# Development of a Cytochrome P450 Expression System with Biomass Scale-up, Streamlined Downstream Processing and Stability Modifications

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# Abstract

Recombinant human P450s are important in preclinical studies to discover possible interactions between drugs when taking medicines. Drugs may be either inhibitors or substrates for P450s. Some P450s also play a major role in cancer and other diseases.

Recombinant P450s offer better activity, availability and consistency than primary hepatocytes. In this thesis, a recombinant P450 expression system in yeast was developed with the aim of providing a cost-effective way of producing human P450 enzymes upon the yeast's endoplasmic reticulum (ER). These ER-bound P450s are usually referred to as microsomal P450s.

A yeast expression system was used to co-express several human P450s and their reductases, essential for P450 activities. Episomal and chromosomally integrating plasmids with auxotrophic markers were used to build the system. Gene expression was by either *GAL1* promoter (galactose inducible) or *ADH2* promoter (ethanol inducible). High level P450 expressions and activities were optimised by the effect of different growth media. It was found that by using different media conditions for recombinant protein expression, P450 activities could be improved.

Efforts were made to produce P450 proteins in fermentors. For these larger-scale cultures, a cheaper, cost effective alternative to lyticase-mediated cell wall lysis was developed; it involves mechanical cell disruption- without degrading the microsomes. For large volumes, fractionation of microsomes by ultracentrifugation was unfeasible so a procedure was established that allows precipitation using polyethylene glycol (PEG).

Recombinant P450s are extremely temperature-sensitive. They are stable only at minus 80°C and rapidly lose activity at room temperature. Lyophilisation is known to extend the shelf-life of recombinant proteins. Although lyophilisation is a harsh procedure which could shear the P450 proteins, from the microsomal membranes, it was found it could stabilise P450 activity so that the enzymes could be kept at room temperatures for weeks. The process was extensively optimised using a variety of different buffers and lyophilisation conditions and it was confirmed that:

    (a) Recombinant microsomal P450s produced from yeast, insect and bacterial cells could be stabilised using lyophilisation;

    (b) The lyophilised P450s could be plated out in 96-well microtitre plates and the plates could be kept at ambient temperature (+21°C) for weeks without losing activity, indicating that the process was robust;

    (c) The lyophilised enzymes and un-lyophilised samples behaved in the same way regarding their, potency towards known inhibitors, in fluorometric/ mass spectrometry assays and shipment to overseas destinations for P450 activity testing.

# Acknowledgements

# List of abbreviations and useful terms

**Abs**         Absorbance of light

**ADE2**        Pertaining to a particular gene or enzyme in the adenine anabolism pathway that when knocked out produces an auxotrophic requirement for plasmid selection.

**ADH**         Pertaining to the alcohol dehydrogenase gene or protein.

**AEBSF**       A protease inhibitor used to prevent P450 degradation.

**AMMC**        Gentest substrate for CYP2D6, 3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin

**APCI-ESI**    Atmospheric-pressure chemical ionization-electrospray ionization method for mass spectrometry.

**$b_5$**          A protein that may donate an electron to a P450.

**$b_5$ reductase**  A redox partner of $b_5$.

**BD (Gentest)**  Becton Dickinson, a major supplier of medical objects and biologicals.

**bp**          Base pair, two complementary opposing nucleotides from each DNA strand that are connected by hydrogen bonds.

**Buffer A/ Sorbitol buffer** A sorbitol buffer used for preparing microsomes.

**Buffer B**    A sorbitol buffer used for yeast lytic enzyme and sonication.

**Buffer C**    A glycerol buffer for storage of microsomes at -80°C.

**CO**          Carbon monoxide gas.

**CEC**         3-Cyano-7-ethoxycoumarin, substrate for CYP1A2.

**CPR**         Cytochrome P450 reductase.

**CYP**         Cytochrome P450.

**DBF**         Dibenzylfluorescein a CYP2C8 substrate

**DBOMF**       An Invitrogen substrate for CYP3A4.

**DTT**         Dithiothreitol, used to stabilise proteins.

**DHAP**        Dihydrooxyacetone phosphate.

| | |
|---|---|
| **DO** | Dissolved oxygen in a solution. |
| **EDTA** | Ethylene diamine tetra acetic acid. |
| **EOMCC** | An Invitrogen 2D6 substrate |
| **EROD** | Ethoxyresorufin, A CYP1 family substrate. |
| **FMN** | Flavin mononucleotide, a prosthetic group of oxidoreductase proteins, enzymes that transfer electrons. |
| **G3P** | Glycerol-3-phosphate. |
| **G6PDH** | Glucose-6-phosphate dehydrogenase, part of P450 artificial regenerating system. |
| **G6P** | Glucose-6-phosphate |
| **GAL** | Pertaining to the galactose metabolism gene, promoter region or transcription factor. |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase. |
| **HIS3** | Pertaining to a particular gene or enzyme in the histidine anabolism pathway that when knocked out produces an auxotrophic requirement for plasmid selection. |
| **HRD** | Human P450 reductase. |
| **KPSI** | Thousands of pounds per square inch. |
| **LB** | Luria-Bertani, bacterial media. |
| **LEU2** | Pertaining to a particular gene or enzyme in the lucine anabolism pathway that when knocked out produces an auxotrophic requirement for plasmid selection. |
| **LEU2d** | *LEU2* gene with a defective promoter to encourage increased copy number of the plasmid that bears it. |
| **LCMS** | A combination of high performance liquid chromatography and mass spectrometry. |
| **NAD$^+$** | β-Nicotinamide adenine dinucleotide, redox agent, oxidising agent, accepts electrons to become reduced. |
| **NADH** | β-Nicotinamide adenine dinucleotide, redox agent, reducing agent donates electrons. |

**NADP**    β-Nicotinamide adenine dinucleotide phosphate, redox agent, accepts electrons to become reduced.

**NADPH**    β-Nicotinamide adenine dinucleotide phosphate, redox agent, reducing agent donates electrons.

**NAD(P)H**    A designation meaning either NADH or NADPH.

**nm**    Nanometres, wavelength.

**OD**    Optical density.

**P450**    An enzyme that was thought of as a Pigment that absorbed strongly in the 450 nm wavelength region.

**PAH**    Polyaromatic hydrocarbons.

**Reductase**    An enzyme that supplies electrons to another protein.

**RF(L)U**    Relative fluorescence (light) units, a dimensionless and arbitrary but proportional amount of light emission detected.

**rpm**    Revolutions per minute.

**S9 fraction**    Cell lysate with large organelles and debris removed

**SD**    A kind of yeast minimal media.

**SOC**    A bacterial media used in plasmid transformations

**Solution A**    Part of the artificial P450 regenerating system containing the enzyme substrate and NADP.

**Solution B**    Part of the artificial P450 regenerating system containing the enzyme for solution A.

**SW6**    A minimal broth of YNB, glucose and casamino acids.

**TRP1**    Pertaining to a particular gene or enzyme in the tryptophan anabolism pathway that when knocked out produces an auxotrophic requirement for plasmid selection.

**TEMED**    *N,N,N',N',*-tetramethylethylenediamine, for the polymerisation of acrylamide gels.

**U**    A Unit of enzyme activity.

**URA3**       Pertaining to a particular gene or enzyme in the uracil anabolism pathway that when knocked out produces an auxotrophic requirement for plasmid selection.

**YNB**        Yeast nitrogen base, mainly an ammonium salt.

**YPG**        A yeast media with yeast extract, peptone and glycerol.

**YRD**        Pertaining to the yeast's P450 reductase

# Contents

# Chapter 1　　Introduction

## 1.0　Statement of purpose

The purpose of this research was to:

    i.    Produce different human cytochrome P450 (CYP) enzymes, bound to microsomal membranes, using a yeast (*Saccharomyces cerevisiae*) expression system;

    ii.    Characterise the produced P450 enzymes for their concentrations and activities;

    iii.    Attempt to reduce the time spent for production of microsome-bound P450 enzymes and, hence, reduce overall costs;

    iv.    Investigate if the fragile microsome-bound P450 (CYP) enzymes were at all capable of retaining activity after lyophilisation and if the process allowed any degree of temperature-stability; P450s are well known for (a) being stable only at minus 80°C (-80°C) and (b) rapidly losing activity at ambient temperature, +21°C.

## 1.1　A brief overview of cytochrome P450s

Cytochrome P450s (CYPs) are enzymes that are found across different biological kingdoms and are of great interest because of their:

    ❖  Use in the modification/removal of xenobiotics, which are exogenous chemicals harmful to humans;

    ❖  Involvement in the turnover of compounds endogenous to the human body and which are crucial for human existence;

    ❖  Use in the development of new medicines for treatment of human disease;

- ❖ Use as biomarkers for gauging pollution and possible environmental bioremediation;

- ❖ Involvement in the genesis of human diseases;

- ❖ Possible use in biotransformation reactions, which are invaluable in the synthesis of high-value chemicals, mimicking the pathways followed by nature.

It has been mentioned that P450s may have been important for development of life on Earth in response to environments that contained toxic molecules (Hasler *et al*., 1999).

Humans are exposed to natural compounds, foods, medicinal plants, medicines and pollution. These contain compounds that are lipophilic and so will not exit the body without conversion to soluble hydrophilic substances and further conjugations that allow easy excretion. The processes of excretion involve Phase I solubilisation reactions which are mediated by P450 enzymes; Phase II metabolism involves conjugation of the solubilised substances to an ionized group that facilitate excretion (Rang, Ritter and Dale, 1999).

It is still unclear why there are so many different kinds of cytochrome P450s within most species. The rice plant (*Oryza sativa)*, for example, has 356 P450s and 99 P450 pseudogenes (Nelson *et al*., 2004). Most plants have 400-500 P450s. Under laboratory-based experimental conditions, many human P450s demonstrate cross reactivity with a wide range of substrates and inhibitors. It is difficult to understand why a particular organism may have so many enzymes with overlapping reactions. However, affinity for specific substrates and the $IC_{50}$ values of inhibition using specific compounds vary greatly indicating that there must be subtle differences in their modes of action (Donato *et al*., 2004).

In humans, many P450s are tissue-specific and often require their genes to be induced implying that they are not constitutively expressed.

## 1.2    Biochemistry of cytochrome P450s

Generally, electron transport chains use cytochromes and their interactions with NAD(P)H and similar molecules to generate energy (Madigan *et al*., 2002). These haem proteins not only assist in the generation of hydrogen ions but can also transport gases within organisms allowing the development of complex life forms (Sandman, 1985). The iron chelated to the porphyrin ring is used for electron transport.

A porphyrin is a ringed group of four pyrroles that forms a macrocycle, Figure 1.1. The metal ion of iron, in a $2^+$ or $3^+$ state, sits at the centre of the ring and chelates with the lone pairs of electrons present on the nitrogen atoms of the four pyrroles (Krishnamurthy *et al*., 2007) to form haem, which acts as a cofactor for the P450 enzymes.

Interaction of a haem with the cytochrome P450s is mediated by chelation of the $Fe^{3+}$ to the 'S' of the –SH (thiol) group of a specific cysteine (an amino acid) contained in the P450. This is depicted in Figure 1.2 (Rabe *et al*., 2008).

**Figure 1.1.   The Porphyrin macrocycle.**
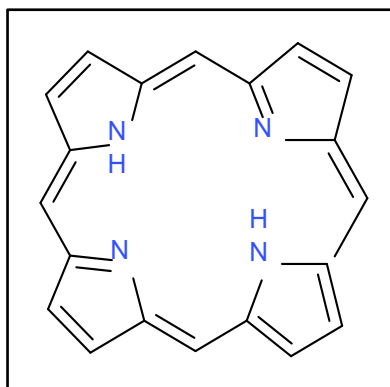


**Figure 1.1**.  The porphyrin ring consists of a group of four pyrroles which form a macrocycle. This goes on to form the haem group through coordination of $Fe^{3+}$ iron with the four nitrogen atoms of the four pyrroles.

**Figure 1.2.   A haem group interacts with P450s through coordination of the iron to the sulphur of a specific cysteine amino acid residue present in all P450s.**
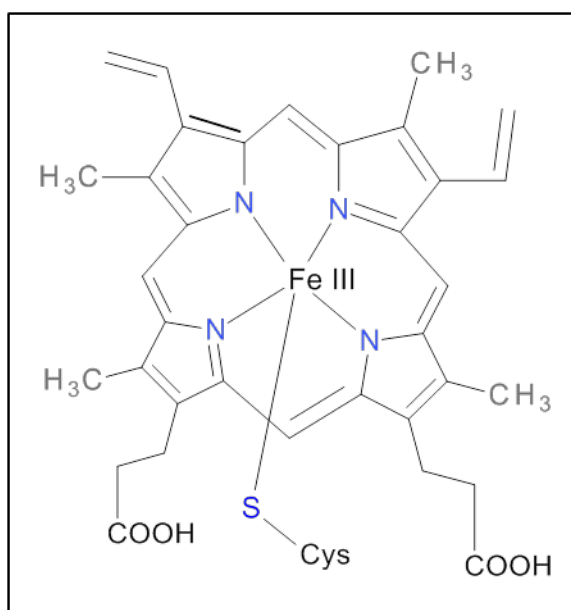


**Figure 1.2**. The porphyrin ring contains four pyrroles. Iron in the $Fe^{3+}$ (Fe III) state chelates with the 'N' atom of the four pyrroles of the porphyrin ring to form haem which ultimately binds to a P450 to form a haem-protein. As an example of how a haem-protein (haem-P450) is formed, the haem group is held in place via hydrogen bonds; in the case of CYP2D6 (P450-2D6), the hydrogen bonds are formed by interactions of CYP2D6's amino acid residues Arg-101, Arg-132, Arg-444 (arginine being a dibasic amino acid), Trp-128 (a pyrrole-containing amino acid), His-437 (an imidazole-containing amino acid) with the –COOH moieties of the haem macrocycle. However, chelation through formation of a 'coordination' bond occurs only when the haem $Fe^{3+}$ chelates with the –SH (thiol group) of the cysteine residue 443 in CYP2D6. Similar interactions with cysteine thiols occur in all P450s.

In eukaryotic cells, P450s are normally membrane bound. When P450s are removed from their membranes their activity is diminished or completely lost (Smith *et al*., 1974). The N-terminal amino acids of P450s are hydrophobic and therefore can embed themselves in lipid membranes, normally upon the smooth endoplasmic reticulum, an intracellular compartment that exists in all eukaryotic cells. The P450 activity is dependent on both lipids and proteins present in the endoplasmic reticular (ER) membranes. Because of this, it has been postulated that ER's lipid components and their proteins have co-evolved together (Simons, 2010). In the laboratory, isolating the fragile ER membranes, embedded with recombinant P450 proteins (known as microsomal P450s), is quite a challenge. The microsomes must be isolated from the cell lysates and separated from the soluble cellular fractions and other intracellular organelles.

The observation that haem proteins possess carbon monoxide (CO) gas-binding properties led to the precursor of the assay that is currently used to quantify P450 concentrations, present in recombinant and human liver microsomes, in the laboratory. In the late nineteenth century, Haldane & Smith (1896) had demonstrated that inhalation of small known amounts of CO gas, from bags, allowed estimation of blood haemoglobin levels. This observation was confirmed in 1919 by Salvesen (Salvesen, 1919).

Spectrophotometric developments in the 1950s (Chance & Legallais, 1951) allowed high quality spectral data to be obtained and created the premise for the discovery by Martin Klingenberg of "the presence of a new CO binding pigment" (Klingenberg, 1958). At the same time, David Garfinkel made a very similar discovery (Garfinkel,

1958). Klingenberg observed an absorbance peak of a pigment at the wavelength of 450 nm (Pigment 450; hence, the name P450) in rat liver microsomes. Garfinkel obtained identical results using pig liver microsomes. Klingenberg had thought the pigment was a single cytochrome similar to cytochrome $b_5$. A few years later, Omura and Sato published their paper describing "the nature and function" of the new pigment (Omura & Sato, 1962).

Cytochrome P450s' role as functional proteins that could be useful for drug metabolism studies was revealed three years later by Cooper *et al* (1965). They demonstrated that several different chemical reactions performed by rat and cow microsomes, on various drug compounds, were mediated only by CO-binding pigments which absorbed light.

## 1.3    Evolution of cytochrome P450s

In 1989, only sixteen P450s were known (Guengerich, 1989). During the 1980s, the idea emerged that all P450 genes diverged from an ancestral gene about 2 billion years ago with the P450 reductase (i.e. an enzymatic protein essential for P450 enzyme activity) possibly being a bridging product (Nerbert *et al*., 1989). It was also suggested that the few mitochondrial P450s that exist in mammalian livers originated earlier, in evolution, than the microsomal ones. The role of the few mitochondrial P450s in mammals is specifically in the syntheses of endogenous steroidal molecules. They are reduced (i.e. activated) by the iron-sulphur protein, adrenodoxin. Plants do not have P450s in their mitochondria. Therefore, it would seem that a significant evolutionary event occurred after animals and plants diverged (Omura, 2006).

It is thought that the diverse role of microsomal P450s may have somehow evolved through the microsomal redox partner (i.e. microsomal P450 reductase) that has such a low specificity; it is the only protein responsible for the activation of numerous (>50) P450s that exist in all mammals (Nelson & Strobel, 1987).

The P450 reductase is conserved across mammals although it may not be perfectly conserved across all species. Despite this, a P450 reductase from one species has been shown to provide electrons to P450s of another; for example, the yeast P450 reductase can activate human P450s in the absence of the human P450 reductase.

In most bacterial cells, P450s exist as fusion proteins, usually an N-terminal P450 being fused to the C-terminal reductase (Lah *et al*., 2008).

## 1.4    Diversity of cytochrome P450s

Figure 1.3 depicts the diversity of the human P450s and Table 1.1 portrays the number of genes that exist in different species in different taxonomic groups (Nelson, 2009).

**Figure 1.3.  A phylogenetic tree of human P450s (Omura, 2006).**
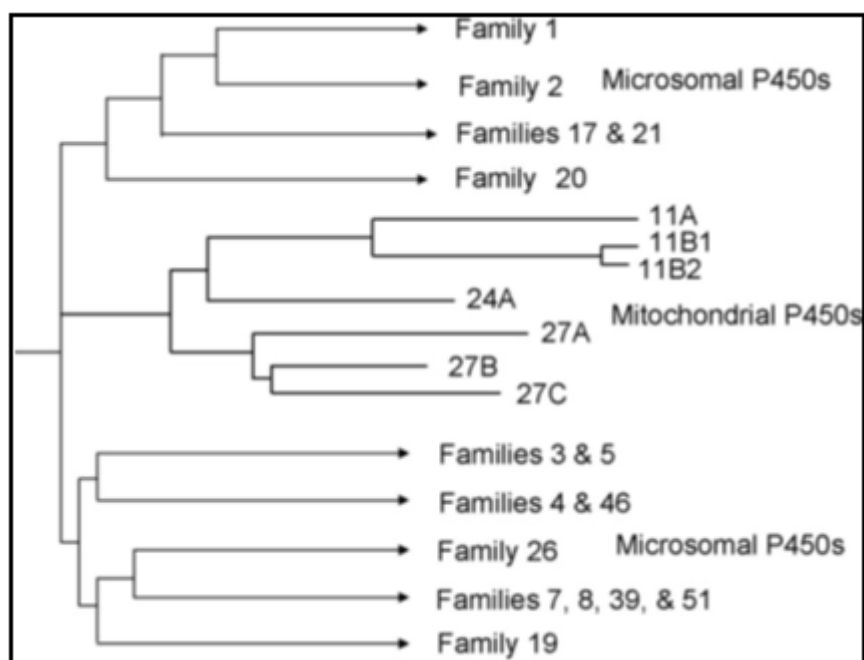


**Figure 1.3**. There are 57 human P450 genes that have been arranged into 15 gene families; amongst them there are 7 mitochondrial genes; there are also 58 human pseudogenes within these groups (Nelson, 2003).

**Table 1.1.    Numbers of P450s which have been discovered per taxonomic group (Nelson, 2009).**

| Taxonomic Group | Number of P450s (% of total P450s discovered) | Number of P450 Families | Number of P450 Subfamilies |
|---|---|---|---|
| Insects | 1,675  (14.8) | 59 | 338 |
| Animals (not insects) | 1,607 (14.2) | 69 | 169 |
| Plants | 4,266  (37.8) | 126 | 464 |
| Fungi | 2,570 (22.8) | 459 | 1011 |
| Protists | 247 (2.2) | 62 | 119 |
| Bacteria | 905 (8.0) | 196 | 409 |
| Archaea | 229 (0.2) | 12 | 14 |
| Virus (Mimivirus) | 2 (0.02) | 2 | 2 |
| Total | 11,294 | 985 | 2,526 |

Nature has produced vast numbers of P450s. It should be noted that the number of P450s listed in Table 1.1 is perhaps influenced by the research interests of scientific investigators, majority of whom prefer certain organisms over others.

## 1.5    Naming of cytochrome P450 genes and their polymorphs

Previously researchers had named cytochrome P450s (CYPs) ad hoc, as they were being discovered. Hence, there were assortments of names which gave rise to a lot of confusion. Matters became further confused when a variety of polymorphisms were discovered in the P450 genes (Anzenbacher & Anzenbacherova, 2001). Because of this, the human cytochrome P450 allele nomenclature committee has now coordinated the naming of P450 genes and their polymorphisms.

Polymorphisms in P450 genes that have emerged do not always result in a diseased state. However, the changes at the genetic level have implications. For example, CYP2A13 is expressed in the respiratory tract and metabolises carcinogens in cigarette/cigar smoke to less toxic compounds but certain polymorphisms in its gene result in reduced function of the CYP2A13 enzyme (Cauffiez *et al*., 2004).

Moreover, too much or unwanted P450 activity may also pose a problem. Excessive P450 activity leading to activation of pro-carcinogens by CYP1 family enzymes is implicated in several human cancers (Su *et al.*, 2009).

## 1.6    Induction of P450s

Many human P450s are tissue-specific.  Most of them are not expressed constitutively because to do so would either cause a burden to the human body or lead to disease. Human P450s are normally expressed only in the presence of a specific substrate or a synthetic compound that mimics the natural substrate; Table 1.2 (Anzenbacher & Anzenbacherova, 2001).

**Table 1.2.    Human P450s and their common inducers and substrates along with information on their location within the body (Anzenbacher & Anzenbacherova, 2001).**

| CYP | Localisation | Typical Substrate | Typical Inducers |
|---|---|---|---|
| 1A1 | Lung, liver, brain, GIT, lymphocytes & heart | PAH | PAH and dioxins |
| 1A2 | Liver | Aromatic amines, PAH & caffeine | PAH, ß-naphthoflavone & smoking |
| 1B1 | Skin, brain, heart, lung, placenta, liver, kidney, GIT & spleen | PAH | Dioxin |
| 2A6 | Liver | Coumarin & steroids | Barbituates & dexamethasone |
| 2B1/2 | Brain | Morphine | Nicotine |
| 2B6 | Liver & heart | Nicotine | Barbituates |
| 2C8 | Liver & kidney | Retinoids & taxol | Unknown |
| 2C9/10 | Liver | Tolbutamide & diclofenac | Barbiturates & rifampicin |
| 2C19 | Liver & heart | (S)-mephenytoin, omeprazole & diazepam | Barbituates & rifampicin |
| 2D6 | Liver, brain & heart | Antidepressants & ß-blockers | Unknown |
| 2E1 | Liver, lung brain endothelium, heart, bone marrow | Ethanol, nitrosamines & acetaminophen | Ethanol & starvation |
| 2F | Lung | Coumarins | Unknown |
| 3A4/5 | Liver, GIT, kidney, lung, brain, endothelium, placenta & lymphocytes | Calcium channel blockers, cyclosporine, acetaminophen, taxol & steroids | Steroids & barbiturates |
| 3A7 | Foetus, placenta & liver | Similar to 3A4/5 | Steroids & barbiturates |
| 4A9/11 | Kidney | Fatty acids | Clofibrate |
| 4B1 | Lung & placenta | Unknown | Unknown |
| 4F2/3 | Kidney | Arachidonic acid derivatives | Unknown |

P450 genes exist in every tissue of the human body; however, the human liver is the key tissue for major P450 activity.  PAH, polyaromatic hydrocarbons; GIT, gastrointestinal tract.

## 1.7  P450 mediated drug-drug interactions (DDI)

Different possible scenarios of P450-mediated interaction between two drugs A and B can be envisaged, a few of which have been listed below:

(1) Drug A inhibits P450 enzyme X which metabolises drug B resulting in the latter being incapable of being excreted from the body and resulting in toxic effects.

(2) Drug A suppresses the transcription of the P450 X gene which would result in P450 enzyme X not being made; thus, drug B which ought to be metabolised by P450 enzyme X cannot undergo excretion from the body resulting in toxic effects.

(3) Drug B induces P450 enzyme Y which metabolises drug A that results in the latter being metabolised far quicker than desired which would result in the prescription of a higher dosage of drug A for it be efficacious.

(4) Drug A acting as a ligand for the activation of receptors that also act as transcription factors; they activate (or suppress) the transcription of P450 gene X which codes for the P450 enzyme X which is responsible for the metabolism of drug B; in the case of activation, more drug would have to be prescribed and, in the case of suppression, less.

(5) Drug A enhances the activity of P450 enzyme X through increased translation that leads to increased formation of toxic metabolites of drug B which the body's excretion system cannot cope with.

In a study of 265 approved drugs it was found that 119 of them were cytochrome P450 inducers and 83 were suppressors at the gene expression level (Lee *et al*., 2006). Current drug approval guidelines clearly state that, before approval of any drug for therapeutic use, P450 inhibition profiles must be submitted. All approved drugs inhibit one or the other major P450 enzymes: 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7. As indicated above, in this Section, use of certain combination of drugs can give rise to low efficacy and, conversely, can be overly effective leading to fatalities.

The problem of drug-drug interaction (DDI) pertains not only to conventional drugs. There has been concern around the interaction of herbal medicines with pharmaceuticals. Use of St. John's Wort (a CYP3A4 inducer) together with the immunosuppressant, cyclosporine, may cause insufficient plasma levels of cyclosporine. Smoking can also increase metabolism of the asthma drug theophylline by significantly enhancing hepatic clearance via induction of CYP1A enzymes (Schrenk, 1998).

