Critical frequency
fm	Frequency of measurement
Cm	Charged membrane per unit area
E_0	Redox potential
C_o	Concentration in the liquid
C_l	Concentration that would be in equilibium with C_o
Kl_a	Mass transfer coefficient
DOT	Dissolved oxygen tension
s'	Conductivity
G	Electrical capacitance
d	Distance
A	Area
E'	Permittivity
c	Conductivity
F	Farads
s	Siemens
K	(d/A) known as the cell constant
T	Time constant
r	Mean radius of cell
C_m	Membrane capacitance per unit area
s'_i	Internal conductivity
s'_o	External conductivity
fc	Characteristic frequency
pi	22/7 (approx.)
P	Membrane enclosed to volume
E'_L	Permittivity at low frequency
E'_{inf}	Permittivity at infinite frequency
s'_L	Conductivity at low frequency