## 1.8 The cytochrome P450–mediated hydroxylation cycle

Cytochrome P450 (CYP) enzymes can perform a variety of complex reactions. However, the most common chemical reaction that probably all P450 enzymes perform is hydroxylation (Guallar *et al*., 2003). This process requires molecular oxygen, CPR (the <u>c</u>ytochrome <u>P</u>450 <u>r</u>eductase), cytochrome b5 and the b5 reductase. Molecular oxygen, $O_2$, is utilised via a dismutation reaction (that involves both oxidation and reduction) and results in the formation of water and reactive oxygen species (ROS) at

the same time. The ROS participates in hydroxylation, inserting an atom from

molecular oxygen on to an unreactive (i.e. inert) carbon atom which could be part of

either an aliphatic –C—H or an unsaturated/aromatic =C—H bond.

Figure 1.4 depicts the hydroxylation of camphor using the bacterial P450cam, the

specific cytochrome P450 that metabolises camphor.

**Figure 1.4.   Metabolism of camphor, a simplified version (Guallar *et al.*, 2003).**
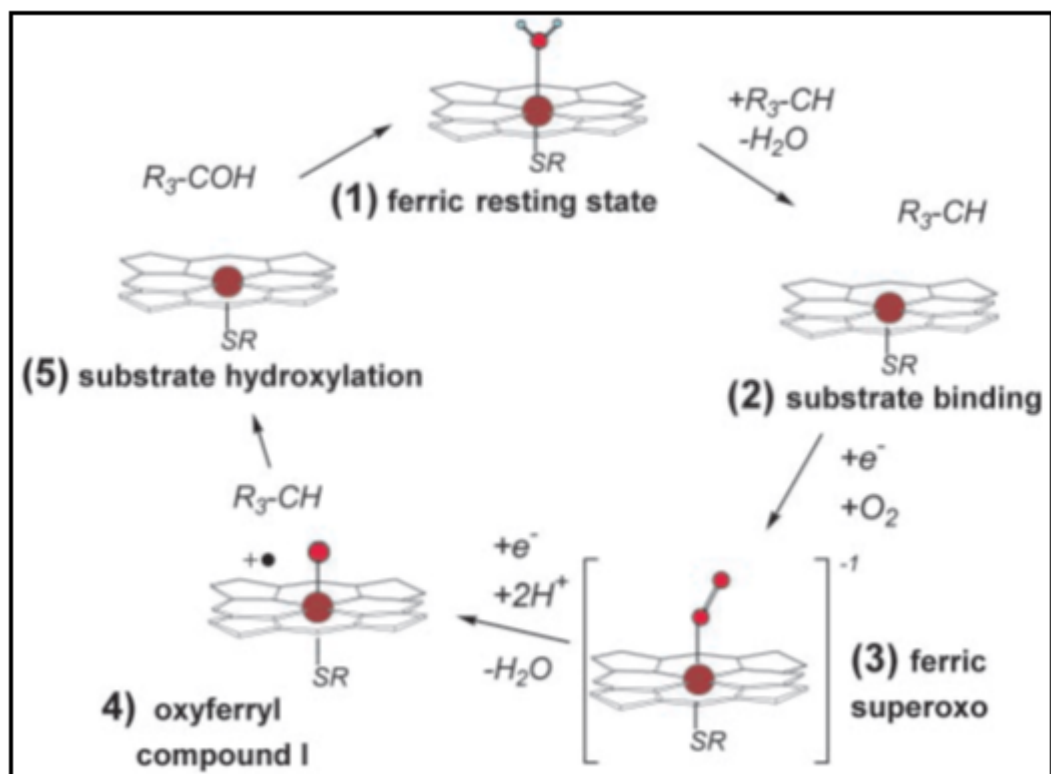


**Figure 1.4**. P450-mediated-hydroxylation of the substrate, camphor. The flat layer of ring structures consist of the porphyrin rings, the larger solid red central sphere is iron. The smaller red and two white spheres in (1) depict water, the two red spheres in (3) is molecular oxygen, and the red small sphere in (4) is singlet oxygen. The substrate, camphor, is denoted by $R_3$-CH. Coordination of the P450 to $Fe^{3+}$ via a thiolate which is being contributed by the –SH of a cysteine in the P450. In multicellular organisms the first and, possibly, the second electron is provided by a P450 reductase. The second may also sometimes be provided by the cytochrome b5/ b5 reductase system.

1) The P450 is bound to the ferric iron Fe (III) of the porphyrin macrocycle in the resting state with water bound and no net charge. Binding of P450 to the iron occurs via the sulfhydryl/thiol (—SH) group of a cysteine residue in the P450.

2) The non-covalent binding of the camphor ($C_{10}H_{16}O$), the substrate, occurs with loss of water. The process allows the complex to receive an electron from NADPH via P450 reductase which reduces the ferric iron Fe (III) to the ferrous Fe (II) state. This causes a structural shift in the porphyrin ring.

3) This new complex receives molecular oxygen which binds to the complex; it is termed as a "ferric superoxo" moiety.

4) A second electron is donated via NAD(P)H by either cytochrome b5 or P450 reductase. This electron reacts with already bound oxygen forming a negatively-charged distal oxygen. It results in changes in the bond between the two oxygen atoms, from a double to a single bond, to provide a stable electron configuration. The proximal oxygen then takes an electron from ferrous iron to make the iron more positively charged, as Fe (III). This double negatively-charged complex attracts a proton as a positively-charged hydrogen ion to the distal oxygen which has an extra electron. This produces a hydroxyl group (–OH) group.

5) The single negatively-charged complex attracts another positively-charged hydrogen ion into its vicinity which allows the complex to substitute itself away from the hydroxyl group to be released as water. The complex is called an oxyferryl moiety with a Fe (IV), the iron being even more positively charged. Since the remaining oxygen requires an electron to fill its outer shell, it forms a double bond with Fe (IV).

6) With the iron moving towards a more positive state it has enough energy to split the stable double bond between two oxygen atoms. The free oxygen atom acts as a free radical, with 2 electrons missing from its outer shell, and inserts itself into the substrate, between the C—H bond, to gain electrons; thus, substrate hydroxylation occurs.

7) With the interaction over and the complex neutrally charged, the substrate has less affinity for the active site.

Using such hydroxylation cycles, P450s can oxidise a diverse range of complex substrates varying from ethylene (28.05 MW) to cyclosporine (1202.61 MW) (Kent *et al.*, 2001).

## 1.9 Drug Metabolism & Elimination

It was shown in Table 1.2 that cytochrome P450s (CYPs) perform their roles in a range of tissues. However, the liver is the primary organ of drug metabolism. It contains the major P450s of interest to pharmaceutical companies, CYP3A4 being the most important, Table 1.3.

**Table 1.3.** **Details of the predominant P450s in the human liver (Anzenbacher & Anzenbacherova, 2001).**

| CYP | Relative content in the liver (%) | Expression variability in fold differences | Estimated fraction of drugs metabolised (%) | Drugs that act as substrates |
|---|---|---|---|---|
| 1A1 | 1 | 100 | < 1 % | None known |
| 1A2 | 12 | 40 | 4 % | Caffeine |
| 2A6 | 4 | 30 | < 1 % | None known |
| 2B6 | 1 | 50 | < 1 % | None known |
| 2C9/2C10/2C19 | 20 | 20 | 11 % | Diclofenac (2C9) (S)-mephenytoin (2C19) |
| 2D6 | 4 | 1000 | 30 % | Sparteine, debrisoquine & dextromethorphan |
| 2E1 | 6 | 20 | 2 | Chlorzoxazone |
| 3A4 | 30 | 60 | 52 | Nifedipine, erythromycin, alprazolam & dexrometorphan |

The key P450s involved in drug metabolism occur in the liver in varying amounts. Certain P450s are highly induced by certain drugs. CYPs 3A4 and 2D6 are of major interest to the pharmaceutical industry. "Not known" denotes that there is no relevant pharmaceutical drug which has been found, as of yet, to be a substrate for the enzyme's liver activity, using analytical tools like Liquid Chromatography/Mass Spectrometry.

Usually, a drug approved for therapeutic use exits the body in a more soluble (polar) form after being metabolised by a P450 enzyme. As described earlier, drug metabolism is split into Phase I and II reactions. In Phase I, a hydroxylation reaction creates compounds that are more reactive and provides a molecular attachment point for molecules such as glucuronide allowing conjugation (a form of esterification) in Phase II. The enzyme UDP-glucuronyl transferase removes glucuronide from a carrier molecule and then transfers the glucuronide to the drug that has been hydroxylated (i.e. metabolised) by a P450. The glucuronide has a carboxyl group (COOH) which is involved in esterification and three polar –OH groups which improve the water solubility of the resultant conjugate (Rang *et al*., 1999).

Other types of Phase II conjugations of hydroxylated drugs, formed via Phase I P450 metabolism, involve reactions that lead to sulphation, methylation, acetylation and glycylation of the hydroxylated product.

## 1.10  Cytochrome P450 (CYP) Modifications

Joo *et al* (1999) have documented P450 mutations in a bacterial P450 that allow peroxide shunt, a partial bypass mechanism in the normal P450 cycle facilitating far easier hydroxylation reactions using hydrogen peroxide than with molecular oxygen. This bypass mechanism does not require the presence of P450 reductase or other chemical cofactors for P450 activity. The authors used alterations in specific amino acids to increase P450 activity 20-fold and were also able to co-express P450s with horseradish peroxidase (HRP) which converts the products of the P450 reaction into fluorescent compounds making them amenable for screening by digital imaging. This allowed rapid assessment of the best constructs through screening of numerous clones using whole cells without having to process them into microsomes, saving time and money.

Unfortunately however, it is nearly impossible to rationally alter known human P450 enzyme activities for further screening, using HRP activity, because 3D structures of most human P450 enzymes are not known in fine detail. If they were to be available, it may allow creation of novel P450 enzymes that could be used for catalysis of intricate bio-organic syntheses that are "difficult to perform using standard organic chemistry"

(Rabe *et al*., 2008), with the possibility of opening up limitless opportunities for biotransformation reactions.

It has been observed that site-directed mutagenesis at or near the active site can yield new functionality in P450s. However, a minor change may also interfere with the whole tertiary structure (Domanski & Halpert, 2001). This problem could possibly be overcome if the relationship between homologous P450 proteins and their substrate specificity could be mapped and then legitimate predictions as to what changes a protein could bear without totally distorting its tertiary structure. As crystal structures of P450s are more widely available, site-directed mutagenesis as a tool to produce novel P450s could be a distinct possibility sometime in the future.

From the crystal structure of CYP2D6 (Figure 1.5), Rowland *et al* (2006) inferred that the negatively-charged amino acids Asp-301 and Glu-216 are the main determinants of substrate and inhibitor binding. This was confirmed by substitution of one or both the negatively-charged amino acids (Aspartic Acid and Glutamic Acid) with a neutral amino acid, Alanine. This caused reduction or loss of activity. It has been suggested that Glu-216 at the top of the active site cavity acts as a "recognition residue" attracting basic molecules into the active site before "ushering" them towards Asp-301, which is within the active site, for any reaction on a substrate to occur. It was noted that Phe-120 aligned the substrate into the correct spatial position within the active site. The authors also confirmed that the reductase binding region is in CYP2D6's C-terminus which is rich in dibasic amino acids; a mutation in this region from strongly basic Arginine-440 to less basic Histidine caused total inactivity.

**Figure 1.5. CYP2D6 structure (Rowland et al., 2006).**
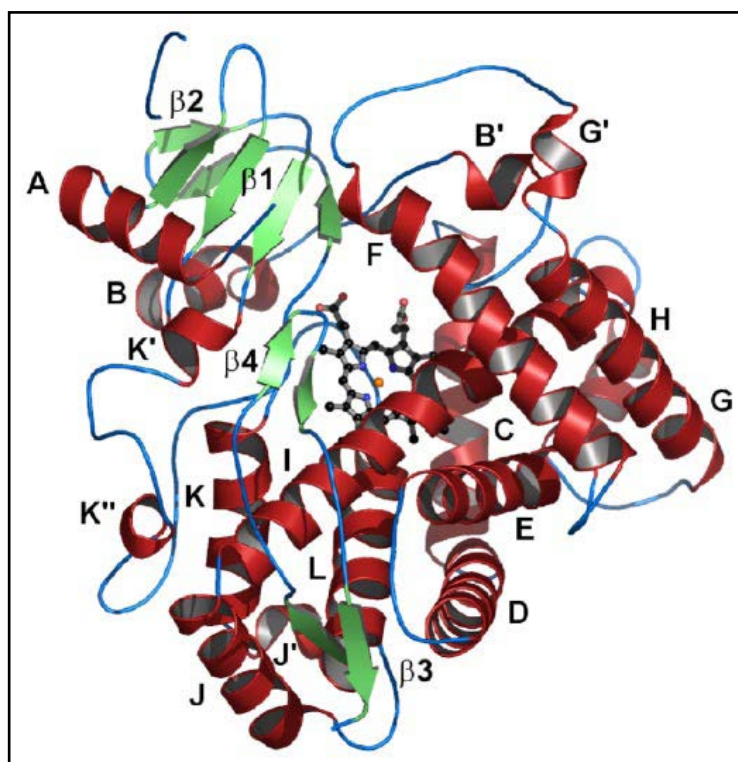


**Figure 1.5**. The 3D structure of CYP2D6 protein has been represented as red helical domains, each one alphabetised for reference; green ß sheets numerically referenced. These domains are joined by blue loops which have flexible structures. The haem group in black and grey is centrally located.

## 1.11 A brief background to cytochrome P450s (CYPs) in the yeast, *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* has three P450 genes: *CYP51F1*, *CYP56A1* and *CYP61A1* (Nelson, 2004). The three genes are essential for yeast's basic existence. They code for mitochondrial enzymes and have highly specific functions that contribute to the yeast cells' structure. They are not involved in the removal of toxic compounds.

Amongst all P450 gene families, the CYP51 family of enzymes is the most widely distributed across fungi (Lepesheva & Waterman, 2004). The *CYP51* gene codes for the enzyme, sterol 14-demethylase, which is important for sterol biosynthesis.

*CYP61* family of genes codes for sterol desaturases. They are involved specifically in ergosterol biosynthesis (Nelson, 1999).

The *CYP56* family of genes is involved in spore wall formation (Nelson, 1999).

## 1.12  Usefulness of yeast, *Saccharomyces cerevisiae,* in the production of human P450s

The yeast *Saccharomyces cerevisiae*, which is as cheap to grow as bacterial cells, has the potential to be an excellent host for expression of human P450s. The full yeast genome has been sequenced and its proteins are well characterised and compared with human proteins, providing a well-defined environment for the expression of foreign proteins (Kellis *et al*., 2003). Moreover, it is a eukaryote and therefore has similar intracellular organelles as human cells. One of these organelles consists of the endoplasmic reticular membranes to which P450s are naturally bound in human cells. An added attraction of yeast, as a possible eukaryotic host, is that the human P450s, when expressed in yeast, are unlikely to be contaminated with significant endogenous P450s that may corrupt an assay for the human enzymes. Hence, yeast-produced P450-bearing microsomes, if it were to be possible to obtain, could be used for screening of potential inhibitors and substrates of human P450 enzymes.

The human P450 reductase (hRD), the enzyme essential for human cytochrome P450 (CYP) activity, closely resembles the rat, rabbit, pig and trout primary protein sequences. Although sequence homology between yeast and human P450 reductases is only about 33%, surprisingly, the yeast P450 reductase has the ability to activate human

P450s. This because the FAD, FMN and NADPH binding regions are highly conserved amongst different species allowing *S. cerevisiae's* endogenous P450 reductase (yRD) to be capable of donating electrons to human P450s (CYPs), in the absence of hRD (Odea *et al*., 1985).

## 1.13 A background to the growth of yeast strains harbouring human P450 genes

Transformation, growth and expression of yeast cells are on a par, in terms of cost, time and complexity, with bacterial systems. Commercial yeast strains containing multiple auxotrophic mutations may allow up to 5 genes to be cloned into the yeast cell offering different possibilities of co-expressing multiple proteins. When an auxotrophic marker is not utilised, the requirement for the missing marker gene is bypassed by adding a relatively cheap supplement to the growth media.

The yeast auxotrophic markers stem from the knockout of genes in the yeast's anabolic pathway that are essential for growth in minimal growth media. The individual gene knockouts remove the ability to manufacture within yeast, for example, (a) an amino acid such as leucine, histidine or tryptophan, and (b) a nucleic acid or nucleic acid precursor such as uracil or adenine. These are nutrients that are essential for yeast's cell growth.

Not all auxotrophic markers are 'equal' implying that introducing the same gene expression cassette at different markers may cause uniquely different burdens to the

cell, depending on the relative importance of the marker in the pathway that leads to the synthesis of an amino acid or a nucleic acid (Amberg *et al.*, 2005).

For regulated expression of heterologous proteins in yeast, two key promoters have often been used to drive the transcription of heterologous genes. One of the promoters that is induced by the addition of galactose is the *GAL1* or *GAL10* promoters. The other promoters from yeast's *ADH1* or *ADH2* genes, are induced by ethanol. In this thesis, an ethanol-inducible 573bp fragment of the *ADH2* promoter is used, instead of the published 300bp *ADH2* promoter sequence to enhance heterologous protein expression. The *GAL1* promoter that has been used has also undergone modifications with the aim of enhancing heterologous protein expression. It has been seen in our laboratory that the variants of the *ADH2* and *GAL1* promoter indeed provide better inducibility and expression of foreign proteins in yeast (unpublished observations). Ethanol-inducibility of the *ADH2* promoter can occur when glucose is gradually exhausted in the media and converted to ethanol, whereas the *GAL1* promoter is induced immediately in the presence of galactose.

The first expression of a cytochrome P450, in yeast, was published in 1985 using the constitutive *GAPDH* promoter (Odea *et al.*, 1985). Later, a paper was published with the aim of maximising expression of P450s in yeast (Urban *et al.*, 1990) using the indicible *GAL1*/*GAL10* promoters. It described that the expressed P450, CYP1A1, in the presence of endogenous yeast P450 reductase (yRD), had poor activity. The P450 activity could be increased by overexpressing yRD up to 5-fold, either from a chromosomal locus or from a multi-copy plasmid; yRD gene expression, in both cases, were driven by the *GAL10* promoter. The paper also stated cryptically that "... a

maximal expression in yeast for a given mammalian P450 activity was not necessarily achieved by maximal P450 expression".

A 1995 paper (Pompon *et al.*, 1995) claimed the development of a third generation yeast expression system which also used the galactose-inducible promoter. The authors tried to split the burdens of exponential growth and expression. They used an intermediate phase of ethanol addition to the medium before switch from glucose to galactose. This method yielded several hundred nano-moles of P450 per litre of culture but the enzymes were poorly active. It appears that the absence of enough yeast P450 reductase was the factor that limited activity. This led to the evaluation of a production system that co-expressed human P450 reductase with a human P450. It was found that expressing the human P450 reductase was much more difficult than the yeast's P450 reductase and the improvement of activity was minimal.

## 1.14 Difficulties with Yeast expression

To maintain a steady utilisation of glucose, microbes are adapted to sensing glucose levels and they respond according to the level of gene expression (Johnston, 1999). *Saccharomyces cerevisiae* is an atypical eukaryotic microbe. It is able to switch between respiration and fermentation depending on glucose concentrations. After sensing glucose, respiratory enzymes, such as (a) electron transport chain cytochromes and (b) enzymes that allow the use of other carbon sources (like sucrose, maltose and galactose), are repressed. Genes encoding glucose transporters and glycolytic enzymes are also expressed.

1 to 2% glucose is usually added to the media when growing yeast in the laboratory. This allows for the production of reasonable biomass. The switching between fermentation and respiration adds extra complexities to optimisation of biomass and expression times.

Another related problem with yeast is petite formation where mitochondria are either damaged or missing. It can be caused by the stresses placed upon cells by expression of foreign proteins (Yaffe, 1991). Cells can appear to be normal when grown in a glucose rich environment. However, without the oxidative pathways the non-fermentable carbon sources, like galactose, cannot be utilised (Goldring *et al*., 1971). Both the rho° and rho⁻ petites, which are intolerant of oxidative stress, are undesirable for the expression of heterologous proteins that need a non-fermentable carbon source for full-blown expression (Jamieson, 1998).

A problem with the fermentative process of growth is that, during glucose fermentation and production of ethanol, excess NADH is produced. Glycolysis occurs, with or without oxygen, in the cytosol, converting glucose to pyruvate. In an oxidative environment the pyruvate produced may enter the mitochondrial Krebs cycle. However, during fermentation of petites, pyruvate formation leads to an excess of pyruvate and NADH that cannot be used. Excess pyruvate in the cell is likely to cause osmotic stress whereas excess NADH can lead to the redox pool running out of NAD. To fix this, some balance would have to be restored through (a) conversion of pyruvate to acetaldehyde via loss of $CO_2$ and (b) NADH consumptive conversion to ethanol formed by alcohol dehydrogenase 1 (ADH1); the formed ethanol can cross membranes freely reducing osmotic stress (Constenoble *et al*., 2000).

A problem can occur if the excess NADP that could be used to supply the electron transport chain cannot cross the mitochondrial membrane, after fermentation is over. The cell employs the glycerol phosphate shuttle, which during fermentation acts as a redox buffer. During respiration, the process can be taken further, shuttling reducing power into the mitochondria in the electron transport chain. This is done by the conversion of the by-product dihydroxyacetone phosphate (DHAP), formed during glycolysis, to glycerol-3-phosphate (G3P) via reduction by glycerol 3-phosphate dehydrogenase (GPDH) by the way of NADP conversion to NAD. G3P can pass into the mitochondria and is reversed backwards in the same way to DHAP to yield NADH for electron transport. Once G3P becomes excessive it is converted by glycerol 3-phosphatase to glycerol.

Even though glycerol is a non-fermentable carbon source it ends up being produced for redox balance. It protects the cells from hyper-osmotic stress caused by high levels of solutes in the media by attempting to match the osmotic pressure to the external surroundings of the cell and therefore protecting proteins and membranes from damage (Michnick *et al*, 1997; Kuroda *et al*., 1994; Remize *et al*., 1999).

All the above factors, which have influence on the growth of yeast, could be further explored for optimal expression of proteins in yeast.

## 1.15  Biotransformation reactions in yeast with P450s

Yeast cells expressing active P450 enzymes and P450 reductases could be potential cellular factories for biotransformation reactions of chemicals. As an example, an

*Arabidopsis* P450 reductase, when expressed in a *CYP61* gene-knockout yeast strain, interfered with the synthesis of ergosterol, forcing the cells to produce the intermediate ergosta-5-enol as a cheap precursor for the mass production of several different steroidal hormones (Kelly & Kelly, 2003). As ergosta-5-enol can replace ergosterol in the yeast membrane, the cells behave normally. By further modifying the cells by introducing different mammalian P450s into the cell, different steroids were produced. This demonstrates that yeast cells could be modified to undergo tailor-made biotransformations which may allow production of medicines with intricate stereochemistry, at very low cost.

## 1.16 Lyophilisation/ stabilisation

Certain purified commercial enzymes, such as glucose-6-phosphate dehydrogenase, are often supplied as dried formulations. This allows shipping at ambient temperature and retention of stability. The P450 enzymes cannot be purified because they are active only when they are bound to endoplasmic reticular membranes. Hence, no one has yet conceived of supplying active membrane-bound P450 enzymes as dried products. Commercial suppliers of P450s require their products to be stored –80ºC and in buffers with high solute concentrations. Depending on the supplier, buffers contain mixtures of glycerol, sucrose, glucose, Tris-EDTA, phosphates and protease inhibitors. They are shipped on dry ice. Maintenance of the cold chain is inconvenient, adds costs and makes most applications difficult, if not impossible.

For preservation over years, proteins are usually stored in buffers which do not have much water, lack oxygen, and are kept at very low temperatures (minus 20°C to minus 80°C). Preservation in the natural environment in the cold is quite well known (Sallon *et al*., 2008). To avoid degradation of protein, inhibitors of proteases are often added.

Lyophilisation is a process that also allows preservation. Lyophilised *Salmonella* vaccines maintain very high levels of viability after years of storage at +4ºC (Khan *et al*., 2007). Attempts have also been made to lyophilise mammalian cells but not with great success.

From efforts to lyophilise cells and soluble proteins, it may seem an obvious step to attempt to lyophilise microsomes (i.e. membrane-bound proteins). Whilst microsomes have been lyophilised, since their discovery, as a means of storage, it is clear that this was used only as a technique for preserving large quantities of animal tissue homogenates for later structural analysis. There was a drive to examine why the stability of drug metabolising enzymes, obtained from animal tissues, had so many variables (Kamataki & Kitagawa, 1974; Aikawa *et al*., 1976). It was shown that microsomes from animal livers were best stored as a lyophilised pellet rather than in solution and that lyophilised S9 fractions were more stable than lyophilised microsomes. There was a significant difference in the stability of microsomes from different animals when tested over a month. It was not clear why this was so but it is possible that differing protein concentrations of the liver preparations affected stability (Tanaka *et al*., 1991).

In the past decade, commercial suppliers have improved P450 products to provide them more as standardised entities with some characterised stability data. However, instability seen during long-term storage and during assays has been brought into focus

by multiple publications (Bernhardt, 2006; Chefson & Auclair, 2006; Urlacher & Eiben, 2006).

Soluble enzymes, upon lyophilisation, lose activity. It is thought that the dehydration of the protein causes a conformational change that is not reversible when rehydrated, although sugars have been usually used as cryo-protectants to prevent loss of activity (Chefson *et al.*, 2007). Chefson expressed CYP3A4 in a yeast expression system and lyophilised the P450s. However, purified proteins (shorn of membranes) were lyophilised, not 3A4-containing microsomes. These had minimal or no biological activity. It has been shown, that in order for the P450 and P450 reductase proteins to retain activity, they must remain attached to the membranes and also retain the haem group.

Nardid, Dyubko and Repina (1997) stated that just freezing and thawing microsomes may cause irreversible conformational changes. This work was conducted with purified P450s from rabbit liver microsomes which were allowed to associate with artificial membranes.

Pearce *et al* (1996) have stated that intact human liver microsomes can tolerate only up to 10 cycles of freeze-thaw without significant changes. Interestingly, in non-processed livers stored at minus 80°C, P450 reductase and key P450 activities diminish up to 40% after only one month.

In summary, the general stability of P450s, before and after lyophilisation, has been found to be poor. The idea that useful levels of P450 activity may be recovered from lyophilised microsomes seemed unlikely but it was still thought to be worthy of further

investigations with recombinant P450-bound microsomes with the aim of allowing the

development of new and exciting applications in drug discovery.

# Chapter 2    Materials and Methods

## 2.1    PCR using the Qiagen Proofstart kit

Qiagen Proofstart kit, catalogue number, 202203.

Invitrogen Custom Primers were used. Primer annealing temperature was calculated as

$((G+C)*41)-675)/ (\Sigma \text{ All bases } A+T+G+C) + 61.8) = °C.$

Control was template substituted for an equal volume of ultrapure water.

Final volume of 50 µl using 0.2 ml PCR tubes (Stratagene, 401425).

- Ultrapure water                                   26 µl
- 10x Proofstart buffer                            5 µl
- 5x Q buffer                                          10 µl
- Forward primer, 0.5 $\mu g.\mu l^{-1}$              2 µl
- Reverse primer, 0.5 $\mu g.\mu l^{-1}$              2 µl
- dNTPs, 20mM                                       3 µl
- Qiagen proofstart taq polymerase        1 µl

The template was either:

1. A small loopful of cells,
2. Previously cloned DNA, 5 $ng.\mu l^{-1}$,
3. Human cDNA liver library, 1 $\mu g.\mu l^{-1}$ , 1 µl

PCR steps;

- 95°C for 5 min,

- 95°C for 45 seconds to melt the strands,

- 55°C (calculated individually) for primer annealing for 90 seconds,

- 72°C for 3 min to elongate the strands,

- 30 cycles of the above 3 steps, 72°C for 10 min,

- 4°C held.

After PCR, the reaction was run on a 1% (w/v) agarose-TAE gel, and relevant bands were excised from the gel using a Qiagen gel-extraction kit.

## 2.2    Agarose gel electrophoresis of DNA

1% (w/v) agarose gels in TAE with 1 µl of 10 mg.ml$^{-1}$ of ethidium bromide was used with the Bio-Rad, Mini-Sub Cell GT and the gels were run at 100 Volts for typically 45 min. Images were captured using the Bio-Rad, XRS Chemidoc system.

Reagents

- Agarose, Gibco, Catalogue number 15510-027,

- 10x or 50x TAE Gel running buffer, pH 8.3,

- Final 1x concentration of 0.4 M Tris-acetate, Fisher, BPE152-1,

- Final 1x concentration of 20 mM Glacial acetic acid, Sigma, 320099,

- Final 1x concentration of 10 mM EDTA, Gibco, 15706-021.


- 10 mg.ml$^{-1}$ Ethidium bromide in ultrapure water, Sigma-Aldrich, Catalogue number E7637.

10x loading buffer in 100 ml of ultrapure water.

- 50 ml of 50% (v/v) Glycerol, Fisher, G/0650/17.

- 2 g Ficoll, Sigma, F-2637.

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- 10 ml of 50 mM EDTA pH 8, Gibco, 1570-6-021.

- 0.5 g of Bromophenol blue, Sigma, 114391.

DNA ladders from New England Biolabs, 1 Kb, N3232 and 100 bp, N3231.

## 2.3    Qiagen Gel-extraction kit

Using the standard supplied protocol, DNA was purified from agarose gels with the aid of the Qiagen gel extraction kit (Qiagen, 28604). The DNA was eluted in 30 µl of TE at pH 8.0.

Reagents

- 10x TE buffer at pH 8.0.

- Final 1x concentration of 100 mM Tris, Fisher BPE152-1.

- Final 1x concentration of 10 mM EDTA, Gibco, 15706-021.

- Made up in ultrapure water.

## 2.4    Restriction digests using NEB and Roche enzymes

Standard supplied protocols were used.

A typical reaction was:

- 2 µg of DNA fragment,

- 0.2 µl of *Bam*HI 10 U.µl$^{-1}$,

- 0.2 µl of *Xba*1 10 U.µl$^{-1}$,

- 1 µl of 10x buffer B,

- 6.6 µl of ultrapure water.

If BSA was required, 0.1 µl of the supplied 100x stock was added.

## 2.5　T4 DNA ligase reactions for DNA molecules using commercial kits from NEB and Roche

Standard supplied protocols were used. Control reactions were also performed with either no ligase or no DNA fragment.

NEB Kit, catalogue number M0202S.

- Ultrapure water　　　12 µl
- 10x Buffer　　　　　2 µl
- Plasmid/Vector　　　1 µl
- Insert fragment　　　4 µl
- T4 Ligase　　　　　1 µl

Roche Kit, catalogue number 716359.

- Ultrapure water　　　7.75 µl
- 10x Buffer　　　　　1.5 µl
- Plasmid/Vector　　　1 µl
- Insert fragment　　　4 µl
- Ligase　　　　　　0.75 µl

Reactions were incubated overnight at +16°C in a water bath in a cold room or kept at +4°C over 48h.

If required, enzymes were heat inactivated before transformation. Where appropriate, the ligation products were run on a 1% (w/v) TAE-agarose gel to check efficiency of ligation and then gel-extracted using the Qiagen gel-extraction kit.

## 2.6  Bacterial competent cell transformation

Competent *E. coli* DH5α cells were sourced from Invitrogen (18265017). The plasmids used were amplified in DH5α before transformation into yeast. Selection was via ampicillin resistance.

Competent cells were thawed on ice for 10 min.

- 7.5 µl of purified plasmid (10-20 ng) or ligation mixes were added to the cells and placed on ice for 10 min.

- The samples were heat shocked at 42ºC for 90 seconds.

- 1 ml of SOC medium (Sigma, S1797) was added to the cells followed by incubation at 37ºC for 1h.

- Cells were concentrated at 13,000 rpm (15,700g) for 1 min allowing the removal of 800 µl of supernatant. Cells were re-suspended in 200 µl, and 100 µl of cells were plated out on to two LB agar plates with ampicillin at 100 $\mu g.ml^{-1}$ of agar.

- Agar plates were incubated overnight at 37ºC.

- Transformants were grown in 10 ml cultures of LB broth with 100 $\mu g.ml^{-1}$ of ampicillin at 37˚C, 180 rpm.

- Glycerol stocks were produced and stored at -80˚C.

- Plasmids were checked using restriction enzyme digestions. See section 2.4.

**Reagents**
- LB broth 25 $g.L^{-1}$ in ultrapure water, Difco, 244620.

- LB agar 20 $g.L^{-1}$ in ultrapure water, Difco, 244520.

- A 100 $mg.ml^{-1}$ stock of ampicillin (Fisher, BPE1760-5) in ultrapure water with a final concentration in the media of 100 $\mu g.ml^{-1}$.

## 2.7 Isolation of plasmids from *E. coli* using the Qiagen Midi prep kit (25 ml cultures)

Standard supplied protocols were used, depending on the amount of DNA required. The Midi preparation (Qiagen, 12143) for 100 μg of plasmids was normally sufficient.

Glycerol stocks for each clone were made if no previous stocks existed.

Each DNA pellet was dissolved based on its size, in a volume of TE (pH 8.0).

Plasmid concentrations were measured by adding 10 μl of plasmids to 1 ml of ultrapure water in a quartz cuvette (14-385-914A).

Plasmid concentration in $\mu g.\mu l^{-1}$ = OD at 260 nm x dilution factor x 0.05,

Plasmid purity = OD at 260 nm/OD at 280 nm.

If the plasmid concentration was too low then the plasmids were concentrated into a smaller volume using a Qiagen gel-extraction spin column. Plasmids could then be re-suspended in a suitable smaller volume.

## 2.8 Transformation of integrating or episomal plasmids in Yeast, DMSO method

- A 10 ml yeast culture was grown in YPD broth, at 30°C, shaking at 220 rpm overnight. Auxotrophic supplements were added at 8.3 μl of stock per 1 ml of media.

- 1.5 ml of the culture was centrifuged for 20 seconds at 13,000 rpm (15,700g) in a microfuge. The supernatant was poured off retaining the pellet.

- 100 μg of salmon sperm DNA from a 2 mg.ml$^{-1}$ stock (50 μl) was added to the pellet. After thawing from –20˚C, the salmon sperm DNA was heat-treated at 95˚C for 5 min.

- The solution and pellet were mixed well.

- Variable step*: (* for integrating plasmids, normally the linearized P450 Reductase, 4-6 μg of DNA was added.; for episomal plasmids, normally encoding P450 genes, 0.5-1 μg of DNA was added).

- The solution was vortexed.

- 500 μl of PEG solution for yeast transformation was added.

- 55 μl of pure molecular grade DMSO (Sigma, D5879) was added.

- Incubated in a Thermomixer for 15 min at 25°C, 400 rpm.

- The samples were heat-shocked for 15 min at 42°C. After 10 min of heat-shocking, 60 μl of pure molecular grade ethanol (Sigma, E7023) was added.

- Cells were centrifuged at 8000 rpm (5900g) in microfuge for 1 min.

- Cells were washed twice in 500 μl in TE, pH 7.5, using above centrifuge settings.

- Cells were re-suspended in 200 μl TE, pH 7.5.

- Agar plates were spread in duplicate using 100 μl of each plate on SD-Minimal agar plates containing the appropriate auxotrophic supplements at 8.3 µl per 1 ml of agar and incubated at 30°C for 3-4 days.

Since yeast cells are capable of growing with damaged or missing mitochondria, growth

on a glycerol media was used to deselect these petites.

- Several individual colonies were transferred on to a glycerol agar (YPG) at 30°C for 3-4 days to deselect petite strains.

- 3 individual colonies were picked directly from the yeast glycerol agar and transferred back upon SD-Minimal agar to maintain plasmid selection.

- Colonies were then picked and grown in 10 ml cultures of YPD broth at 30˚C in a shaking incubator at 220 rpm.

- Glycerol stocks were produced from individual colonies for -80°C storage.

**Reagents**

PEG solution for yeast transformation, final concentrations in ultrapure water

- 40% (w/v) PEG-3350 MW, Sigma, P4338,

- 0.1 M Lithium acetate, pH 7.5, Sigma, L4158,

- 10 mM Tris, pH 7.5, Fisher, BPE152-1,

- 1 mM EDTA, pH 7.5, Gibco, 1570-6-021.

**Salmon sperm** (Sigma, D1626)

- 200 mg of salmon sperm in 100 ml of TE, pH 8.

- DNA was pipetted up and down.

- Left overnight in a 4°C cold room with vigorous magnetic stirring.

- Aliquoted and stored at -20°C.

- Heated to 99°C in a thermomixer for 5 min before use.

**S-Minimal agar**, in 500 ml, in ultrapure water

- 3.35 g of YNB, Difco, 291940,

- 10 g of Glucose, Sigma, G5400,

- 7.5 g of Agar, Oxoid, LP0013,

- 1 pellet of Sodium hydroxide as supplied by Fisher, BP359-500.

**YPG agar**, in 500 ml, in ultrapure water

- 5 g of Yeast Extract, Oxoid, LP0085,

- 10 of Peptone, Oxoid, LP0021,

- 15 ml of Glycerol, Fisher, G/065/17,

- 10 g of Agar Oxoid, LP0013.

**YPD broth** 25 $g.L^{-1}$, Difco, 242810.

## 2.9 Growth and expression of P450s in Yeast Strains using YPGE media for galactose promoter clones

Strains were grown from glycerol stocks and grown on SD-Minimal agar with the required supplements at 30ºC for 3 days.

### Growth of strains, Day 1

A 50 ml flask was inoculated with 10 ml of modified SD-Minimal broth with the required supplements. 83 µl of stock solution was added for each required supplement (adenine 125 µl) and 100 µl of Casamino acids stock solution. The starting OD at 600 nm ($OD_{600}$) was measured and the cells incubated at 30ºC for 16h at 220 rpm.

### Growth of strains, Day 2

$OD_{600}$ was measured. Once the culture reached approximately 6 ODs, it was placed at +4ºC to cool. This culture was then used to inoculate a 100 ml YPGE culture in a 500 ml flask; see Table 2.1 for inoculum volume for $OD_{600}$ ranges.

**Table 2.1.    Inoculum volume for $OD_{600}$ ranges.**

| Ranges of OD at 600 nm | Inoculum volume in ml |
|:---:|:---:|
| 4.5 – 5.5 | 1.5 |
| 6 – 6.7 | 1.2 |
| 7 | 0.8 |
| 7+ | 0.5 |

830 µl of stock solutions were added for each required supplement (adenine being an exception, 1250 µl). The starting $OD_{600}$ was measured and the cells incubated at 30ºC for 16 to 24h at 220 rpm.

## Expression of strains, Day 3

The $OD_{600}$ of the 100 ml culture was measured. Once the culture reached over 5 ODs at 600 nm, it was placed at +4°C to cool.

10 ml of 20% (w/v) filter-sterilised galactose solution was added, for induction. 415 μl of stock solutions of each required supplement was added (adenine 625 μl). The $OD_{600}$ was measured and the cells incubated at 30ºC for 14 to 24h at 220 rpm.

## Harvest of strains, Day 4

$OD_{600}$ was measured for the 100 ml culture. Once the culture reached over 17 ODs at 600 nm, then it was placed at +4°C to cool. The cultures were transferred to centrifuge buckets and centrifuged at 3620 rpm (2828g), in a Sorvall Legend RT, for 12 min at 4°C.

The supernatant was poured away and the pellet was re-suspended gently in 100 ml of Sorbitol buffer A. The process was repeated twice more at the same settings.

The pellet weight was recorded and the pellet stored at –80°C.

**Reagents**

**SD Minimal Agar** (500 ml)

- 3.35 g YNB, Difco, 291940,
- 10 g Glucose Sigma, G5400,
- 7.5 g Agar, Oxoid, LP0013,
- 1 Pellet of Sodium hydroxide as supplied by Fisher, BP359-500.

**Casamino acids**

Stock concentration 1 g.10 ml$^{-1}$ of Casein hydrolysate, N-Z-Case, Sigma, C-7585

**SD Minimal broth** (1 Litre)

- 6.7 g YNB Difco, 291940,
- 40 ml of 50% (w/v) glucose, Sigma, G5400.

Additions

- Adenine Stock concentration 5 mg.ml$^{-1}$ Sigma, A3159,
- Histidine Stock concentration 2.4 mg.ml$^{-1}$ Sigma, H8125,
- Leucine Stock concentration 1.8 mg.ml$^{-1}$ Sigma, L-8000,
- Tryptophan Stock concentration 4.8 mg.ml$^{-1}$ Sigma, T0254, Filter sterilised,
- Uracil Stock concentration 2.4 mg.ml$^{-1}$ Sigma, U1128.

**YPGE broth** (1 Litre)

- 900 ml of ultrapure water
- Yeast Extract 10 g, Oxoid, LP0085,
- Bactopeptone 10 g, Oxoid, LP0021.

The components were autoclaved except where detailed. Components below were

added after autoclaving

- 10 ml 50% (w/v) Glucose solution, Sigma, G5400,
- 30 ml, 100% Ethanol, Sigma, E7023,

Final volume adjusted to 1 Litre with sterile ultrapure water.

**Galactose**

10 ml of 20% (w/v) Galactose, 20 g.100 ml$^{-1}$ and filter sterilised, Fisher, 150610010.

**Sorbitol buffer A**

- 162.5 ml of 4 M Sorbitol solution, Melford, MB1015,

- 10 ml of 1M Tris pH 7.5, Fisher, BPE152-1,

- 200 μl of 0.5 M EDTA pH 8, Gibco 1570-6-021,

- 827.3 ml of sterile ultrapure water.

## 2.10 Growth and expression of P450s from Yeast Strains, using YP media for *ADH2* promoter clones

Strains were grown from glycerol stocks on SD-Minimal agar with the required supplements (see Section 2.8) at 30ºC for 3 days.

### Growth of strains, Day 1

A 50 ml flask was inoculated with 10 ml of modified SD-Minimal broth with the required supplements. 83 μl of stock solution was added (adenine 125 μl) and 100 μl of Casamino acids stock solution was added. The start $OD_{600}$ was measured and the cells incubated at 30ºC for 16h at 220 rpm.

### Growth of strains, Day 2

$OD_{600}$ was measured for the 10 ml culture. Once the culture reached approximately 5 to 10 ODs, it was used for inoculating a 100 ml YP culture in a 500 ml flask; see Table 2.2, for inoculum volume of $OD_{600}$ ranges.

**Table 2.2    Inoculum volume for $OD_{600}$ ranges.**

| Ranges of OD at 600 nm | Inoculum volume in ml |
|:---:|:---:|
| 4.5 – 5.5 | 1.5 |
| 6 – 6.7 | 1.2 |
| 7 | 0.8 |
| 7 to 10 | 0.5 |

830 µl of stock solutions were added (adenine 1250 µl). The starting $OD_{600}$ was measured and the cells incubated at 30ºC for 16 to 24h at 220 rpm.

## Harvest of strains, Day 3

$OD_{600}$ was measured for the 100 ml culture. Once the culture reached over 12-21 $OD_{600}$, it was cooled, and harvested. The cultures were centrifuged at 3620 rpm (2828g), in the Sorvall Legend RT, for 12 min at 4ºC.

The supernatant was poured away and the pellet re-suspended gently in 100 ml of Sorbitol buffer A. The process was repeated twice more, at the same settings.

The pellet weight was recorded and the pellet stored at –80ºC.

**Reagents**

See Section 2.9.

## 2.11 Lyticase treatments

The cell pellets from expressions were removed from -80°C storage. Depending on size, pellets were kept on ice for 10 min followed by 10 min at room temperature.

Sorbitol buffer B, at room temperature, was added (2.5 ml per 100 ml culture) to the cell pellet. The sorbitol buffer B contained the protease inhibitor 1 mM AEBSF, and 0.1 mM DTT.

The pellet was gently re-suspended and transferred to a 50 ml conical flask and incubated at 30°C in an orbital-shaking incubator at 90 rpm.

Spheroplasts were produced by adding lyticase. The amount of lyticase (ICN Biomedicals, 152270) added was calculated as below,

(Final culture OD 600 nm X Total culture volume in ml X 75) / 2000 = mg of lyticase added to the 50 ml flask of re-suspended cells.

The $OD_{600}$ was observed by removal of a few µl to track the lysis process as a decrease in $OD_{600}$. Then the spheroplasts were poured into pre-chilled 50 ml centrifuge tubes and 1 ml sorbitol buffer B was used to wash out the remains from the flask. The spheroplasts were pelleted, in the Sorvall Legend RT, at 3500 rpm (2643g) for 10 min at +4°C.

The following steps were performed in a cold room on ice. The pellet from 400 ml of original culture was re-suspended in 5 ml of sorbitol buffer A which contained 1 mM AEBSF and 0.1 mM of DTT. The pellet was gently re-suspended using a glass rod and pipetted up and down with a 10 ml pipette.

The spheroplasts were sonicated for 10 seconds; this was done eight times with 3 min gaps, in-between using the sonicator (UP50H, Dr Hiescher); settings cycle 1 at 30% amplitude.

The cells were centrifuged again at 3500 rpm (2643g) for 10 min at +4°C. The supernatant was transferred to Beckman JA17 tubes and centrifuged at 10,000 rpm (13,776g) for 10 min at +4°C. This was repeated with fresh tubes, 3 times.

The supernatant was ultracentrifuged in the type 70 Ti rotor at 45,000 (208,429g) for 90 min at +4°C. Finally, the microsomal pellet surface was washed twice using 500 µl of buffer C. The pellet was removed from the tube with micro-spatulas and placed into a glass homogeniser with a PTFE (Teflon) pestle. The microsomes were homogenised in as small as possible volume of Buffer C, aliquoted and stored at -80°C.

## 2.12  Bradford assay of the total microsomal protein content

The supplied microplate protocol was used. The BioRad Bradford Assay Dye reagent concentrate (Cat No 500-0006) and the Biotek Synergy HT plate reader with standard flat bottomed, clear 96 well plates (Appleton Woods, 9017) were used for determining protein concentrations.

**BSA Stock**

A bovine serum albumin (BSA) stock solution (Bio-Rad Protein Assay Standard II, 500-0007) was diluted in water and the solution was aliquoted and stored at –21°C. The assay measured end-point absorbance at 595 nm.

## 2.13 CO-difference binding assay

Absorbance was measured in microsomal samples between 400-500 nm to calculate the P450 content. This was performed using the Shimadzu UV2401PC dual-beam spectrophotometer relative to a base-line of the microsomal sample before CO was added and with reference to a cuvette containing only buffer.

900 μl of 100 mM phosphate buffer pH 7.4 with 20% (v/v) glycerol was added to two cuvettes. 5 mg of sodium hydrosulphite (i.e. sodium bisulphite; Sigma, S-1256) was added and the sample was mixed gently by inversion and left for 1 min. 1.5 mg of microsomal protein was added and gently mixed by inversion and held for 1 min. The volume should be adjusted with buffer to a 1 ml final volume.

Samples were set to zero, followed by reading of the spectral baseline.

A 50 litre CO lecture bottle (Sigma, 29,5116) was used with a fine control two-stage BOC regulator ending in a glass Pasteur pipette to deliver CO. The cuvette's solution was bubbled with CO at the base of the cuvette, at the rate of 1 bubble per second for 60 seconds. The pipette was removed and the sample held for a 1 min. The sample was set to zero followed by the spectral reading.

**Calculations**

- The extinction coefficient at 450 nm for these samples was 91 $mM^{-1}.cm^{-1}$.
- The extinction coefficient at 420 nm (misfolded P450) = 110 $mM^{-1}.cm^{-1}$.
- ((Absorbance at 450 - absorbance at 490) / 91) * 1000 = P450 content in $nM.ml^{-1}$ in the diluted sample.
- (P450 $nM.ml^{-1}$ * dilution factor)/ total protein = Specific Content in $nM.mg^{-1}$
- Dilution factor was total volume / volume of microsomes used for assay.

**Reagents**

Potassium phosphate buffer

- A solution of 1 M (174.18 g.L$^{-1}$) $K_2HPO_4$ (Anhydrous, Dibasic) was produced, Sigma, P-8281.

- A solution of 1 M (136.06 g.L$^{-1}$) $KH_2PO_4$ (Anhydrous, Monobasic) was produced, Sigma, P-5379.

- 100 mM Potassium phosphate buffer at pH 7.4 with 20% (v/v) glycerol.

- 80.2 ml of 1 M $K_2HPO_4$ (Dibasic) and 19.8 ml of 1 M $KH_2PO_4$ (Monobasic) were mixed together and 400 ml of 50% (v/v) glycerol and was then topped up to 1 litre with ultrapure water.

50% (v/v) glycerol, Fisher, Cat No. G/0650/17; autoclaved and stored at room temperature.

## 2.14 P450 fluorescent activity assays

P450 activity was measured by conversion of a substrate into a product and that the product was fluorescent with a light intensity directly proportional to the concentration of the enzyme. These assays were performed in 96-well plates and analysed using the Biotek Synergy HT plate reader. Substrates were presumed to be light-sensitive, hence covered with foil and kept at -20ºC.

### Coumarin (Sigma, Fluka, 28150)

3 mM stock solution in water, diluted to 50 µM solution in water on the day of use.

### 7-Ethoxy-3-cyanocoumarin (CEC, Sigma, C2612)

10 mM stock solution in pure acetonitrile (Fisher, A/0638/17), diluted to 1.23 mM in 1% (v/v) acetonitrile on the day of use.

### 7-Ethoxyresorufin (7-ER, Sigma, E3763)

1 mM stock solution in pure DMSO (Sigma, D5879), diluted to 0.1 mM in 1% (v/v) DMSO on the day of use.

**Dibenzylfluorescien (DBF, Sigma D7191)**

2 mM stock solution in pure acetonitrile, diluted to a 0.1 mM working solution in 1%

(v/v) acetonitrile on the day of use.

**3-[2-(N,N-Diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC, BD Gentest, 451705)**

10 mM stock solution in pure acetonitrile, diluted to 25 µM working solution in 1%

(v/v) acetonitrile on the day of use.

**Vivid EOMCC (Invitrogen, P3024)**

2 µM stock solution in pure acetonitrile, used directly.

**Vivid DBOMF (Invitrogen, P2974)**

2 µM stock solution in pure acetonitrile, used directly.

**Regenerating System**

The regenerating system was stored at –20ºC. The system was stored as two

components, solutions A and B, to aid stability.

**Regenerating System-Solution A**

65.42 mM Magnesium Chloride solution containing 26.13 mM $NADP^+$ and 65.77 mM

of Glucose-6-Phosphate.

**Regenerating System-Solution B**

5 mM Tribasic sodium citrate solution contain 40 Units per ml of glucose-6-phosphate

dehydrogenase was then added.

**Alternative Solution A (A1) for CYP2A6 and CYP2D6 with AMMC**

65.42 mM magnesium chloride solution containing 1.31 mM $NADP^+$ and 65.77 mM of

glucose-6-phosphate.

**Reaction conditions and dilution of the P450s**

The P450s were diluted in solutions described in Table 2.3, parts A and B. P450s were

diluted to give the correct amount of P450 in pmol in a 10 µl volume per reaction well.

After this, 90 µl of reaction mix was added.

**Table 2.3A.   Reaction conditions for each P450, part A.**

| Enzyme | Substrate | Product | Excitation (Bandwidth) | Emission (Bandwidth) |
|--------|-----------|---------|------------------------|----------------------|
| CYP1A1 | 7-ER | Resorufin | 530 nm (25 nm) | 590 nm (20 nm) |
| CYP1A2 | CEC | CHC | 409 nm (20 nm) | 460 nm (40 nm) |
| CYP1B1 | 7-ER | Resorufin | 530 nm (25 nm) | 590 nm (20 nm) |
| CYP2A6 | Coumarin | 7-HC | 400 nm (20 nm) | 460 nm (40 nm) |
| CYP3A4 | DBOMF | Green Standard | 485 nm (20 nm) | 530 nm (25 nm) |
| CYP2C8 | DBF | Fluorescein | 485 nm (20 nm) | 538 nm (25 nm) |
| CYP2D6 | EOMCC | Blue Standard | 400 nm (20 nm) | 460 nm (40 nm) |
| CYP2D6 | AMMC | AHMC | 400 nm (20 nm) | 460 nm (40 nm) |
| CYP2E1 | EOMCC | Blue Standard | 400 nm (20 nm) | 460 nm (40 nm) |

**Table 2.3B.   Reaction conditions for each P450 per well, part B.**

| [Final Substrate] | [CYP per reaction] | [NADP$^+$] | Potassium Phosphate Buffer, pH 7.4 |
|-------------------|--------------------|-----------|------------------------------------|
| 5 µM | 1.5 pmol | 1.3 mM | 100 mM |
| 16 µM | 1.0 pmol | 1.3 mM | 100 mM |
| 5 µM | 1.5 pmol | 1.3 mM | 100 mM |
| 3 µM | 1.0 pmol | 0.065 mM | 100 mM, Tris pH 7.5 |
| 2 µM | 1.0 pmol | 1.3 mM | 100 mM |
| 1 µM | 4.0 pmol | 1.3 mM | 50 mM |
| 10 µM | 2.5 pmol | 1.3 mM | 100 mM |
| 1.5 µM | 2.5 pmol | 0.065 mM | 100 mM |
| 10 µM | 2.5 pmol | 1.3 mM | 200 mM |

Glucose-6-phosphate 3.3 mM, G-6-P dehydrogenase 0.04 Units and MgCl$_2$ 3.3 mM were used throughout as part of the regenerating system.

The reactions were rapidly mixed and the kinetic reaction followed.

## 2.17 Detection of proteins using Western blotting

This method used a vertical one-dimensional Bio-Rad Mini Trans Blot Gel System with the Bio-Rad Mini-Protean 3 system and an acrylamide gel to separate proteins relative to their molecular weights. This was followed by semi-dry blotting. After blotting, membranes were probed with antibodies as required.

The secondary antibodies were HRP-conjugates and were used with luminol detection in conjunction with the Bio-Rad XRS Chemidoc system.

Samples were prepared as 1.5 - 3 μg of total microsomal protein per well.

- Microsomes were thawed on ice.

- Samples containing 100 μg of microsomes were prepared,

- Water was added to make up to 400 μl after addition of the volume of microsomes which was variable.

- 6x SDS Buffer 66.7 μl

- Total Volume 400 μl

Samples heated to 99°C in a thermomixer for 5 min.

6 µl or 12 µl of samples contained 1.5 or 3 µg of total cellular protein, respectively.

10 µl of protein markers (ladders) with major bands at 75 and 50 KDa (Bio-Rad, 161-0373) were used. Negative control microsomes (in-house clone YW41, containing no P450 gene), and relevant positive controls from BD Gentest were used.

**Casting gel**

Resolving gels of 1.5 mm thickness were cast using water-saturated butanol solution(Sigma, B-1417) applied on top.

Resolving gel composition for 2 gels

- 17.8 ml of ultrapure water,

- 11.25 ml of 1.5 M Tris, pH 8.8, Fisher, BPE152-1,

- 15.1 ml of 30% Acrylamide/bis solution 29:1 Bio-Rad, 161-0156.

- 450 µl of 10% (w/v) SDS, Gibco, 15711-021

- 225 µl of 10% (w/v) Ammonium persulphate (APS), Sigma, A3678.

- 22.5 µl of TEMED, Gibco, 15713-019.

Once set, a stacking gel was applied on top, and comb added.

**Stacking gel**

- 7.5 ml of ultrapure water,

- 3.15 ml of 0.5 M Tris, pH 6.8,

- 1.875 ml of 30% Acrylamide/bis solution 29:1,

- 125 µl of 10% (w/v) SDS,

- 51 µl of 10% (w/v) APS,

- 13.5 µl of TEMED.

Samples were run at 90 Volts through the stacking gel and 110 Volts through the resolving gel for 120 min

Gels were removed from the tank system and cut to a relevant size.

A semi-dry blotter (Phase) was used for protein transfer. Three large rectangles of filter paper (Whatman, 3030 335; 16.4 cm by 11 cm) were soaked in cathode buffer until saturated and placed upon the metal base plate. The gel (8.2 cm by 5.5 cm) was then placed on top and smoothed out after soaking in cathode buffer.

A gel-sized Immobilon-P, PVDF membrane (Millipore, IPVH20200) was cut and briefly dipped in methanol (Sigma, 154903) and transferred to anode II buffer for 2 min.

The membrane was placed upon the gel and air bubbles removed.

Three gel-sized pieces of filter paper, also saturated in anode buffer II, were applied on top and rolled flat. A further 3 pieces of filter paper of the same size, saturated in anode buffer I, were rolled on top.

The semi-dry blotter was run at 0.8 mAmps per $cm^2$ of each gel for about 75 to 90 min for a 1.5 mm gel thickness (Bio-Rad, Power Pac 200).

After blotting, the membrane was removed with forceps and soaked in a trough containing PBS, 0.2% (v/v) Tween. Then the membrane was placed upon a plate rocker for 10 min at a low speed sufficient to cause gentle agitation. All the following steps were carried out at room temperature and using a plate rocker.

- The PBS, Tween solution was poured off and 10 ml of 10% (w/v) powdered milk was added and agitated for 1h.

- Next the milk was removed, a 15 min wash with PBS 0.2% (v/v) tween was carried out as 3, 5 min washes.

- Following this step, primary antibodies as (1:1000 volume for volume) were applied to the blot as a 25 ml solution of 1% (w/v) milk in PBS 0.2% (v/v) Tween. Antibodies were incubated for 1h at room temperature or 4ºC overnight.

- The blot was washed 3 times with PBS 0.2% (v/v) Tween for 10 min.

- Next the blot was incubated in 25 ml of a secondary antibody solution made as above except at 1:2000 antibody dilution for 1h.

- Then the blot was washed in twice in PBS 0.2% (v/v) Tween for 5 min then again for 5 min in PBS with 0.01% (v/v) Tween, again in PBS for 5 min and finally very briefly washed in ultrapure water.

- Next an ECL kit (Santa Cruz, sc-2048) was used mixing 2.5 ml of solution A and B together. This was added to the membrane carefully and wrapped in cling film.

- Bands were detected by the Bio-Rad XRS Chemidoc using the chemiluminescence settings.

**Reagents**

**6x Sample buffer, 25 ml**

- 17.5 ml of 0.5 M Tris, pH 6.8, Fisher, BPE152-1,

- 7.5 ml of Glycerol, Fisher, G/0650/17,

- 2.5 g of SDS, Gibco, 15711-021,

- 2.3 g of DTT, Melford, MB1015,

- 3 mg of Bromophenol blue, Sigma, 114391.

**5x Running buffer**

- 75.5 g Tris, Fisher, BPE152-1,

- 360 g Glycine, Gibco, 15709-017,

- 25 g SDS, Gibco, 15711-021,

- Ultrapure water up to 5 litres.

**Anode Buffer I per litre, pH 10.4**

- 36.3 g Tris, Fisher, BPE152-1,

- 100 ml Methanol, Sigma, 154903,

- Ultrapure water up to 1 litre.

**Anode Buffer II per litre, pH 10.4**

- 3.03 g Tris, Fisher, BPE152-1,

- 100 ml Methanol, Sigma, 154903,

- Ultrapure water up to 1 litre.

**Cathode Buffer per litre, pH 9.4**

- 5.2 g of aminocaproic acid, Sigma, A2504,

- 3.03 g Tris base, Fisher, BPE152-1,

- 100 ml Methanol, Sigma, 154903,

- Ultrapure water up to 1 litre.

**Blocking Solution 5%**

- 2.5 g Dried milk powder (Bio-Rad, 170-6404) in 50 ml of PBS or PBS Tween

**PBS**

- 1 Tablet added to 200 ml ultrapure water, Sigma, P4417-100TAB.

**PBS Tween 20 0.2%**

2 ml of tween in 1 litre of PBS, Sigma, P2287.

For details of the antibodies used, see Table 2.4, Antibodies used for Western blot.

## Table 2.4.    Antibodies used for Western blot.

| P450 | Primary Antibody | Raised against | Raised in | Secondary Antibody | Raised in | Positive control |
|------|------------------|----------------|-----------|---------------------|-----------|------------------|
| CYP 1A2 | Gentest 1A1, 458124 | Rat CYP1A1 | Goat | Santa Cruz, sc2304 | Donkey | Gentest 1A2, 456203 |
| CYP 2D6 | Gentest 2D6, 458246 | Human CYP2D6 | Mouse | Santa Cruz, sc2302 | Goat | Gentest 2D6, 456217 |
| CYP 2E1 | Gentest 2E1, 458216 | Rat CYP2E1 | Goat | Santa Cruz, sc2302 | Goat | Gentest 2E1, 456206 |
| CYP 2A6 | Gentest 2A6, 458106 | Human CYP2A6 | Mouse | Santa Cruz, sc2302 | Goat | Gentest 2A6, 456254 |
| CYP 2C8 | Abcam 2C8, AB22596 | Human Synthetic Peptide | Rabbit | Santa Cruz, sc2301 | Goat | Gentest 2C8, 456252 |
| CYP 3A4 | Gentest 3A, 458254 | HLM fraction | Mouse | Santa Cruz, sc2302 | Goat | Gentest 3A4, 456202 |

## 2.18  Growth of Yeast Fermentation Seed Cultures

Strains were grown from glycerol stocks to agar to 10 ml cultures to 100 ml seed cultures.

### Growth of Strains from Glycerol Stocks

Strains were streaked out from glycerol stocks and grown on SD-Minimal agar with the required supplements at 30ºC for 3 days.

### Growth of Strains

A 50 ml flask was inoculated with 10 ml of yeast seed SD-Minimal broth and required supplements at 83 μl of stock solutions (adenine 125 μl) per flask. Also 100 μl of 10% (w/v) Casamino acids stock solution was added.

Starting $OD_{600}$ was measured. OD measurements were performed using the WPA Biowave CO800 Cell Density Meter and standard disposable plastic microcuvettes (Fisher, FB55147).

Cells were incubated at 30ºC for around 14 to 16h at 220 rpm, Sanyo Orbisafe TS.

### Growth of Strains (Time, 24h)

The $OD_{600}$ of the 10 ml culture was measured. Once the culture reached 6 to 8 ODs, it was placed at +4ºC to cool. This culture was then used to inoculate a 100 ml yeast seed broth in a 500 ml flask. This was made in the same way as above; see Table 2.5 for inoculum volume.

**Table 2.5.    Inoculum volume for OD at 600 nm ranges.**

| Ranges of OD at 600 nm | Inoculum volume in ml |
| --- | --- |
| 4.5 – 5.5 | 1.5 |
| 6 – 6.7 | 1.2 |
| 7 | 0.8 |

830 μl of stock solutions was added for each required supplement (adenine 1250 μl) to each flask. Also 1 ml of 10% (w/v) Casamino acids was added. Starting $OD_{600}$ was measured. Cells were incubated at 30ºC for approximately 16h at 220 rpm.

## Growth of Strains (Time, 48h)

The OD at 600 nm was measured from the 100 ml culture. Once the culture reached approximately 6 to 8 ODs at 600 nm, it was placed at +4ºC to cool. This culture was sufficient to inoculate a 10 litre fermentor.

**Reagents, Media and Chemicals**

**SD-Min Agar (500 ml)**

- 3.35 g YNB, Difco, 291940,
- 10 g of D-(+)-Glucose, Sigma, G5400,
- 7.5 g of Agar, Oxoid, LP0013,
- 1 pellet of Sodium Hydroxide as supplied by Fisher, BP359-500.

Autoclaved under standard conditions, 121ºC for 20 min.

**SD Min broth (1 Litre)**

- 6.7 g of YNB, Difco, 291940,
- 40 ml of a pre-autoclaved 50% (w/v) Glucose solution using the above glucose.

Autoclaved under standard conditions, 121ºC for 20 min.

**Yeast Seed broth (also known as SW6 media)**

Yeast seed broth was transferred from SD-Minimal broth by the addition of Casamino acids stock, as below at 10 $\mu l.ml^{-1}$. Stock concentration was 1 $g.10ml^{-1}$ in ultrapure water, filter sterilised with a 0.2 μm filter and stored at +4ºC (Sartorius Minisart Cat No. 16534, Appleton Woods).

**Casamino acids stock**

Casein Hydrolysate, N-Z-Case, Sigma, Cat No. C-7585.

**Auxotrophic supplements**

- Adenine stock concentration 2.5 g.500ml$^{-1}$, Sigma, A3159,

- L-Histidine stock concentration 1.2 g.500ml$^{-1}$, Sigma, H-8125,

- L-Leucine stock concentration 3.6 g.500ml$^{-1}$, Sigma, L-8000,

- L-Tryptophan stock concentration 2.4 g.500ml$^{-1}$, T0254,

- Uracil stock concentration. U-1128. 1.2 g.500ml$^{-1}$, U1128.


## 2.19 Fermentor Protocol for the Growth of a *GAL1* Promoter Driven Expression in Yeast

This method used the New Brunswick Scientific Bioflow 110, 10 litre and 1 litre water-jacketed vessels with four feed pumps, temperature probe, oxygen probe, pH probe, antifoam probe, gas on/off controller and gas flow regulator. Turbidity probes were also added (Solvias, OFS-125-297HA). Compressed air was from a Jun-Air laboratory compressor and chilled water for the vessel jackets and condensers from a Grant, RC1400G chiller.

### Preparation of 10 litre or 1 litre Vessel for Autoclaving

All parts were well cleaned with ultrapure water to remove cleaning detergents.

Media was weighed dry into the fermentor and topped up with ultrapure water. 600 ml was used for 1 litre fermentation and 6 litres for 10 litre fermentation.

Before autoclaving pH probes were calibrated using standard pH 7.0 and pH 4.0 calibration solutions by connecting to the fermentor controller system.

Probes were autoclaved *in situ*, filters (Corning, 0.2 µm, 50 mm diameter, Appleton Woods, 431227) were protected with foil. A port was left loose for venting pressure.

A validated autoclave cycle was found to ensure 121°C for 20 min, the run times for a 150 litre top-loader (Priorclave) autoclave was found to be 121°C for 115 min for the 10 litre vessel. 121°C for 60 min was sufficient for the 1 litre.

**Preparing the fermentor to start batch fermentation (Time, 24h)**

The coolant system was set to run at 12°C and the vessel temperature was set to 30°C two hours prior to starting the fermentor run. The filtered compressed air, parallel supply, was sparged at 5 litres per min for the 10 litre fermentor and 2.5 litres per min for the 1 litre fermentor.

One hour prior to the start time 40 ml of a 50% (w/v) glucose solution was added (400 ml for 10 litre fermentation).

Auxotrophic supplements were added in the following amounts.

- 15 ml of double strength adenine. (150 ml for 10 litre fermentation),
- 16.9 ml of histidine, leucine, tryptophan or uracil. (169 ml for 10 litre fermentation).

100 µl of antifoam (Sigma antifoam 204, A8311), 1 ml for 10 litre fermentation was finally added.

The pH was adjusted to 6.6 by adding a 5 M NaOH solution with agitation at 500 rpm. Dissolved oxygen (DO) was adjusted after addition of antifoam. To calibrate the DO probe, the air flow was set to 10 litres per min and the impeller speed set to 500 rpm and this was set as 100%.

To start the run, the airflow was set to either 5 litres per min for the 10 litre fermentor (2.5 litres per min for the 1 litre) and the agitation maintained at 500 rpm.

When the experiment was ready to start, between 80 ml-120 ml (10 litre fermentor) of yeast seed broth culture was added.

### Automatic Nutrient Feed, (Time, 48h)

After 12h of fermentation the program delivered (at 10 ml per min) the following feed.

For the 10 litre fermentor this comprised of

- 300 ml of 50% (w/v) Glucose, Sigma, G5400,
- 300 ml of ethanol, BP grade, Fisher, E/0650DF/25,
- 150 ml of double strength Adenine,
- 169 ml of Histidine, Leucine, Tryptophan or Uracil.

### Induction (Time, 48h)

After 11h of further fermentation, 300 g of galactose (Fisher, 150610010) was added as a thick suspension with one of the other liquid additions. The galactose was added slowly to avoid osmotic shock. For the 10 litre fermentor this comprised of,

- 150 ml of double strength adenine,
- 169 ml of histidine, leucine, tryptophan or uracil,
- 200 ml of absolute ethanol,
- 1 ml of antifoam.

### Harvest (Time, 72h)

After 36h, the fermentation was stopped.

The system was set to +4°C and agitation reduced to 50 rpm. Air flow was reduced to one litre per min. The culture was pumped into cold buckets and centrifuged at 4500 rpm, (4495g) for 20 min at +4°C, using the Sorvall Legend RT.

The cell pellets were washed three times with sorbitol buffer A (see section 2.9). Each time the pellet was gently re-suspended. Pellets were weighed and were stored dry for short periods at –80°C.

**Fermentor Media Components and Chemicals**

- YP Media,

- 200 g of Yeast Extract, Oxoid, LP0085,

- 200 g of Peptone, Oxoid, LP0021.

**Additions**

- D-(+)-Glucose min 99% GC 50% (w/v) solution, Sigma, G5400,

- Ethanol, Sigma, E7023,

- D-(+)-Galactose 99+% Fisher, 150610010,

- 5 g.500ml$^{-1}$ (1 x) Adenine, Sigma, A3159,

- 1.2 g.500ml$^{-1}$ L-Histidine, Sigma, H-8125,

- 3.6 g.500ml$^{-1}$ L-Leucine, Sigma, L-8000,

- 2.4 g.500ml$^{-1}$L-Tryptophan, Sigma, T0254,

- 1.2 g.500ml$^{-1}$ Uracil Sigma, U-1128,

- 5 M Sodium Hydroxide solution, Fisher, BP359-500,

- Antifoam 204, Sigma, A8311.

## 2.20 Microsome Preparation from 10 Litre Fermentation Cultures Using the Constant Systems Cell Disruptor

Pellets were removed from –80°C and kept on ice for 30 min, then at room temperature for 30 min. Sorbitol buffer A at +4ºC (see section 2.9) was added on top of the pellet in each bucket at the ratio of 1 ml per g of wet weight of pellet. Next the buckets were placed on a slow rocker until the pellets were fully defrosted.

After stirring, the pellet was re-suspended gently with a pipette. Buffer A also contained 0.275 mM DTT and 2.75 mM of AEBSF to stabilise the P450s and prevent protease activity.

The cells were disrupted using the Constant Systems Basic Z model with a continuous jacketed processing head connected to a chiller at +4°C.

A chilled bucket was placed at the machine outlet to collect the lysate. The pressure was set to 20 KPSI. The lysate was then centrifuged at 4600 rpm (4495g) for 20 min at $^+$4°C using the Sorvall Legend RT.

The lysate supernatant was poured into fresh buckets and centrifuged repeatedly (as above) until the remains of unbroken cells were removed.

## Large scale production method

Supernatants were further processed with a High-Speed Beckman Coulter centrifuge Avanti J-20XP, JLA10.5 rotor at 10,000 rpm (18,600g) for 20 min at 4°C. This removed cell debris, nuclei, peroxisomes, lysosomes and mitochondria. This step was repeated into clean-cooled buckets until no significant pellet was produced. The product of this step is known as S9 fractions which were poured into fresh buckets and centrifuged at 10,000 rpm (18,600g), for about 21h at $^+$4°C in the JLA10.5 rotor. This step was to pellet the microsomal fraction of the cell.

The following steps were all performed on ice in a cold room.

- The supernatants containing the soluble cytosolic fraction were removed and the surface of the pellets was washed twice with 10 ml of microsome buffer C.
- The pellets were transferred into a 30 ml glass homogeniser with PTFE pestle (Fisher, FB56769). A minimal amount of microsome Buffer C was added depending on size of pellet to allow initial homogenisation. The volume was

enough to homogenise with no clumping of pellet. The batches of homogenised microsomes were pooled together and were gently pipetted up and down.

- Microsomes were aliquoted into 50 ml tubes and stored at –80°C. A few separate small volumes were kept for assaying.

- After assaying they were thawed on ice and diluted to 1 nmol of P450 per ml and stored in small volumes at -80ºC.

## Reagents

- 4 M (728.68 g.L$^{-1}$) D-Sorbitol, Melford, 50807,

- 1 M (121.14 g.L$^{-1}$) Tris, pH 7.5 Fisher, BPE152-1,

- 0.5 M (186.12 g.L$^{-1}$) EDTA, pH 8, Gibco, 15706-021,

- 50% (v/v), Glycerol, Fisher, G/0650/17.

## Sorbitol buffer A

- 162.5 ml of 4 M Sorbitol final concentration 0.65 M, Melford, MB1015,

- 10 ml of 1 M Tris pH 7.5, final concentration 10 mM. Fisher, BPE152-1,

- 200 µl of 0.5 M EDTA pH 8.0, final concentration 0.1 mM, Gibco, 1570-6-021,

- Final volume was to 1 litre with ultrapure water.

## Buffer C

- 1 ml of 1M Tris, pH 7.5 final concentration 10 mM, Fisher, BPE152-1,

- 200 µl (0.5 M EDTA pH 8.0) final concentration 1.0 mM, Gibco, 1570-6-021,

- 40 ml of 50% (v/v) Glycerol final concentration 20% Fisher, G/0650/17,

- Topped up to 100 ml with ultrapure water.

## 100 mM AEBSF (4-(2-Aminoethyl)-benzenesulfonyl fluoride, HCl) Protease Inhibitor Working Solution

- 239.7 mg of AEBSF in 10 ml of ultrapure water. Melford, MB2003.

## DL-Dithiothreitol (DTT) Working Solution (100 mM)

- 154.2 mg in 10 ml of ultrapure water. Melford, MB1015.

## 2.21 PEG and Calcium chloride precipitation

### PEG precipitation

The samples were processed to the S9 fraction (after high speed centrifugation, see section 2.11). The volume of S9 fraction in ml was multiplied by 3.75. This value gave the amount of cold TES buffer to add in ml. Next, the volume of cold 5 M sodium chloride solution was calculated in ml by dividing of the TES volume required by 40.

After this, the volume of ice cold PEG was calculated in ml by multiplication of the volume of 5 M NaCl required by 10 for the amount of ice cold PEG 3350 50% (w/v) to be added. This was added drop-wise, left for 20 min, and centrifuged in the Beckman JA17 rotor, 9,400 rpm (12,000g) at +4 for 10 min; microsomal pellets were produced and processed using glass homogenisers.

### Calcium chloride precipitation

The samples were processed to the S9 fraction. The volume of S9 fraction in was used to calculate the amount of calcium chloride to add to the S9 fraction to the volume so that the final concentration was 18 mM $CaCl_2$. The samples were kept at +4°C for 30 min. The microsomes were then centrifuged in the Beckman JA17 rotor, 10,800 rpm (16,000g) for 15 min and microsomal pellets were produced and processed using glass homogenisers.

**Reagents**

- 10 ml of 1 M Tris pH 7.5, BPE152-1,
- 400 µl of 0.5 M EDTA, pH8.0, Gibco, 1570-6-012,
- 30 ml of  4 M Sorbitol, Melford, MB1015,
- Topped up to 200 ml with ultrapure water,

- 50% (w/v) PEG 3350 made up in ultrapure water, Sigma, P4338,

- 5 M Sodium chloride in ultrapure water, Sigma, S-3014,

- 2 M Calcium chloride in ultrapure water, Sigma, C1016.

## 2.22 LC-MS procedure for detection of CYP2D6 dextromethorphan metabolism using lyophilised samples

Dextromethorphan hydrobromide (MW 370.3), (Sigma, D9684-5g) is converted by CYP2D6 to dextrorphan (MW 356.3) by the dextromethorphan O-demethylase reaction mediated by CYP2D6. From this, CYP2D6 activity may be measured.

### Sample preparation

Dextromethorphan hydrobromide substrate was made as a 10 mM stock in pure ethanol. Working stocks were diluted in ultrapure water to 100 µM. 1.25 µl was further diluted in ultrapure water to 10 µl (1/8 dilution) to give a 12.5 µM solution. From this, 1 µl was added to a 100 µl reaction (1/10) making the final concentration 125 nM.

100 µl reactions were mixed in glass tubes, Fisher, FB71810. Volumes per reaction,

- 400 mM Potassium Phosphate, pH 7.6       50 µl,
- Ultrapure water                          25 µl,
- Solution A                               10 µl,
- Solution B                               2 µl,
- 3 pmol of CYP2D6 microsomes              3 µl,
- Substrate                                10 µl.

Tubes were incubated for 10, 20 and 30 min at 30°C in a thermomixer at 200 rpm.

Solutions A and B were identical to the ones described in the P450 fluorescent activity assays; see section 2.14.

Reactions were stopped by adding 100 µl of 0.05% (w/v) formic acid in acetonitrile and kept on ice for 10 min.

Tubes were centrifuged in an Eppendorf 5702R at 17°C, 4400 rpm (3000g) for 15 min.

The supernatant was removed for analysis, pipetted into standard brown HPLC vials with septum lids and placed in the 4°C auto-sampler. 20 µl injection volumes were used.

## 2.23  HPLC-Mass Spectrometry settings

The Agilent HPLC 1100, LC/MSD SL with multimode head machine was used.

### LC-MS settings

- m/z 258 analyte, 272 substrate- fragmentor-240, APCI-ESI,
- Range 100-1000 -fragmentor-240, APCI-ESI,
- Spray chamber dry gas flow rate 12 L.min$^{-1}$,
- Capillary voltage 5400,
- Nebuliser pressure 50 PSI,
- Corona current 1 Amp,
- Dry gas temperature 300°C,
- Charge voltage 1000,
- Vaporiser temperature 200°C.

The HPLC column was an Agilent eclipse XDB Zorbax C18 (993967-902) kept at 30°C.

Two solvents were used as the flow rate of 1 ml.min$^{-1}$ with a maximum pressure of 400 PSI. Table 2.9 shows solvent gradient during a typical HPLC/MS run,

- A 0.05% (w/v) formic acid (Fluka, 94318) in ultrapure water.

- B 0.05% (w/v) formic acid in acetonitrile (Fisher, A/0638/17).

**Table 2.6.**    **Solvent gradient during HPLC/MS run.**

| Time in min | % Solvent A | % Solvent B |
|---|---|---|
| 0 | 90 | 10 |
| 2 | 90 | 10 |
| 14 | 10 | 90 |
| 17 | 10 | 90 |
| Post 17, Stop and wash through | 90 | 10 |

Typical column retention times were substrate 8.51 min and analyte (i.e. metabolite) 6.71 min.

## 2.24  Method for determination of $IC_{50}$

1 pmol (1µl) of yeast CYP1A2, 2 pmol (2 µl) of yeast CYP2D6 and 1 pmol (1µl) of yeast CYP3A4 in a buffer mix containing 100 mM potassium phosphate buffer and 20% (w/v) sucrose (total volume 10 µl) were aliquoted into wells of microtitre plates then lyophilised. After lyophilisation, plates were kept for 21 days at 21°C. The wells were re-hydrated with 10 µl with ultrapure water.

Yeast (prepared during the course of work conducted for this thesis) and insect cell derived microsomes obtained from Gentest, both of which had been stored at -80°C, were used as controls for testing the activities of the lyophilised microsomes. The plate layouts included 6 inhibitor concentrations to be tested in quadruplicate with further

wells used for the positive control (i.e. wells containing uninhibited P450s plus solvent, which showed 0% inhibition) and a negative control (i.e. wells containing reactions that were stopped before P450 was added, which showed 100% inhibition).

Furafylline (Sigma Q-3625), a published CYP1A2 inhibitor, was used at a final well concentration of between 3125 µM and 0.0305 µM of furafylline and ~0.5% DMSO.

Quinidine (Sigma Q-3625), a published CYP2D6 inhibitor, was used at a final well concentration of between 3.4 µM and 0.00017 µM and ~0.5% DMSO.

Ketoconazole (Sigma K-1003), a known CYP3A4 inhibitor, was used at a final well concentration of between 1.95 µM and 0.061 µM and ~0.5% DMSO.

Inhibitors were pre-incubated with the P450s for 20 min at 21°C in 100 mM potassium phosphate buffer at pH7.4. The reaction was initiated with standard regenerating system components A and B with the substrates: CEC for CYP1A2, EOMCC for CYP2D6 and DBF for CYP3A4. After 10 min at 30°C the reactions were stopped with 20% 0.5M Tris base (pH not altered) with 80% acetonitrile and analysed for end point RFLUs.

## 2.25 Lyophilisation reagents

Below is a list of equipment and reagents used in the process.

- Glass ampoules, SLS, VIA1000,
- Heto Drywinner lyophilisation machine, CT/DW60E,
- BOC-Edwards vacuum pump, RV5,
- Twin jet ampoule sealer, Adelphi manufacturing, 6002100, 6002120 and 6002110,
- Bioteck Synergy HT plate reader with KC4 software version 3.9, revision 12,

- A list of various commercial P450 used throughout the work follows,

- Glycerol, Fisher, G/0650/17,

- Mannose, Sigma, M6020,

- Trehalose, Sigma, T9531,

- Maltose, Sigma, M5885,

- Sucrose, Sigma S0389,

- Galactose, Sigma, G6404,

- Raffinose, Sigma, R-0514,

- Sorbitol, Melford, MB1015,

- Glucose, Sigma, G5400.

**BD Gentest, Supersomes (Insect cell derived)**

- CYP1A1, 456211,

- CYP1A2, 456203,

- CYP1B1, 456220,

- CYP2A6, 45254,

- CYP2C8, 456252,

- CYP2D6, 456217,

- CYP2E1, 456206,

- CYP3A4, 456202,

- CYP2D6, 455117 Lympoblastoid derived.

**Cypex (Bacterial cell derived)**

- CYP2D6, CYP/EZ007,

- CYP1A2, CYP/EZ001,

- CYP2E1, CYP/EZ036,

- CYP3A4, CYP/EZ005.

**Invitrogen (Insect cell derived)**

- CYP1A2, P2797,

- CYP2E1, P2948,

- CYP2D6, P2283,

- CYP3A4, P2377.

# Chapter 3.    Construction and characterisation of plasmids, optimisation of media components

## 3.1    Creating promoter driven P450 reductase constructs with an auxotrophic marker for chromosomal integration

A galactose-inducible (*GAL1*) promoter fragment, Gal1MS (*GAL1* promoter with contiguous *c*-myc tag and *SUC2* terminator) with *Sac*I and *Xho*I ends was isolated from plasmid pBGAL1MS (Figure 3.1). This 3942 base pair plasmid contained a ColE1 origin of replication and an ampicillin marker. The ~1 Kbp GAL1 promoter-*c*-myc tag-SUC2 terminator fragment was isolated.

**Figure 3.1    Plasmid Map of pBGAL1MS.**
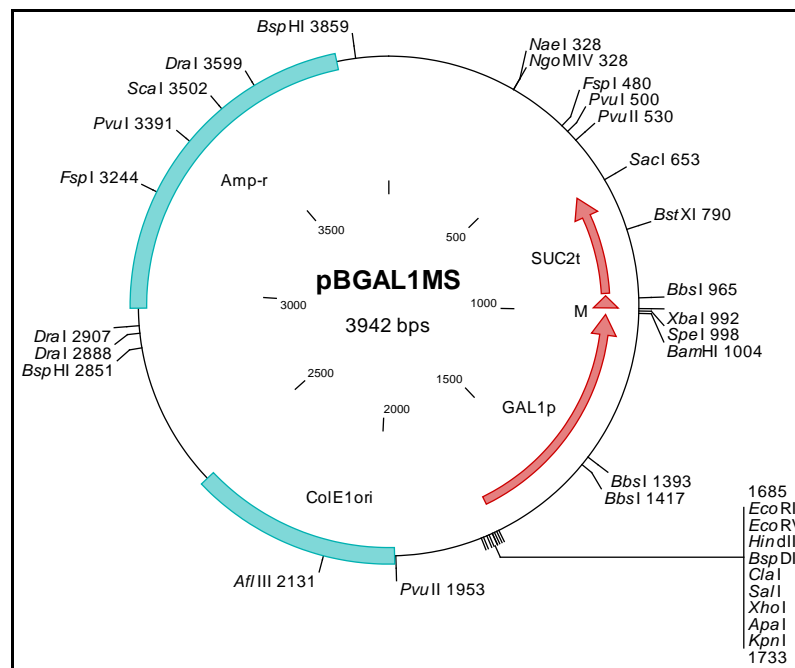


This is a bacterial vector with the β-lactamase gene as ampicillin resistant marker (Amp-r). It has been used to amplify the *GAL1p-SUC2t* cassette for further sub-cloning in yeast integrating vectors so that a P450 reductase gene could be cloned under the control of the galactose-inducible *GAL1* promoter.

pRS403 (ATCC, #87477) is a yeast integrating plasmid of 4456 base pairs with a ColE1 origin of replication, bacterial ampicillin resistance gene and the yeast *HIS3* gene as a yeast selection marker. The plasmid was linearised at the multi-cloning site by digesting with *Sac*I and *Xho*I. A linear fragment of approximately 4.4 Kbp was isolated, Figure 3.2.

**Figure 3.2** *Sac*I and *Xho*I digested pRS403.
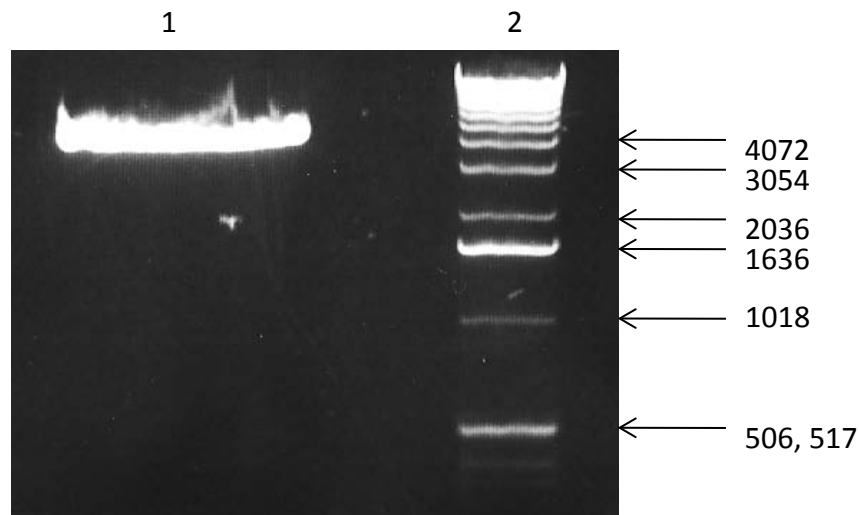


This is a 1% agarose-TAE gel. Lane 1, the pRS403 plasmid (ATCC, 87477) digested with *Sac*I and *Xho*I to allow ligation of the GAL1-SUCt cassette from pBGAL1MS. Lane 2, a DNA ladder with the main DNA markers highlighted in base pairs (bps).

The fragments from pBGAL1MS and the linearised plasmid pRS403 were ligated using T4 DNA ligase. Six different bacterial clones from the transformation were amplified in small bacterial cultures and the DNA obtained was analysed using restriction enzyme digests. The DNA from the clones was digested with *Sac*I and *Xho*I to confirm that they yielded the expected fragments, 4365bp (Vector) and 1069bp (Insert, *GAL1p-SUC2t*), Figure 3.3. All six clones were correct. One correct clone was designated YIHisGAL1MS, the plasmid map for which is shown in Figure 3.4.

**Figure 3.3** *Sac*I-*Xho*I digest of YIHisGAL1MS.
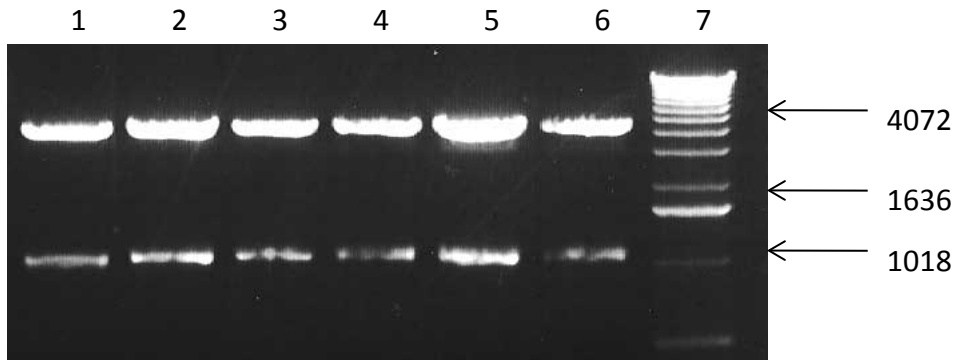


This is a 1% agarose-TAE gel. Lanes 1 to 6, pRS403 vector (upper band) and *GAL1p-SUCt* insert (lower band), that had been isolated from pBGAL1MS. The digest shows correct ligation to form the plasmid, YIHisGAL1MS. Lane 7, main DNA markers shown in base pairs (bps).

**Figure 3.4.** **Plasmid Map of YIHisGAL1MS.**
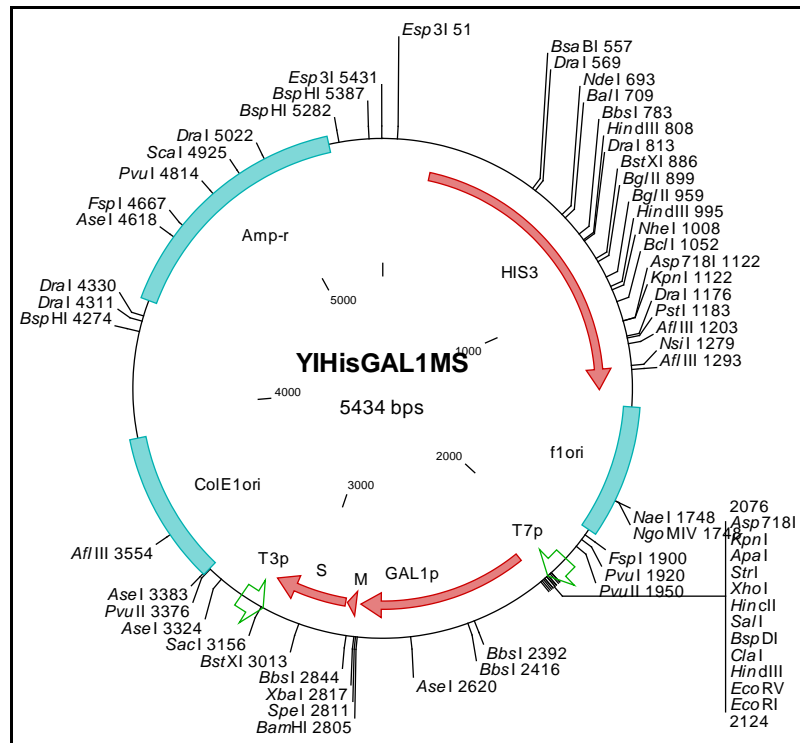


YIHisGAL1MS plasmid is a shuttle vector that can be amplified in bacterial cells and can be used for integration into a particular chromosomal locus (where the *HIS3* gene resides) of the yeast, *S. cerevisiae*. The plasmid was later used to clone P450 reductase genes between the *BamH*I-*Xba*I restriction sites.

Next, a *BamH*I-*Xba*I fragment of the human cytochrome P450 reductase gene (hRD) and a *Bgl*II-*Xba*I yeast reductase (yRD) fragment were cloned into the new YIHisGAL1MS plasmid so that the two reductase genes could be driven by the *GAL1*

promoter. The hRD fragment was isolated from a human liver cDNA library and the yRD fragment was isolated from a yeast genomic library, using the polymerase chain reaction (PCR) and a proof-reading Taq polymerase. YIHisGAL1MS plasmid was cut with (*BamH*I-*Xba*I) and then ligated together with the hRD and yRD fragments (*BamH*I and *Bgl*II restriction sites having compatible overhangs) to create the new integrating plasmids YIHisGAL1MS/hRD (Figure 3.5) and YIHisGAL1MS/yRD (Figure 3.6). The two plasmids were further amplified in *E. coli* so that they could be used for integration in yeast.

**Figure 3.5.   YIHisGAL1MS/hRD.**
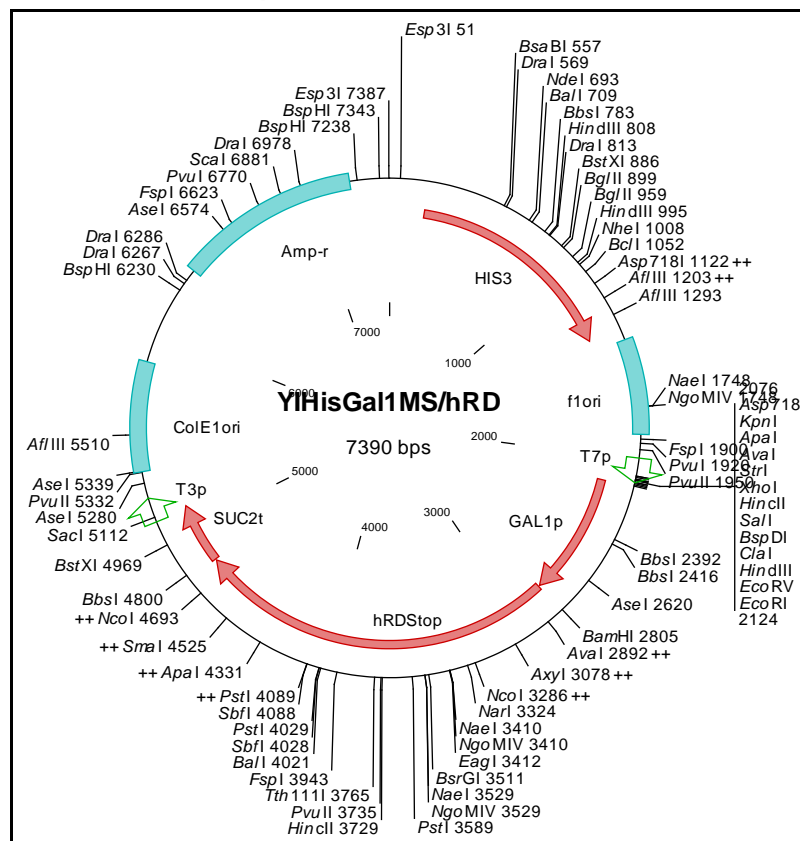


The human reductase gene (hRDStop) was inserted into the YIHisGAL1MS plasmid to form the new plasmid YIHisGAL1M/hRD. This plasmid was used for integration of the hRD expression cassette, driven by the galactose-inducible *GAL1* promoter, into yeast's *HIS3* chromosomal locus.

**Figure 3.6   YIHisGAL1MS/yRD.**
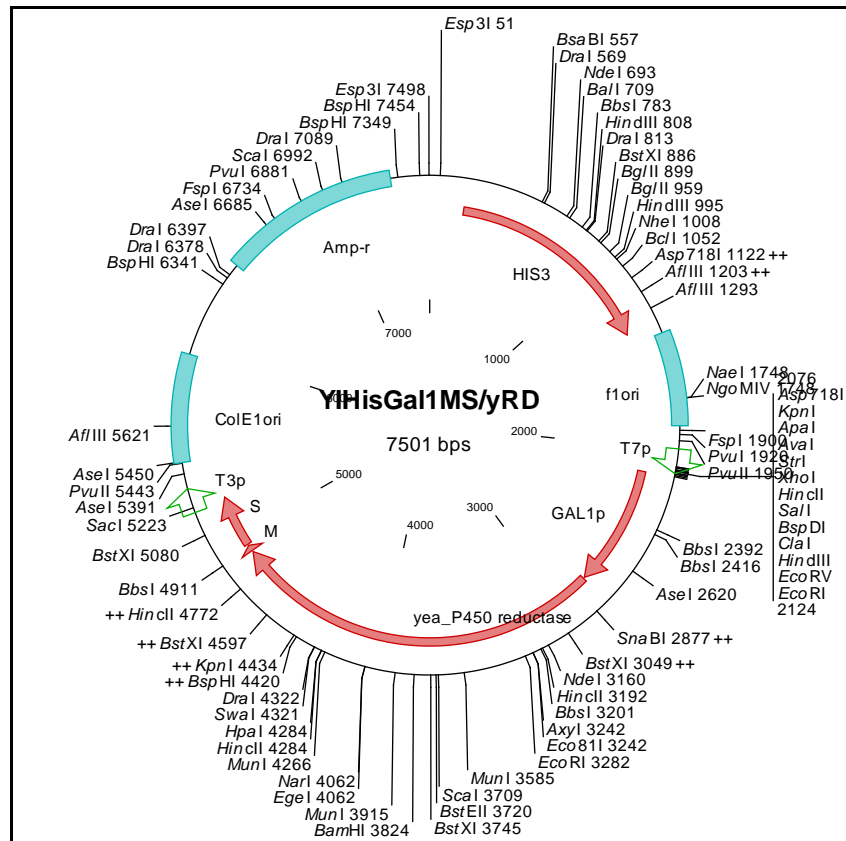


The yeast reductase gene (yea_P450 reductase) was inserted into the YIHisGAL1MS plasmid to form the new plasmid YIHisGAL1M/yRD.  This plasmid was used for integration of the yRD expression cassette, driven by the galactose-inducible *GAL1* promoter, into yeast's *HIS3* chromosomal locus.


A *Sal*I-*Xba*I digest of the YIHisGAL1MS/hRD and YIHisGAL1MS/yRD plasmids

excised the reductase genes from the plasmid and this was confirmed by gel

electrophoresis, Figure 3.7.

**Figure 3.7** *Sal*I-*Xba*I digest of YIHisGAL1MS/hRD and YIHisGAL1MS/yRD.
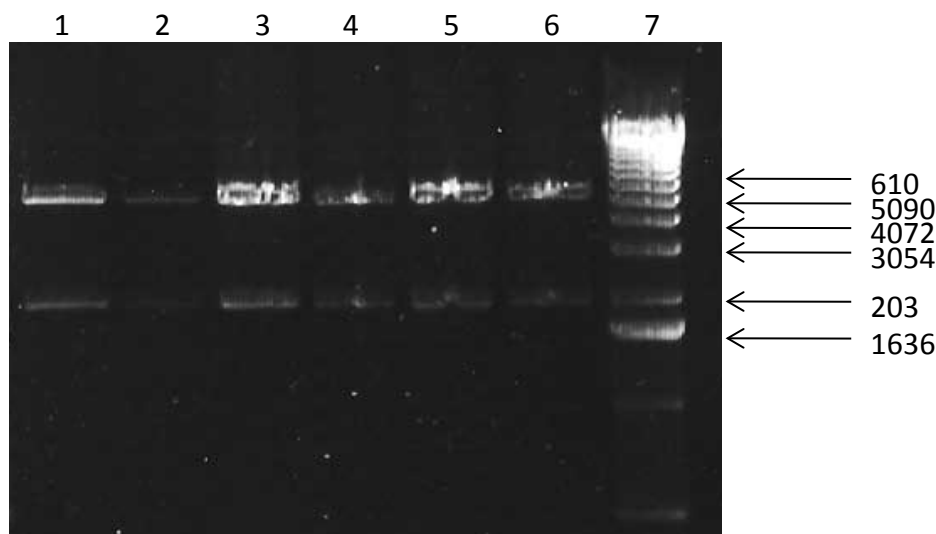


Lanes 1 to 3, YIHisGAL1MS/hRD and lanes 4 to 6, YIHisGAL1MS/yRD, digested with *Sal*I-*Bam*HI which yields two bands to confirm correct ligation. The upper band is the plasmid and the lower the P450 reductase insert. This 1% agarose-TAE gel has the DNA ladder, in lane 7; the sizes of the main DNA markers are highlighted.

The YIHisGAL1MS/hRD and YIHisGAL1MS/yRD plasmids were used separately for integrations into the yeast strain W303Matα which lacks a functional copy of the *HIS3* gene. Both plasmids were linearised at the *Nhe*I restriction site which is within the *HIS3* gene. The sites for linearization are shown in Figures 3.8 and 3.9. Upon homologous recombination, the linearised plasmid should provide a functional, repaired copy of the *HIS3* gene.

After linearization with *Nhe*I, the linearized DNA was isolated for integration into yeast, Figure 3.10.

**Figure 3.8   Map showing where *Nhe*I linearises the plasmid YIHisGAL1MS/hRD.**
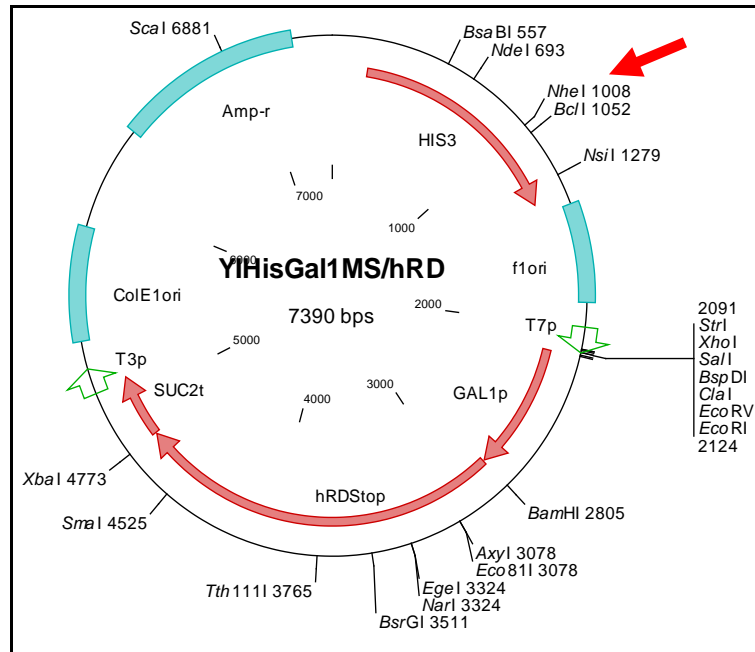


The YIHisGAL1MS/hRD plasmid required linearisation at the *Nhe*I site (shown with a red arrow) to allow chromosomal integration to occur at the *HIS3* site.

**Figure 3.9   Map showing where *Nhe*I linearises the plasmid YIHisGAL1MS/yRD.**
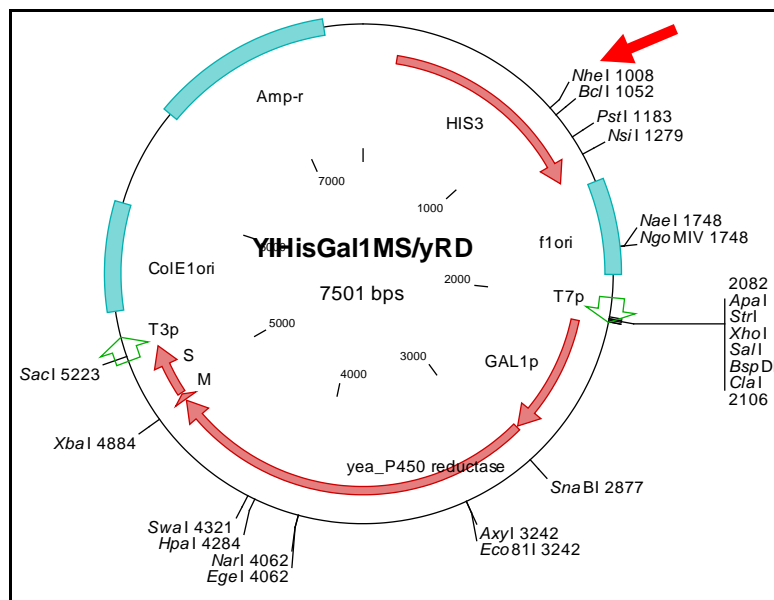


The YIHisGAL1MS/yRD plasmid required linearisation at the *Nhe*I site (shown with a red arrow) to allow chromosomal integration to occur at yeast's *HIS3* chromosomal locus.

**Figure 3.10 YIHisGAL1MS/hRD and YIHisGAL1MS/yRD *Nhe*I linearisation.**
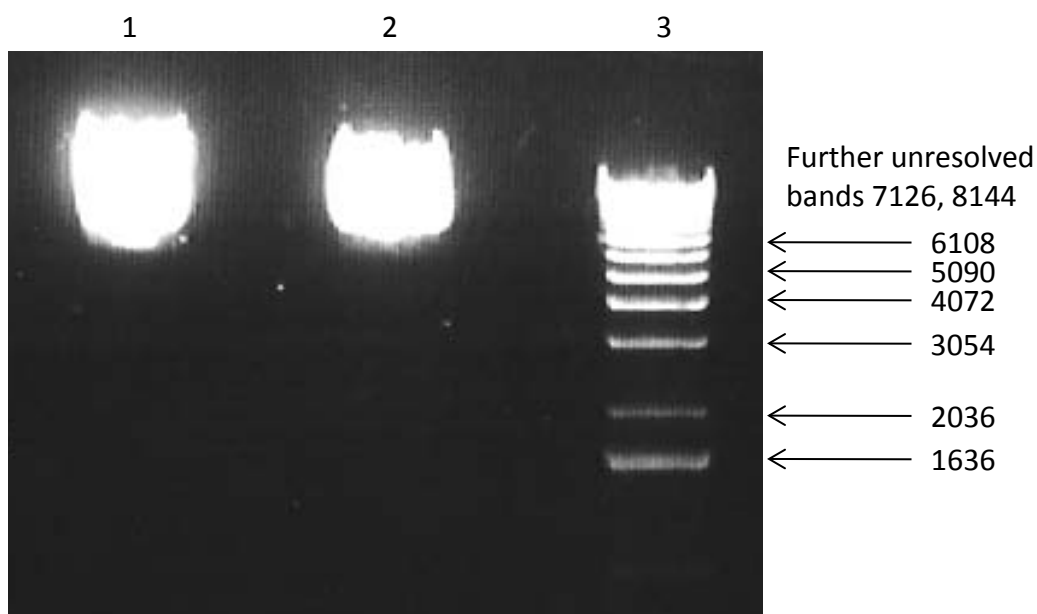


Lanes 1 and 2, Plasmids YIHisGAL1MS/hRD and YIHisGAL1MS/yRD linearised with the restriction enzyme *Nhe*I. The linearized DNA fragments were isolated and purified for further integration into yeast. This is a 1% agarose-TAE gel, with the DNA ladder in lane 3; the main DNA markers are highlighted on the side.

Later, an integrative vector encoding the human *cytochrome b5* gene was constructed which would allow integration in yeast's *TRP1* locus. The methods used to construct the plasmid were similar to that used for the construction of the *reductase* gene encoding *HIS3* integrating plasmids. The *cytochrome b5* gene was also under the control of the *GAL1* promoter. Besides *his3* and *trp1*, the yeast strain W303Matα contains three other auxotrophic markers. They are *ade2*, *leu2* and *ura3*. A *URA3* bearing yeast episomal plasmid was used to create derivatives that encode different *P450* genes. These plasmids complement the *ura3* auxotroph. The two *reductase* genes have also been cloned in *ADE2* and *LEU2*-bearing yeast integrating plasmids in order to determine if the two *reductase* genes placed in different chromosomal loci would express the reductase proteins differentially.

The *P450* and *reductase* genes were cloned in episomal and integrative plasmids that contained a diverse range of promoters. Different promoter bearing plasmids were created essentially through exchange of promoters. Besides the *GAL1* promoter driven constructs, plasmids bearing the ethanol-inducible *ADH2* promoter have widely been used in this thesis.

All the human P450 genes were isolated from a cDNA library derived from a human liver using PCR and a proof-reading Taq polymerase.

## 3.2    Creating a P450 episomal plasmid

Various transformations using episomal P450 plasmids were made searching for clones with high expression levels.

Using a human liver cDNA library as the PCR template, the 1506bp *BamH*I-*Xba*I fragment of the human *CYP2D6* gene was amplified using the following two primers:

- Forward primer, 5'-ATG GAT CCA AA**A TG**G GGC TAG AAG CAC TGG TGC CC-3' (OBC016A); it has an AAA sequence, which is thought to enhance gene expression in yeast, between the *BamH*I restriction site (underlined) and the ATG start site (bold letters), and

- Reverse primer, 5'-CTA GTC TAG ACT AGC GGG GCA CAG CAC AAA GC-3' (2D6STOP) which contains the *Xba*I site (underlined).

Figure 3.11 shows amplification of the human *CYP2D6* gene from the human liver

cDNA library. Figure 3.12 shows isolation of the restriction enzyme digested human

*CYP2D6* gene fragment which is ready for cloning in a vector of choice.

**Figure 3.11** **PCR to isolate the CYP2D6 gene from a human liver cDNA library.**
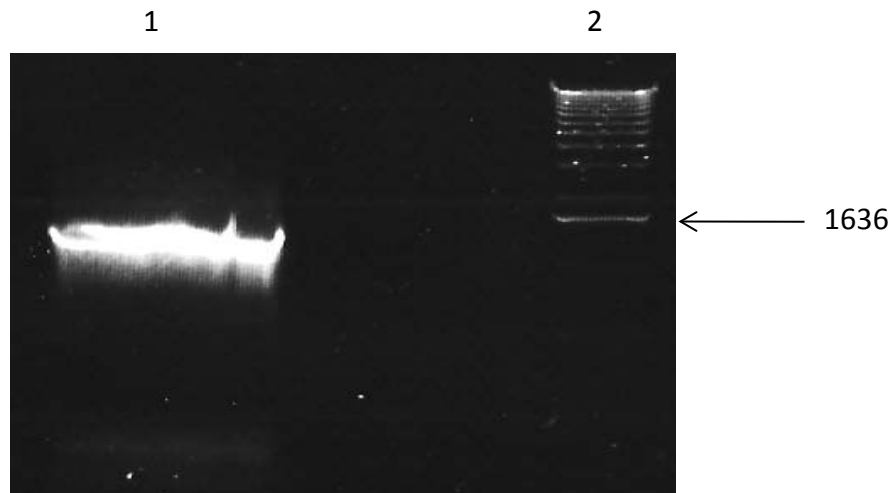


Lane 1, the CYP2D6 gene was amplified by PCR from a human liver cDNA library so that the amplified fragment contains the required restriction sites. This is a 1% agarose-TAE gel which has the DNA ladder in lane 2; the 1636bp DNA marker is highlighted on the side.

**Figure 3.12 Restriction of CYP2D6 gene PCR product with *BamH*I and *Xba*I.**
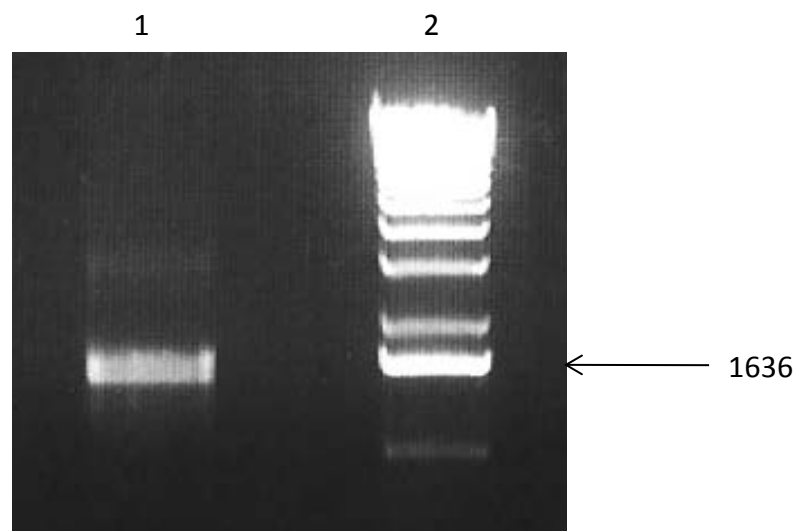


Lane 1, the *BamHI-XbaI* digested CYP2D6 gene fragment was isolate and purified for cloning into episomal vectors. This is a 1% agarose-TAE gel which has the DNA ladder in lane 2; the 1636bp DNA marker is highlighted on the side.

The *BamH*I-*Xba*I digested human *CYP2D6* gene, from Figure 3.12, was ligated to a 2964bp pBluKS+ plasmid, also digested with *BamH*I and *Xba*I, to create the 4455bp plasmid which was designated pBluKS/BamHI-XbaI/3A-CYP2D6 (Figure 3.13).

**Figure 3.13          pBluKS/*BamH*I-*Xba*I/3A-CYP2D6.**
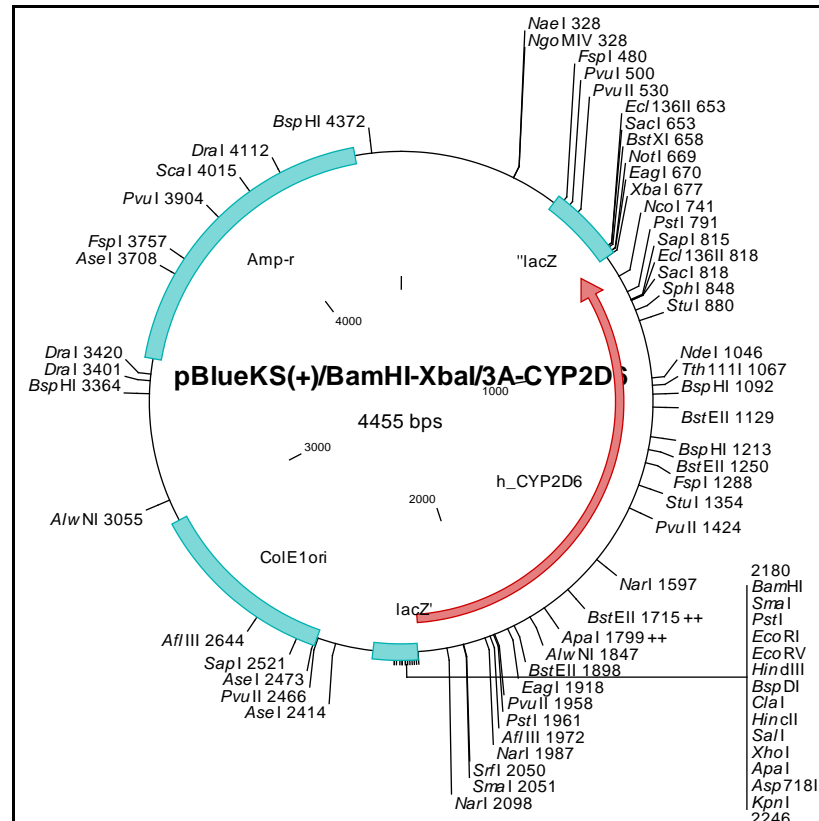


The *BamH*I-*Xba*I restricted *CYP2D6* PCR product was ligated into the vector pBlueKS(+) in its multi-cloning site. The resultant clones were selected in bacterial cells in the presence of ampicillin (for selection of ampicillin-resistant clones) and X-Gal/IPTG blue/white screening (for confirming that the *CYP2D6* gene had indeed disrupted the *lacZ* gene on the parent plasmid).

The plasmid was checked with a *BamH*I-*Xba*I restriction digest which yielded a 2952 base pair vector fragment, as seen below the 3054 base pair DNA ladder marker with a very faint ~1500 base pair CYP2D6 fragment just beneath the 1636 base pair marker; see Figure 3.14.

**Figure 3.14 BamHI-XbaI digest of pBluKS/*BamH*I-*Xba*I/3A-CYP2D6.**
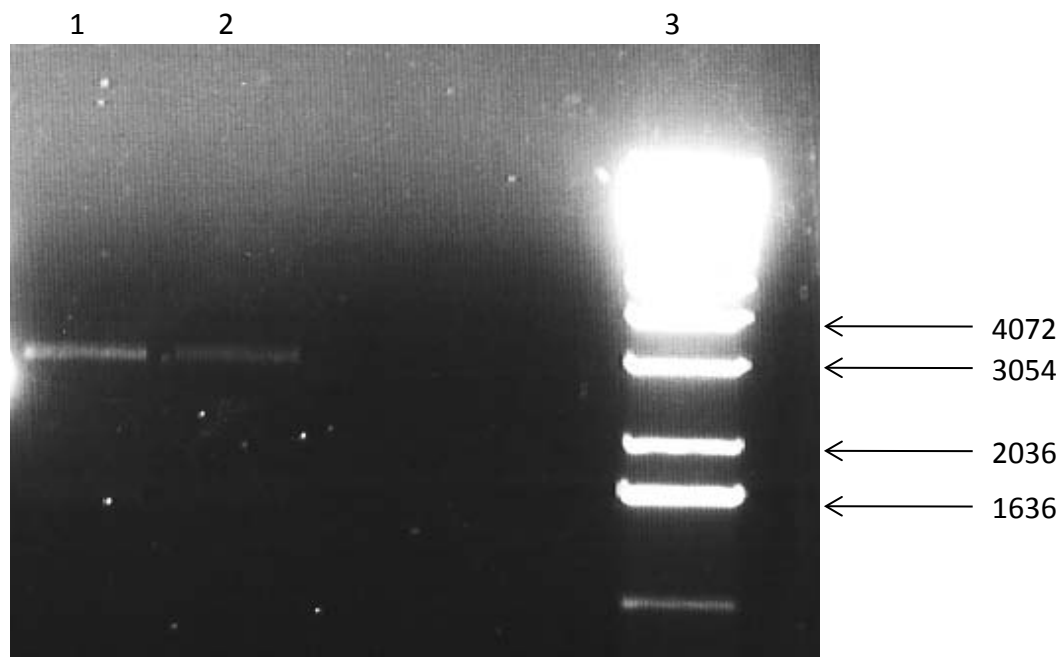


Lanes 1 and 2, the pBluescript plasmids were digested to show that it contained the *CYP2D6* gene insert. This is a 1% agarose-TAE gel which has the DNA ladder lane in 3; the main DNA markers are highlighted on the side. The vector is visible at 3054bp but the *CYP2D6* gene fragment was very faint.

The pBluKS/BamHI-XbaI/3A-CYP2D6 plasmid and the pSYE224 (a yeast shuttle vector with the *URA3* marker, a partial 2μ sequence and the *Gal1p-CYC1t* promoter-terminator cassette) were both separately digested with *BamH*I and *Xba*I. The products were ligated, amplified in bacterial cells, and plasmid DNA was purified. The new plasmid was designated pSYE224/hCYP2D6 (Figure 3.15) and it was used to transform different W303Matα yeast strains which harboured different P450 reductases for expression with active human CYP2D6 enzyme.

**Figure 3.15**      **The episomal plasmid, pSYE224/hCYP2D6.**

*Plasmid Map showing restriction sites that occur only once.*
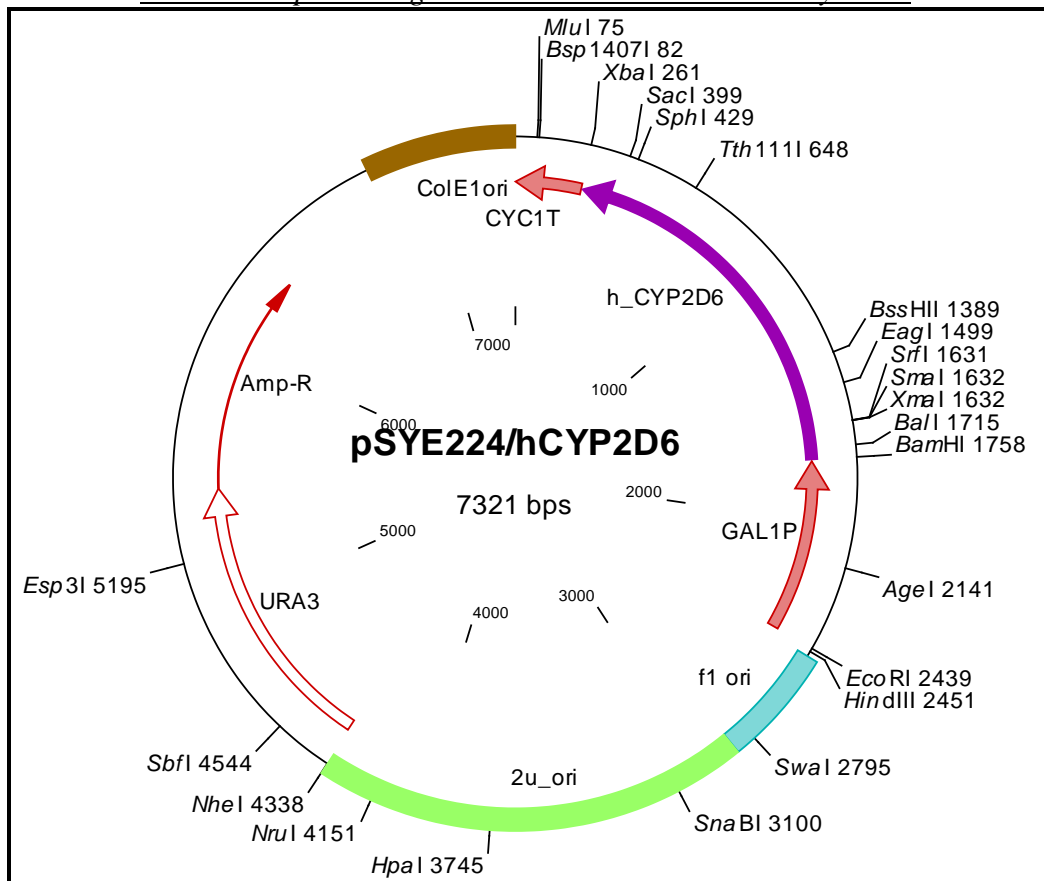


The *BamH*I-*Xba*I human *CYP2D6* gene fragment (h_CYP2D6) isolated from the plasmid pBluKS/*BamH*I-*Xba*I/3A-CYP2D6 was ligated to the pSYE224 plasmid digested with *BamH*I and *Xba*I. The resultant plasmid was designated pSYE224/hCYP2D6. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP2D6 protein could be expressed. The *CYP2D6* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2μ_ori) controls plasmid copy number in yeast.

The *BamH*I-*Xba*I human *CYP2D6* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP2D6.

A diagnostic for correct ligation of *CYP2D6* gene to the vector was carried out via

digestion with *BamH*I and *Xba*I, Figure 3.16. The *CYP2D6* gene fragment was around

the size of the 1517bp DNA marker; the vector backbone was above. Lanes one to six

are six clones from the same transformation procedure.

**Figure 3.16**         *BamH*1 and *Xba*1 digest of pSYE224/2D6.
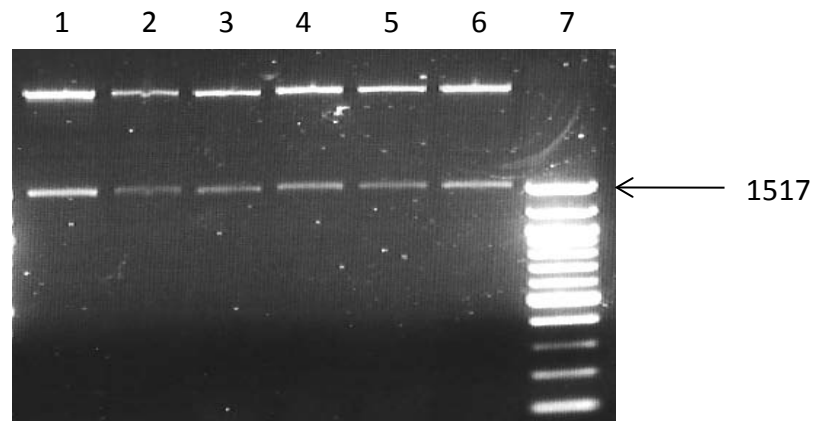


Lanes 1 to 6, the DNA isolated from the clones, obtained from the ligation of CYP2D6 gene to pSYE224, were digested to show correct ligation. Analysis of all six clones confirmed the formation of the plasmid, pSYE224/2D6. The upper band is the plasmid backbone and the lower, *CYP2D6*. This is a 1% agarose-TAE gel which has the DNA ladder in lane 7; the 1517bp marker highlighted on the side.

The episomal plasmid used contained only a 1471bp region of the yeast 2-micron (2μ) sequence which contains the autonomous replication sequence (ARS), origin of replication and the *REP3* segregation element (STB).

In the 2μ plasmid, the *REP3* element co-expresses with two other genes (*REP1* and *REP2*) which form a complex that controls segregation to daughter cells. As the host yeast strain already contains an endogenous, full 2μ plasmid (cir$^+$), the minimal 1471bp Ori-STB sequence allows only replication in yeast without allowing recombination with the endogenous 2μ plasmid. However, the regulation of the copy number of the episomal plasmid is probably still provided by the endogenous 2μ plasmid.

Similarly, five other episomal plasmids encoding the human *CYP1A2*, *CYP2A6*, *CYP2C8*, *CYP2E1* and *CYP3A4*, all being driven by the yeast *GAL1* promoter, were created. Their plasmid maps are shown below (Figures 3.17, 3.18, 3.19, 3.20, 3.21).

## 3.3. Maps of CYP gene-encoding *GAL1* promoter-driven episomal plasmids

### Figure 3.17 The episomal plasmid, pSYE224/hCYP1A2.

*Plasmid Map showing restriction sites that occur only once.*
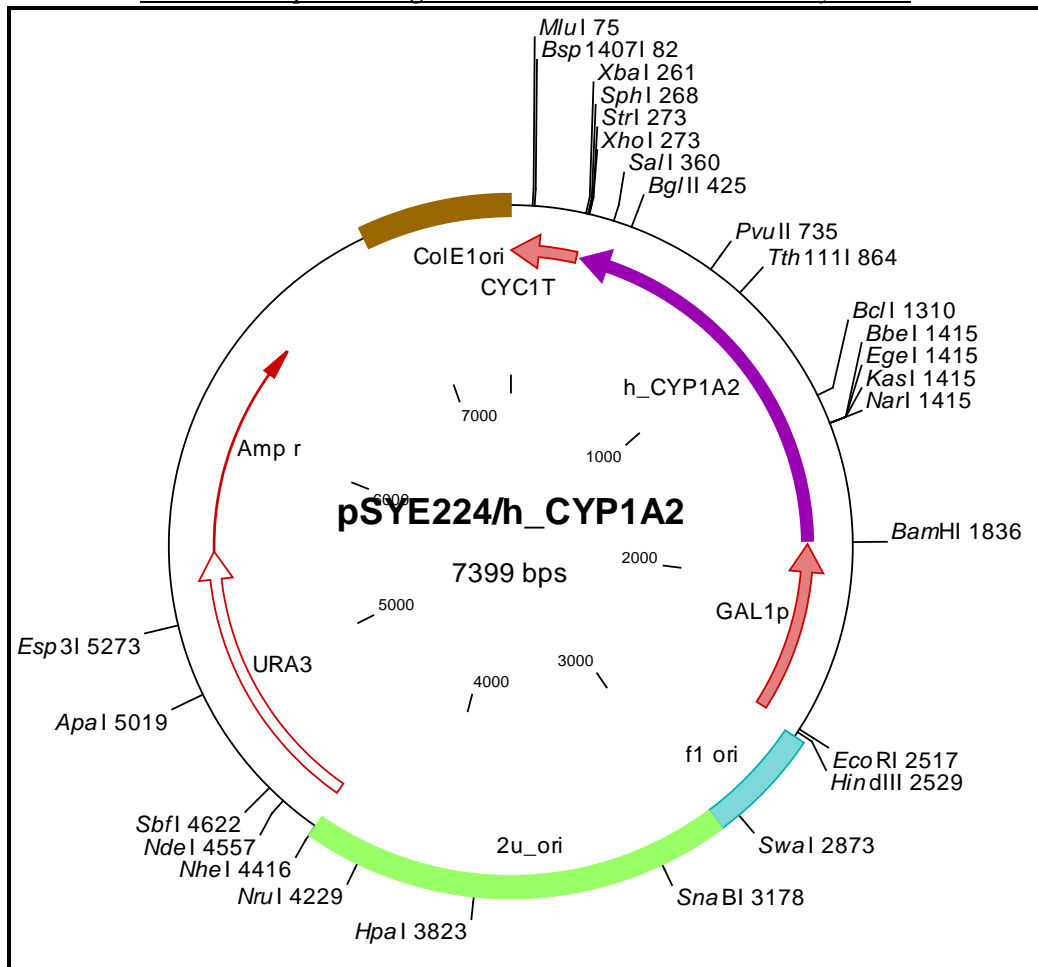


The *BamH*I-*Xho*I human *CYP1A2* gene fragment (h_CYP1A2) isolated from the plasmid pBluKS/*BamH*I-*Xho*I/6A-*CYP1A2* was ligated to the pSYE224 plasmid digested with *BamH*I and *Xho*I. The resultant plasmid was designated pSYE224/CYP1A2. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP1A2 protein could be expressed. The *CYP1A2* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2μ_ori) controls plasmid copy number in yeast.

The *BamH*I-*Xho*I human *CYP1A2* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP1A2.

**Figure 3.18 The episomal plasmid, pSYE224/hCYP2A6.**

*Plasmid Map showing restriction sites that occur only once.*
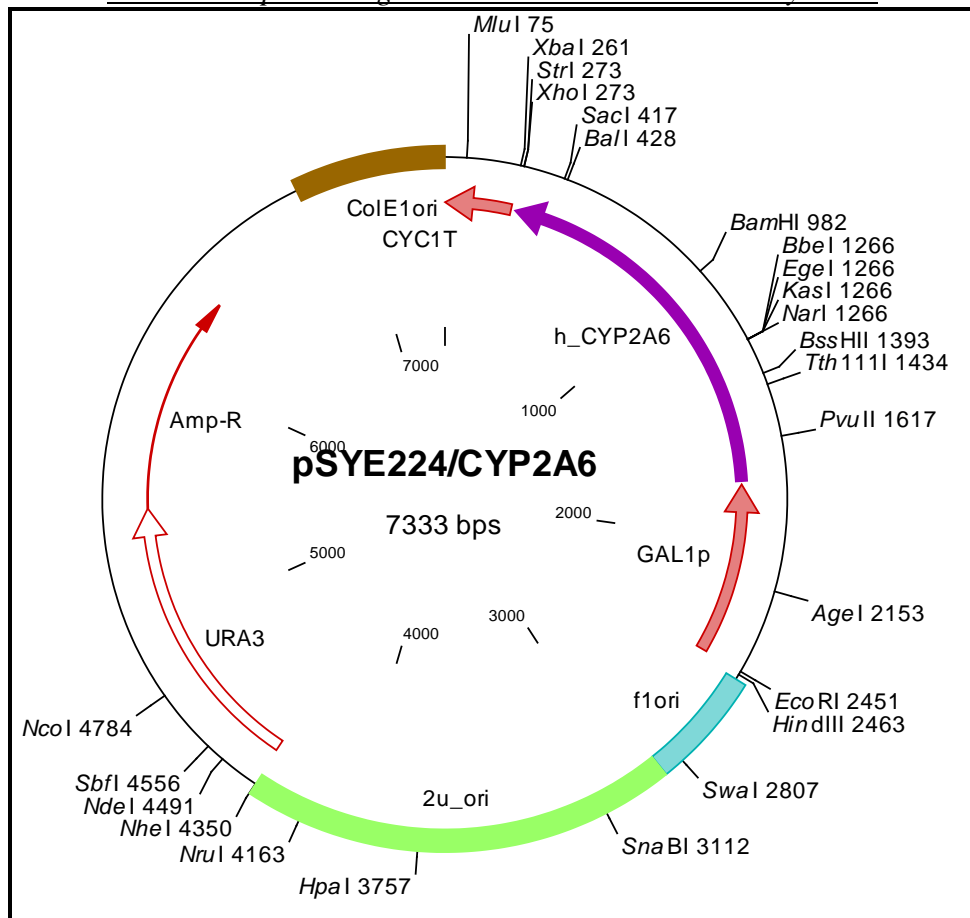


The *Bgl*II-*Xho*I human *CYP2A6* gene fragment (h_CYP2A6) isolated from the plasmid pSP73/*Bgl*II-*Xho*I/6A- *CYP2A6* was ligated to the pSYE224 plasmid digested with *BamH*I and *Xho*I. The resultant plasmid was designated pSYE224/CYP2A6. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP2A6 protein could be expressed. The *CYP2A6* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2μ_ori) controls plasmid copy number in yeast.

The *Bgl*II-*Xho*I human *CYP2A6* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP2A6.

**Figure 3.19 The episomal plasmid, pSYE224/hCYP2C8.**

*Plasmid Map showing restriction sites that occur only once.*
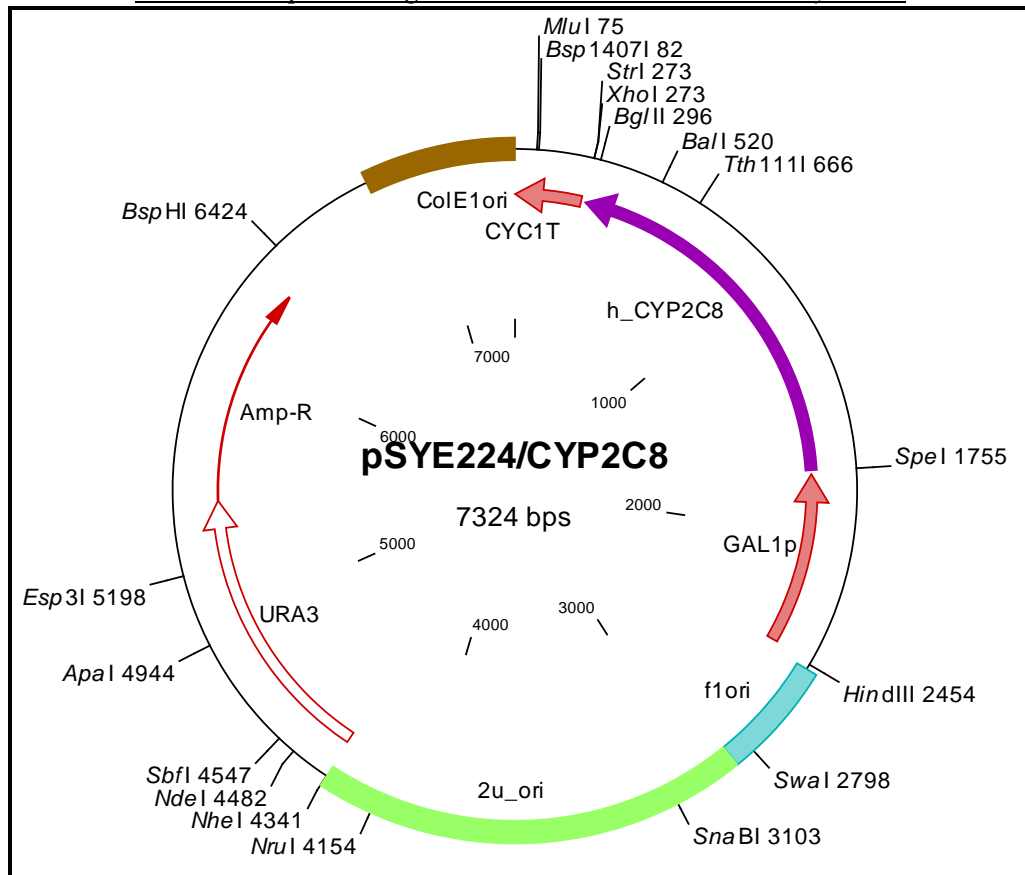


The *Spe*I-*Xho*I human *CYP2C8* gene fragment (h_CYP28) isolated from the plasmid pBluKS+/*Spe*I-*Xho*I/6A- *CYP2C8* was ligated to the pSYE224 plasmid digested with *Spe*I and *Xho*I. The resultant plasmid was designated pSYE224/CYP28. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP28 protein could be expressed. The *CYP2C8* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2μ_ori) controls plasmid copy number in yeast.

The *Spe*I-*Xho*I human *CYP2C8* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP2C8.

**Figure 3.20 The episomal plasmid, pSYE224/hCYP2E1.**

*Plasmid Map showing restriction sites that occur only once.*
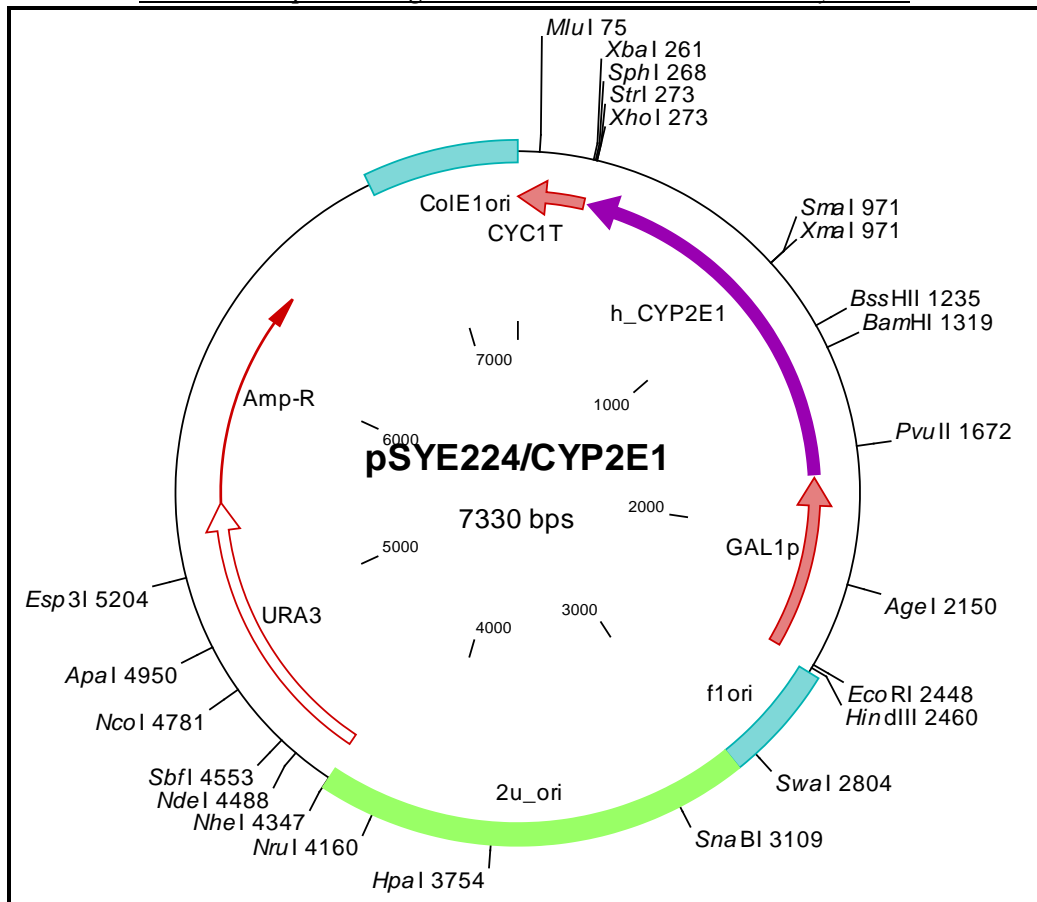


The *Bgl*II-*Xho*I human *CYP2E1* gene fragment (h_CYP2E1) isolated from the plasmid pSP73/*Bgl*II-*Xho*I/6A- *CYP2E1* was ligated to the pSYE224 plasmid digested with *BamH*I and *Xho*I. The resultant plasmid was designated pSYE224/CYP2E1. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP2E1 protein could be expressed. The *CYP2E1* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2µ_ori) controls plasmid copy number in yeast.

The *Bgl*II-*Xho*I human *CYP2E1* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP2E1.

**Figure 3.21 The episomal plasmid, pSYE224/hCYP3A4.**

*Plasmid Map showing restriction sites that occur only once.*
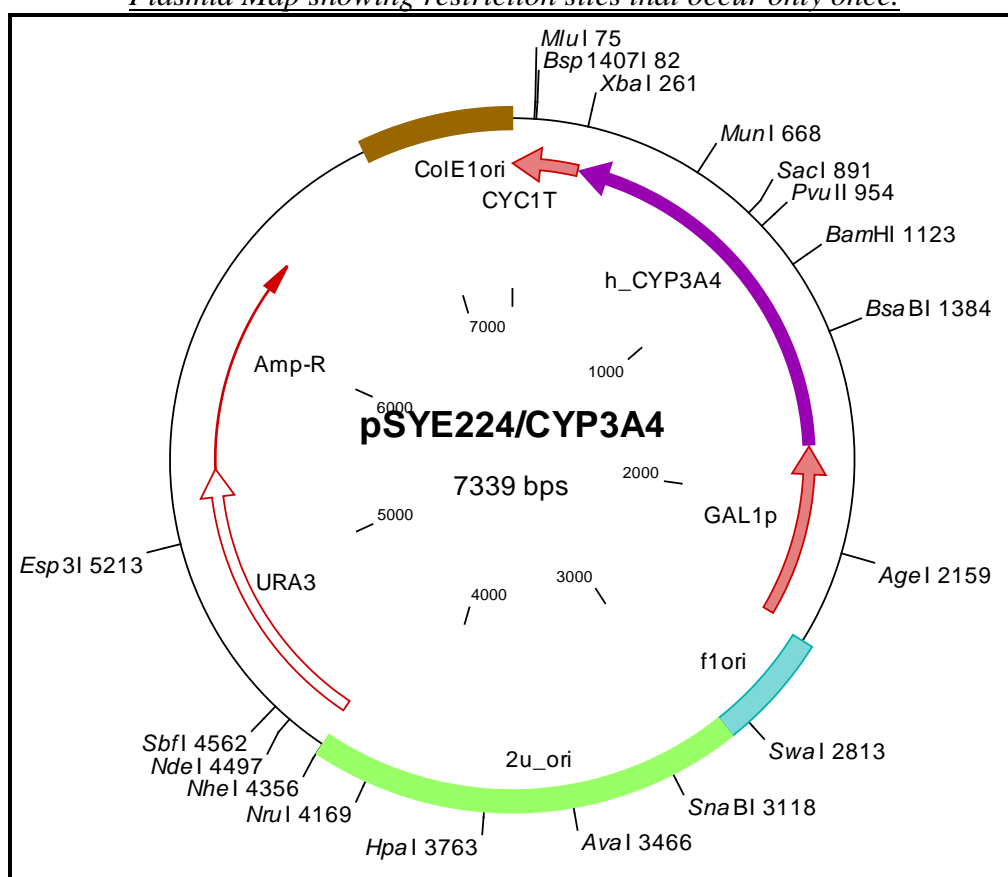


The *Bgl*II-*Xba*I human *CYP3A4* gene fragment (h_CYP3A4) isolated from the plasmid pSP73/*Bgl*II-*Xba*I/6A- *CYP3A4* was ligated to the pSYE224 plasmid digested with *Bam*HI and *Xho*I. The resultant plasmid was designated pSYE224/CYP3A4. This episomal plasmid with a uracil marker (*URA3*) was amplified in *E. coli* for the plasmid to be transformed in yeast so that CYP3A4 protein could be expressed. The *CYP3A4* gene fragment was under the control of a galactose-inducible promoter (Gal1p) with a terminator from a cytochrome C gene (CYC1t). The 2 micron origin of replication from yeast (2μ_ori) controls plasmid copy number in yeast.

The *Bgl*II-*Xba*I human *CYP3A4* gene fragment was similarly cloned in the episomal plasmid pSYE263, to allow *ADH2* promoter-driven expression of CYP3A4.

## 3.4   Western Blots

Western blots were performed to confirm the presence of specific P450s. Tables and

Western Blots 3.1 to 3.6 are linked to the expression of the CYP1A2, CYP2A6,

CYP2C8, CYP2D6, CYP2E1 and CYP3A4 proteins, respectively. Since microsomes do not contain housekeeping proteins, CYP expression levels from different constructs were compared using equal amounts of microsomal protein.

For all Western blots, total proteins from microsomal samples were transferred from a 10% SDS-polyacrylamide gel. Each lane contains 3µg of total protein that had been prepared from microsomes using the cell disruption method. For all CYP expressions, cells were grown in the non-selective, YP medium, in shake flasks.

In the case of CYP expression driven by the *ADH2* promoter, the carbon source was glucose and expression was induced with the exhaustion of glucose.

For *GAL1* promoter driven CYP expression, the promoter was induced by the addition of galactose.

All the CYPs were expressed in yeast from a *URA3*–based episomal plasmid. The CYP proteins were co-expressed with two different P450 reductases:

(a) ΔhRDmyc (ΔhRDM) which represent a human P450 reductase (hRD) variant which lacks first N- terminal 24 amino acids of hRD but contains an additional 11 amino acids from the *c*-myc tag at its C-terminus.

(b) The yeast reductase (yRD) represents the wild type reductase.

Both the reductase gene expression cassettes, *ΔhRDM* and *yRD*, were integrated into yeast's *LEU2* chromosomal locus. The empty strain (W303Matα) which neither contained a CYP-bearing plasmid nor a P450 reductase was used as a negative control.

Western Blot 3.1 shows the presence of CYP1A2, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP1A2 protein.

**Table and Western Blot 3.1 CYP1A2.**

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 2 | - | - | - | YW41 | Untransformed Negative Control |
| 3 | 1A2 | *ADH2* | ΔhRDM | YY43 | LEU2 P450 reductase |
| 4 | 1A2 | *ADH2* | Yeast reductase (yRD) | YAC50 | LEU2 P450 reductase |
| 5 | 1A2 | *GAL1* | ΔhRDM | YAG09 | LEU2 P450 reductase |
| 6 | 1A2 | *GAL1* | Yeast reductase (yRD) | YAF76 | LEU2 P450 reductase |
| 7 | 1A2 | - | - | - | Gentest Positive Control |
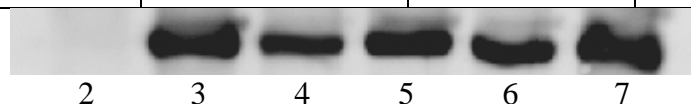


2    3    4    5    6    7

The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP1A2-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP1A2-bearing microsomes (obtained from Gentest).

Western Blot 3.2 shows the presence of CYP2A6, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP2A6 protein.

## Table and Western Blot 3.2    CYP2A6.

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 2 | - | - | - | YW41 | Untransformed Negative Control |
| 3 | 2A6 | ADH2 | ΔhRDM | YAD48 | LEU2 P450 reductase |
| 4 | 2A6 | ADH2 | yRD | YAD53 | LEU2 P450 reductase |
| 5 | 2A6 | GAL1 | ΔhRDM | YAG33 | LEU2 P450 reductase |
| 6 | 2A6 | GAL1 | yRD | YAG17 | LEU2 P450 reductase |
| 7 | 2A6 | - | - | - | Gentest Positive Control |
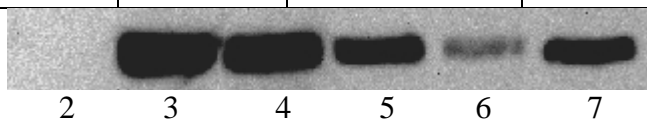


The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP2A6-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP2A6-bearing microsomes (obtained from Gentest).

Western Blot 3.3 shows the presence of CYP2C8, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP2C8 protein.

## Table and Western Blot 3.3    CYP2C8.

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 2 | - | - | - | YW41 | Negative Control |
| 3 | 2C8 | ADH2 | ΔhRDM | YAC11 | LEU2 P450 reductase |
| 4 | 2C8 | ADH2 | yRD | YAC16 | LEU2 P450 reductase |
| 5 | 2C8 | GAL1 | ΔhRDM | YAG37 | LEU2 P450 reductase |
| 6 | 2C8 | GAL1 | yRD | YAG21 | LEU2 P450 reductase |
| 7 | 2C8 | - | - | - | Gentest Positive Control |
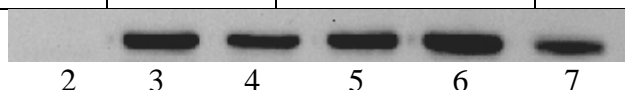


The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP2C8-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP2C8-bearing microsomes (obtained from Gentest).

Western Blot 3.4 shows the presence of CYP2D6, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP2D6 protein.

**Table and Western Blot 3.4     CYP2D6.**

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 2 | - | - | - | YW41 | Negative Control |
| 3 | 2D6 | *ADH2* | ΔhRDM | YY51 | LEU2 P450 reductase |
| 4 | 2D6 | *ADH2* | yRD | YAC52 | LEU2 P450 reductase |
| 5 | 2D6 | *GAL1* | ΔhRDM | YAG13 | LEU2 P450 reductase |
| 6 | 2D6 | *GAL1* | yRD | YAG01 | LEU2 P450 reductase |
| 7 | 2D6 | - | - | - | Gentest Positive Control |
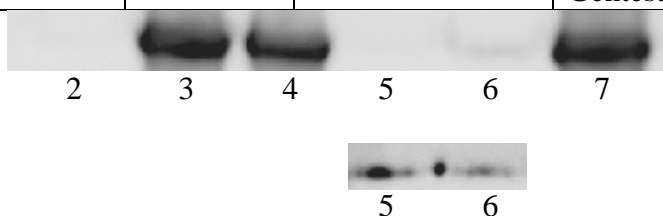


The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP2D6-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP2D6-bearing microsomes (obtained from Gentest). Proteins in lanes 5-6 could be detected only when the total microsomal proteins were 6 µg, instead of the normally used 3 µg.

Western Blot 3.5 shows the presence of CYP2E1, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP2E1protein.

## Table and Western Blot 3.5    CYP2E1.

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 2 | - | - | - | YW41 | Negative Control |
| 3 | 2E1 | *ADH2* | ΔhRDM | YAC67 | LEU2 P450 reductase |
| 4 | 2E1 | *ADH2* | yRD | YAC72 | LEU2 P450 reductase |
| 5 | 2E1 | *GAL1* | ΔhRDM | YAG41 | LEU2 P450 reductase |
| 6 | 2E1 | *GAL1* | yRD | YAG25 | LEU2 P450 reductase |
| 7 | 2E1 | - | - | - | Gentest Positive Control |



2    3    4    5    6    7

The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP2E1-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP2E1-bearing microsomes (obtained from Gentest).

Western Blot 3.6 shows the presence of CYP3A4, in the 55KDa region, in all yeast

microsomal samples that contained the human CYP3A4 protein.

## Table and Western Blot 3.6    CYP3A4.

| Lane | P450 | Promoters | Reductase | Strain Reference | Other Information |
|------|------|-----------|-----------|------------------|------------------|
| 1 | - | - | - | - | Marker |
| 2 | - | - | - | YW41 | Negative Control |
| 3 | 3A4 | *ADH2* | ΔhRDmyc | YAC05 | LEU2 P450 reductase |
| 4 | 3A4 | *ADH2* | Yeast reductase | YAC09 | LEU2 P450 reductase |
| 5 | 3A4 | *GAL1* | ΔhRDmyc | YAG45 | LEU2 P450 reductase |
| 6 | 3A4 | *GAL1* | Yeast reductase | YAG29 | LEU2 P450 reductase |
| 7 | 3A4 | - | - | - | Gentest Positive Control |
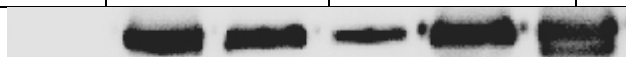


2    3    4    5    6    7

The Figure shows Western blot of total proteins from microsomal samples. Lanes 3-6, yeast CYP3A4-bearing microsomes; lane 2, the negative control (microsomes from an empty strain); lane 7, insect CYP3A4-bearing microsomes (obtained from Gentest).

## 3.5   Testing media components for optimal P450 expression

### 3.5.1  Strain and clone background

W303Matα, a haploid *Saccharomyces cerevisiae* strain, was used for CYP expression studies. It has five auxotrophic markers (*ade2*, *his3*, *leu2*, *trp1*, *ura3*) which were generated by knocking out dominant genes in biochemical pathways that lead to the synthesis of adenine, histidine, leucine, tryptophan and uracil (Sikorski & Hieter, 1989). A W303Matα derivative that expresses human CYP2C8 protein was selected for this study because the strain produced high levels of P450. This may allow effects on expression levels due to changes in specific components of the growth media to be more easily monitored.

The yeast strain (*LEU2*::*P450 reductase*, *TRP1*::*cytochrome b5*), transformed with a *URA3*-bearing episomal plasmid encoding the human *CYP2C8* gene and driven by the *ADH2* promoter, is referred to as YAC16. The strain YAC16 requires additions of histidine and adenine for growth in minimal media. As the strain simultaneously expresses three heterologous proteins, CYP2C8, P450 reductase and $b_5$, it was thought that optimisation of media conditions may be able to somewhat overcome the extra burden of tripartite expression and thereby benefit the levels of CYP2C8 expression.

### 3.5.2  Growth Media

Growing auxotrophic yeast cells in minimal media is not as simple as providing complex rich nutrients in excess in full media. In glucose (a carbon source), auxotrophic yeast grows via fermentation rather than respiration. This leads to reduction of

mitochondrial activity, oxygen utilisation and growth rate. Nitrogen sources also impact

growth and strain selection. Although a minimal ammonium salt media provides an

environment for plasmid selection, it does not provide growth conditions for optimal

expression of heterologous proteins. Complex and undefined media are nutrient rich and

are conducive to optimisation of levels of protein production.

Keeping the carbon source [1% (w/v) glucose] constant, alterations to the other

components of complex media were investigated.

Table 3.1 contains a breakdown of the components that were varied.

**Table 3.1      Breakdown of components, in the media, which were varied.**

| Media | Yeast extract $g.l^{-1}$ | Peptone $g.l^{-1}$ | Tryptone $g.l^{-1}$ | Total $g.l^{-1}$ |
|---|---|---|---|---|
| Peptone rich media | 10 | 20 | 0 | 30 |
| Tryptone rich media | 10 | 0 | 20 | 30 |
| Equal media | 10 | 10 | 10 | 30 |
| Yeast extract rich media | 20 | 10 | 0 | 30 |
| Yeast extract rich & Peptone rich media | 20 | 20 | 0 | 40 |
| Exclusive yeast extract media | 30 | 0 | 0 | 30 |
| Exclusive Peptone media | 0 | 30 | 0 | 30 |
| Exclusive Tryptone media | 0 | 0 | 30 | 30 |
| Exclusive Casamino acids | 30 | | | 30 |

Different complex media, where the components were varied, were used for testing for optimal production levels in an *ADH2* promotor-driven expression of CYP2C8. The different media were given names to assist in their use. All media components were per litre of ultrapure water and autoclaved under a standard procedure, 121ºC for 20 min. The exclusive "casamino acids media" contained none of the other components.

### 3.5.3  Dissection of the media components for optimal P450 activities

CYP2C8-bearing microsomes were prepared after growth in each of the complex media

in shake flasks (see Section 2.10). The activities of these samples were determined by

fluorescent microplate assay. As each sample had its own specific P450 content (as

measured according to the protocol detailed in Section 2.13), the volume of sample per

well was adjusted to 4 pmol per microplate-well, Table 3.2. The results from the

fluorescent microplate assay show that CYP2C8 prepared using different media gave a

wide range of activities, Graph 3.1.

**Table 3.2    Volumes of microsomes, equating to 4 pmol of P450, that were used per well of a 96-well microtitre plate.**

| Media | Volume per reaction in µl |
|---|---|
| Exclusive Casamino acids | 13.0 |
| Exclusive Peptone media | 3.7 |
| Exclusive Tryptone media | 3.0 |
| Peptone rich media | 2.7 |
| Equal media | 2.4 |
| Tryptone rich media | 2.1 |
| Yeast extract rich & Peptone rich media | 2.6 |
| Exclusive yeast extract media | 2.4 |
| Yeast extract rich media | 0.9 |

The CYP2C8-bearing yeast strain, YAC16, was initially grown on SD-Min media solid-agar plate (containing histidine and adenine) for 3 days at 30°C. A pre-culture was grown in SW6 (casamino acid) media, overnight at 30°C in shake flasks at 220 rpm. These cells were used to inoculate the different complex media types for expression of protein in 1% glucose; cells were grown overnight at 30°C in shake flasks at 220 rpm. The cells were harvested to produce microsomes using the mechanical cell disruption method and assayed for total protein content by Bradford assay and specific P450 content was derived from the CO-binding assay. These measurements provided the amounts of P450 (CYP2C8) that were added to different wells of the microtitre plate for the fluorescent-based activity assays.

**Graph 3.1    Graph showing the CYP2C8 activities obtained in different complex media.**
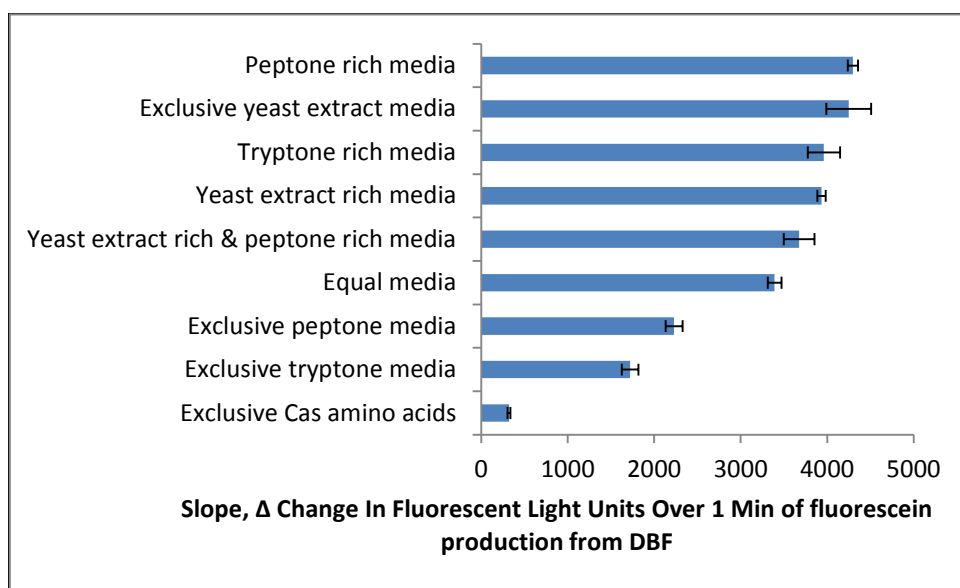


CYP2C8 activities obtained from different complex media. Bars show the changes in relative fluorescence units (RFLUs) that occurred over 1 min of assay. The activity was calculated from the linear part of the kinetic reaction that produces fluorescein from dibenzylfluorescein (DBF). The assay was performed, in quadruplicate, after shaking before each read in 96-well black microplates, at 30°C for 30 minutes. Each well contained 4 pmol of CYP2C8 in a 100 mM phosphate buffer, pH7.4, [DBF] 1 µM. The components of the regenerating system used were at the standard concentration. Final reaction volume was 100 µl; water was used to make up the P450 volume differences. Fluorescence measurements were made at 485/538 nm. The error bars are +/- 1x standard deviations.

Whilst the overall P450 activity was important, the activities in Graph 3.1 were observed with CYP2C8 enzymes which were made up in very different volumes to obtain 4 pmol of CYP2C8. These were then normalised for a fixed volume of CYP2C8 (1µl), Table 3.3.

**Table 3.3    Activities in Graph 3.1, normalized per microlitre of sample.**

| # | Media | Activity per min per 1 µl of CYP2C8 |
|---|---|---|
| 1 | Exclusive Casamino acids | 24.69 |
| 2 | Exclusive Tryptone media | 574 |
| 3 | Exclusive Peptone media | 602.73 |
| 4 | Equal media | 1413.33 |
| 5 | Peptone rich media | 1592.24 |
| 6 | Tryptone rich media | 1886.59 |
| 7 | Yeast extract rich & Peptone rich media | 2297.38 |
| 8 | Exclusive yeast extract media | 3034.37 |
| 9 | Yeast extract rich media | 4370.40 |

Values of CYP2C8 activities were taken from Graph 3.1 and were divided by values in Table 3.2, i.e. the volumes of CYP2C8 enzymes, per 4 pmol, which were added per reaction for each media type. These new values gave an activity for a fixed 1µl volume of microsomes.

From Table 3.3, one observes that the "Peptone rich media" (# 5) had 36.4% of the activity of the "Yeast extract rich media" (# 9), volume for volume, even though the reactions overall contained the same amount of P450. The two samples had similar protein concentrations but the specific activity from the "Peptone rich media" was 0.081 nmol.mg$^{-1}$ and from the "Yeast extract rich media" 0.161 nmol.mg$^{-1}$, which is twice as much. This means that the "Yeast extract rich media" (# 9) produced about twice the P450 amount, without a corresponding doubling of the protein concentration. So the "Peptone rich media" (# 5) contained more non-P450 related protein per mole of P450 than the "Yeast extract rich media" (# 9).

### 3.5.4  Discussion of the dissection of media components for optimal P450 activities.

Regarding the make-up of these media, Proteose Peptone was derived from meat treated with enzymes of pancreatic origin. The Tryptone was made from a pancreatic digest of casein. The Casamino acids were also produced from casein but with the use of hydrochloric acid causing a complete hydrolysis of all peptide bonds and giving rise to

all the essential amino acids excepting tryptophan which is destroyed during acid-hydrolysis (Mueller *et al.*, 1941). Yeast extract contains B vitamins and these are immensely important for growth of yeast. Yeast requires thiamine (B$_1$) as a cofactor for pyruvate decarboxylase that allows fermentation of pyruvate to ethanol (Dyda *et al.*, 1990). As yeast ferments at the start of expression due to glucose levels, presence of B vitamins may be a major advantage.

"Normal" media contains 30 g of powder which is comprised of 10 g of yeast extract and 20 g of peptone per litre (a standard Becton Dickinson recipe). This media had been used by others for P450 production and the expression of many proteins in yeast (Yang *et al.*, 2008 and Garcia-Ruiz *et al.*, 2010). Equal quantities of yeast extract and peptone have also been used for production of proteins in shake flasks, including expression of P450s in yeast (Bellamine *et al.*, 1998). The recipe mentions using 10 g.L$^{-1}$ of yeast extract and peptone. Again during expression studies, McLellan *et al* (2000) used equal quantities of the two but at 20 g.L$^{-1}$.

There is no evidence that others have used media where the yeast extract content was greater than the peptone.

The data shows that simple variations in the composition of the media alter the potency of CYP2C8 in CYP-bearing yeast microsomes. This observation can be useful. Obtaining greater P450 activity in smaller volumes is important for the miniaturisation of P450 assays in microplates and possibly microarrays. This would allow reduction of costs and time for screening of potential P450 substrates, inhibitors and metabolic conversion products. With a media that provides high activity of CYP enzymes, biotransformation reactions could also be carried out with superior efficiencies.

# Chapter 4    Large-scale Production of P450s

## 4.1    The use of fermentation

Production of large quantities of P450s is required to reduce batch to batch variations and produce quantities that may be of use for testing in a whole new variety of applications. Hence, attempts were made to have high yields of P450s with the help of fermentors. But with so many variables involved in fermentation, a starting point was required. Although there are published examples of P450s that have been produced in fermentors, using both bacterial and yeast cells, procedural information is limited.

The aim of the experiments was to perform a feasibility study using small-scale fermentors with the hope that this would be a starting point for larger-scale production, if required, at a later date.

## 4.2    Optimising of conditions for growth of yeast in a fermentor

The temperature, to be used for fermentation, is perhaps directly transferable from growth of yeast in shake flasks to fermentors. Some papers cite 30ºC as the best temperature for growth (van Dijken *et al*., 2000 and Blatiak *et al*., 1987). However, other groups have grown P450 gene-bearing yeast in fermentors at a lower temperature, 28ºC (US Patent 6117649, Denis Pompom's laboratories) and 28.5ºC (McLellan *et al*., 2000).

Conditions for base input, gas-flow, agitation, amount of additions of specific reagents and timing of their additions would have to be optimised for growth of yeast in

fermentors to see if yields of P450s could be increased from what was obtained in shake

flasks. These parameters were tested and optimised using publications as starting points.

## 4.3 Effects of dissolved oxygen (DO) on growth, during fermentation

In the absence of a known oxygen concentration that could be used, a range of

concentrations were tried using three different gas-flow rates and allowing agitation to

vary so that a fixed dissolved oxygen concentration (DO) of 30%, 50% or 70% could be

maintained throughout fermentation. The air flow rates were 1.0, 1.5 or 2.5 Litres per

min. The $OD_{600}$s were used to assess the differences in growth; see Graph 4.1.

**Graph 4.1**   **$OD_{600}$ obtained during the *ADH2* promoter-driven expression of CYP1A2 from a yeast strain, at fixed DO concentrations and gas-flow rates.**
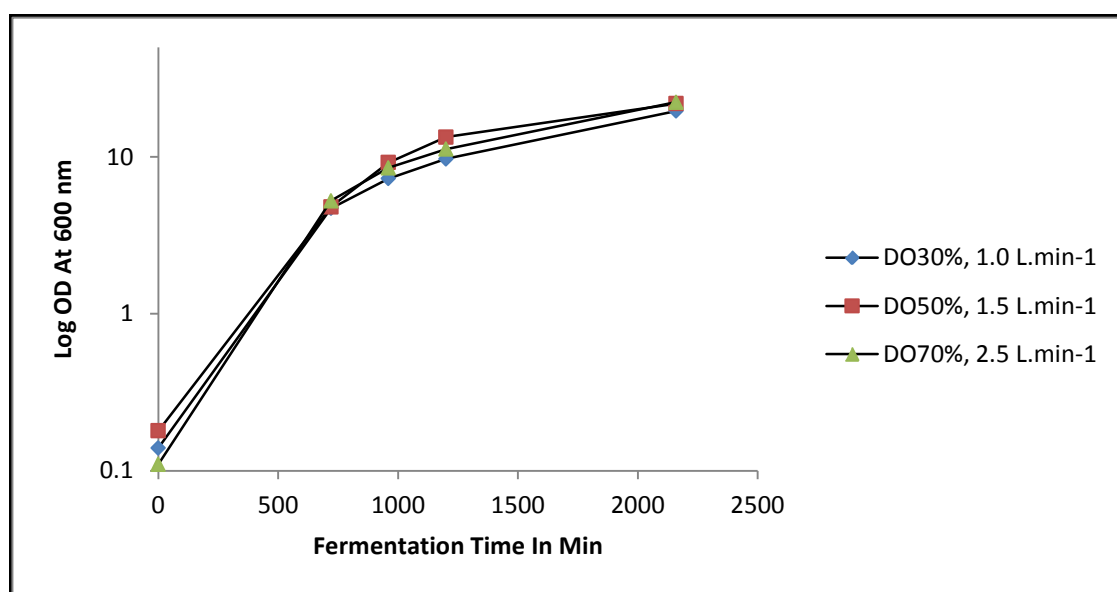


Measurements of $OD_{600}$ during fermentation of the strain YR42, in a one-litre ferrmentor. YR42 harbours a plasmid that bears an *ADH2* promoter-driven *CYP1A2* expression cassette. The strain was grown in non-selective YP media, containing 1% glucose, pH held at 6.6. Identical fermentations were performed with differing compressed air gas-flow rates. Agitation in impellor (via rpm) was allowed to automatically adjust the relative dissolved oxygen (DO) level to reach 30, 50 or 70% for the corresponding gas-flow rates. Despite the different oxygenation levels there was no impact to the optical density, $OD_{600}$ (OD at 600 nm), values.

The $OD_{600}$s suggested that there were no differences in growth that occurred during the fermentations. After the fermentations, the cells were centrifuged and the pellets weighed. However, the wet weight pellets were unexpectedly not equal,

(a) DO 30%, 1.0 L.min$^{-1}$ was 30.5 g,

(b) DO 50%, 1.5 L.min$^{-1}$ was 46 g, and

(c) DO 70%, 2.5 L.min$^{-1}$ was 39.0 g.

There was a mismatch in the results as changes in dissolved oxygen (DO) gave rise to a change in biomass but not in OD. However, wet weights are not as accurate as taking dry weights. Based on OD, clamping the fermentor DO seemed unnecessary.

It was then decided that the cells should be left to take what they required during growth from a fixed agitation impellor rpm. It would be similar to growth in shake flasks where the cells also take what they require from a fixed agitation.

## 4.4 Removal of dissolved oxygen (DO) control

Fermentation without clamping of dissolved oxygen (DO) concentration whilst holding a fixed agitation at a particular rpm was then tested. The DO concentrations were measured; see Graph 4.2.

**Graph 4.2    Relative percentages of DO over a fermentation time course without controlling DO, during the *ADH2* promoter-driven expression of CYP1A2 from a yeast strain.**
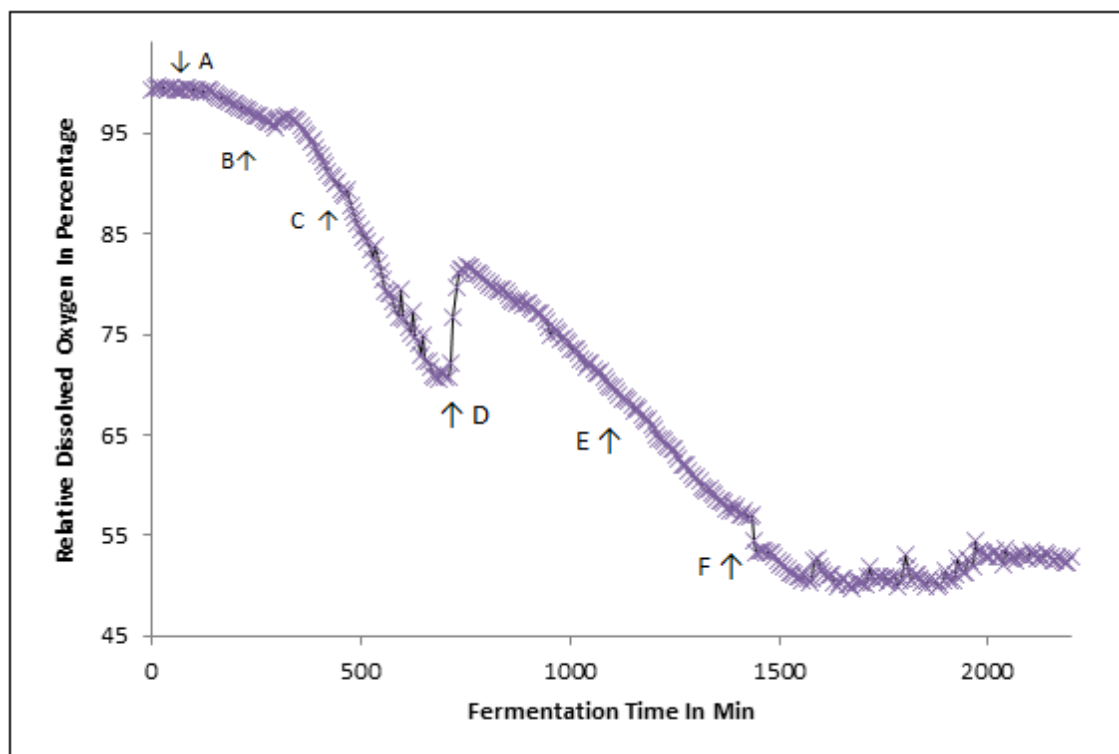


Measurements of dissolved oxygen (DO) during fermentation of the strain YR42 in a one-litre ferrmentor. YR42 harbours a plasmid that bears an *ADH2* promoter-driven *CYP1A2* expression cassette. The strain was grown in non-selective YP media containing 1% glucose, pH held at 6.6. Agitation and compressed gas flow was fixed at 500 rpm and 0.5 L.min$^{-1}$. At point A, there were few cells and the oxygenation levels were high. At point B, about 250 min, the cells underwent slight alteration of the rate at which oxygen was utilised. At point C, about 480 min, further glucose was added and this caused oxygen utilisation to reverse at point D, 600 min. At point E, at 1080 min, glycerol and ethanol were added which continued to complement the rate of oxygen utilisation. After point F at 1400 min the oxygen utilisation seemed to stabilise.

It was clear that the cells had different oxygen requirements at different times of growth. As the glucose reduced, oxygen utilisation from the media increased and reversed upon addition of more glucose. The glycerol and ethanol addition did not reverse oxygen utilisation. This was expected as these carbon sources require oxygen.

In the same way as shake flasks were used at a fixed speed throughout growth, it was found that the best approach was to keep both the gas flow rpm constant, simply allowing the yeast cells to utilise oxygen as they require it.

## 4.5   Modifications of a published fermentation protocol

Merely growing cells did not equate to successful expression of P450s. Optimisation was difficult since no P450s were produced.

A paper from McLellan *et al* (2000) had described expression of P450s in yeast in fermentors, under the control of the *GAL* promoter. Having failed to obtain any measurable P450 from an *ADH2*-driven construct, attempts were made to express CYP1B1 from the *GAL1* promoter. The yeast strain, YH50, had earlier produced CYP1B1 reliably, in shake flasks, from a *GAL1* promoter-driven construct. Hence, the strain YH50 was used in fermenters in conjunction with the parameters published by McLellan *et al* (2000), with the hope that these parameters could offer a platform for further improvements. The YH50 strain was extensively tested in fermentors and modifications of the published protocol made until a successful method was found. In a one-litre fermentor, an $OD_{600}$ of 96 and a wet weight pellet of 103.6 g were ultimately obtained; see Graph 4.3.

**Graph 4.3    $OD_{600}$ obtained during the *GAL1* promoter-driven expression of CYPB1 from a yeast strain**
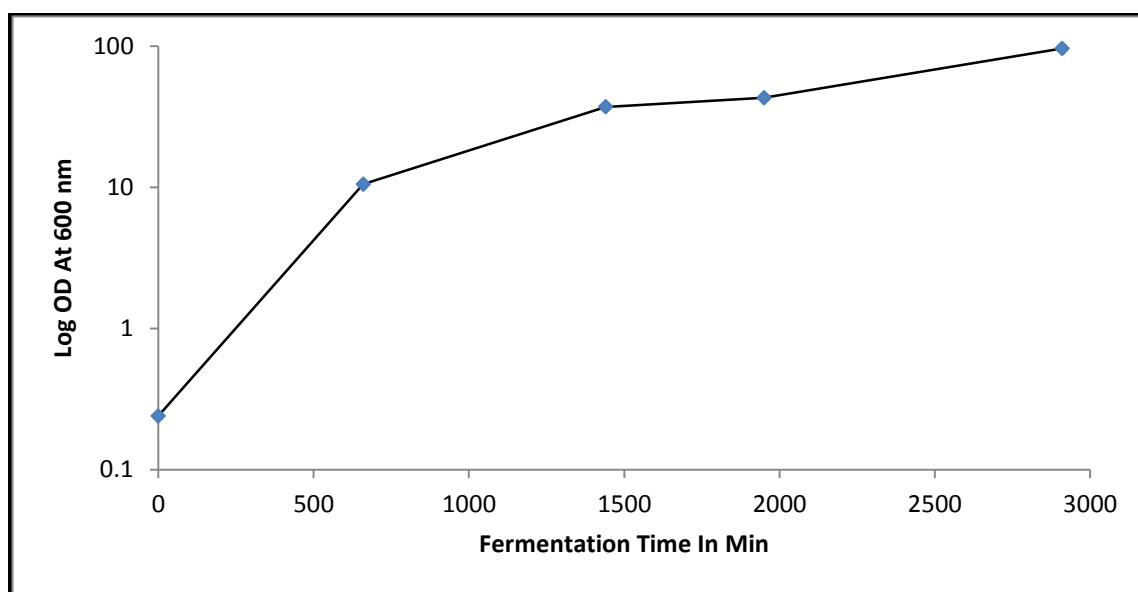


The growth curve, during fermentation, of the strain YH50, in a one-litre ferrmentor. YH50 harbours a plasmid that bears a *GAL1* promoter-driven *CYPB1* expression cassette. The graph initially demonstrates a typical log-phase growth. This was followed by a gradual change to stationary phase. The optical densities became extremely high. Growth started from a 10 ml seed culture grown in selective media, in a shake flask, overnight at 30°C at 220 rpm. The selective media contained, 6.7 $g.L^{-1}$ Yeast Nitrogen Base, 1 $g.L^{-1}$ Casamino acids, 20 $g.L^{-1}$ Glucose, 8.3 $ml.L^{-1}$ and 20 $mg.L^{-1}$ of auxotrophic supplements. After seeding all 10 ml to the 1 litre fermentor, cells were grown in media that contained 16 $g.600\ ml^{-1}$ yeast extract, 16 $g.600\ ml^{-1}$ peptone, 32 ml of 50% (w/v) glucose, 12 ml of adenine 2.5 $g.250ml^{-1}$, 13.5 ml of other auxotrophic supplements (1.8 $g.250ml^{-1}$), together with 45 µl of antifoam and amounts of 5M NaOH so that pH 6.6 is attained. After 12h of fermentation, 24 ml of 50% (w/v) glucose and 24 ml of pure ethanol was added. The same amount of auxotrophic supplements, as above, was also added. After a further 9h of fermentation, 24 g of galactose powder suspended in 12 ml of ethanol and the required auxotrophic supplements were added in the same amounts as above. After a further 16h of induction, fermentation was halted by cooling the vessel to 4ºC. The culture from the fermentor was centrifuged and processed to prepare microsomes using the mechanical cell disruption method.

This culture in the one-litre fermentor was able to produce P450, as seen on the CO-difference assay spectra; see Graph 4.4. 80 units of P450 were produced, as calculated from the CO-binding assay.

However, the total protein content in the microsomes was quite high, 49.2 $µg.µl^{-1}$. The specific content of P450, CYP1B1, was calculated to be 0.119 $nM.mg^{-1}$.

**Graph 4.4    CO-difference assay of CYP1B1, obtained from the strain YH50 (which harbours an episomal plasmid that bears a *GAL1* promoter-driven *CYPB1* expression cassette).**
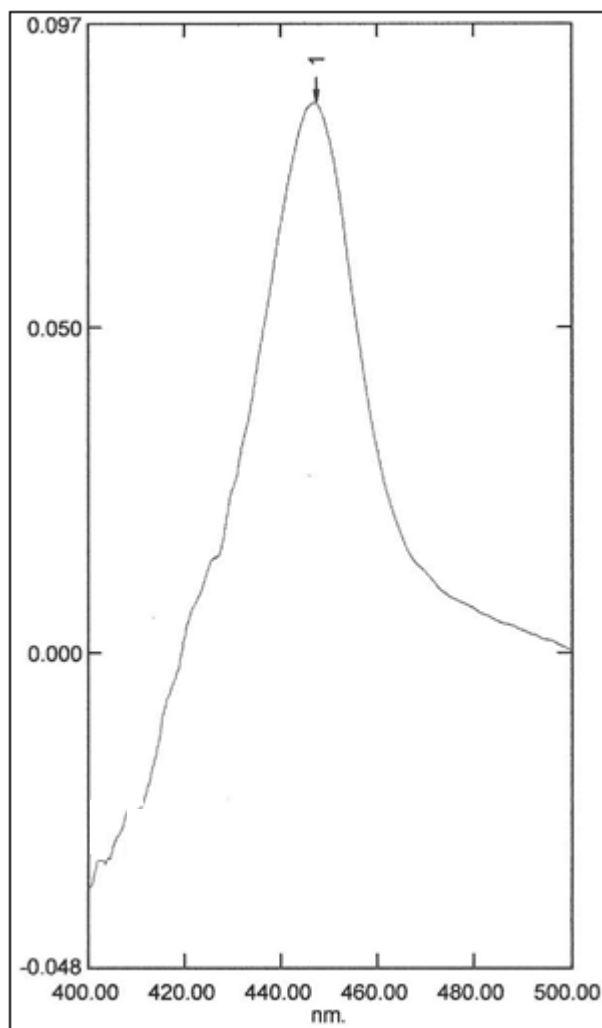


The formation of a CO-P450 peak (position 1, on the spectra) obtained from the CYP1B1, produced in the strain YH50 (harbouring an episomal plasmid that bears a *GAL1* promoter-driven *CYPB1* expression cassette). The microsomes were prepared from the culture grown in a 1 litre fermentor (Graph 4.3). The spectral analysis was performed in a cuvette. 1.5 mg of microsomes were topped up to 1 ml with 100mM potassium phosphate buffer (pH 7.4,) containing 20% glycerol; 5 mg of sodium hydrosulphite was added and scanned from 400 to 500 nm (i.e. this is the 'blank'). After bubbling at 1 bubble per second for 1 min with carbon monoxide (CO), the sample was re-scanned at 400 to 500 nm. The corresponding units were derived from the y-axis absorbance at 450 nm minus the absorbance at the base of the peak towards the 490 nm reference position. There was minimal disturbance of the line at 420 to 430 nm which infers minimal formation of misfolded P450 protein during the production of microsomes.

## 4.6   Summary

The cells from a one-litre fermentor were easily accommodated by the centrifugation methods normally used for shake flasks. The microsomal pellet also had the correct appearance in colour and viscosity, based on previous experience.

Fermentation was extremely successful in terms of biomass. The P450 content was also encouraging. However, the scale-up of the process of fermentation proved to be a bottleneck because it is not feasible to use

(1) Gram quantities of lyticase to lyse large volumes of cells obtained from a fermentor, and

(2) An ultracentrifuge to fractionate the large volumes of lysates obtained from the cells after fermentation.

Therefore, a new protocol involving PEG precipitation was developed to avoid the use of lyticase, in Chapter 5, to help remediate this problem.

The ability to demonstrate that the fermentation process could be scaled-up and downstream processing could be optimised would reduce optimisation time, if circumstances required much larger productions, in the future.

# Chapter 5　　Down-stream processing

## 5.1　Introduction

The aim of this Chapter was to produce a downstream processing protocol that was cost-effective, user-friendly and suitable for larger-scale operations. This would avoid the two critical steps in the preparation of microsomes:

(1) Lyticase treatment, lyticase being extremely expensive for lysis of a large volume of cells, and

(2) Ultracentrifugation, which would be impossible to use with very large volumes of cell lysates.

Although it is known that proteins, which are not bound to membranes, could be conveniently precipitated using a variety of methods, it was suspected that these methods may not be suitable for precipitation of microsomes because of their extremely fragile nature.

## 5.2　An alternative to lyticase treatment

For small scale expressions, up to a couple of litres of culture, it is possible to prepare microsomes using a yeast lytic enzyme (lyticase). Lyticase treatment is usually followed by:

(a) A number of short bursts of sonication in a cold room,

(b) High speed centrifugation, which is followed by

(c) Ultracentrifugation.

Use of yeast lytic enzyme is a problem because it is very expensive and the process of sonication could be lengthy when using large volumes.

There are a wide range of physical and chemical methods that could be used for cell disruption. It was thought that a method, which allows mechanical cell disruption and would disrupt the yeast outer cell wall keeping the fragile microsomal membrane intact, would be preferred to the combined processes of lytication and sonication.

Hence, attempts were made to use a mechanical cell disruptor in order to avoid lyticase treatment and sonication. The cell disruptor was kept chilled at +4ºC and was operated continually for large volumes of samples.

## 5.3 An alternative to ultracentrifugation

A straightforward alternative to ultracentrifugation would be to precipitate the membrane-bound P450s using calcium chloride ($CaCl_2$). In order to precipitate proteins, different final $CaCl_2$ concentrations, centrifugation speeds and run times for centrifugation have been suggested (Peyronneau *et al*., 1992; Scheller *et al*., 1996).

PEG (6000 MW) precipitation of P450s from rabbit liver microsomes has also been known since 1974 (van der Hoeven *et al.*). However, the researchers had noted lack of activity and the P450s had to be reconstituted with lipids. Later, Sadler et al (1985) also attempted PEG precipitations of yeast-produced P450s with no great success.

The published PEG procedures were modified. Precipitation of microsomal P450s was attempted with PEG-3350 (MW 3350) instead of PEGs with higher molecular weight.

## 5.4   Results

### 5.4.1  Results from testing an alternative to yeast lytic enzymes and sonication

The lyticase incubation caused a time-dependent drop in $OD_{600}$ (OD at 600 nm) that corresponded to the gradual destruction of the cell wall, over an hour, with a 79.9% drop in OD.

In contrast, the drop in $OD_{600}$, using mechanical cell disruption, averaged 44.4% for pressures between 15 and 30 KPSI. The cell disruptor is capable of being set at too high a pressure which would destroy all intracellular membranes. It was a case of finding the pressure that caused maximal disruption with minimal microsomal membrane damage.

After sonication of the lyticase treated cells, the samples were processed first with high-speed centrifugation and then ultracentrifugation.

Active CYP1B1 enzymes were produced from CYP1B1-expressing cells (from yeast strain YH50) using

(1) Lyticase/sonication treatment, and

(2) Cell disruption (using a mechanical cell disruptor).

The amounts of P450s obtained were assayed via P450 CO-difference spectroscopy; see Table 5.1.

**Table 5.1    Levels of P450 produced from cell disruption (at different pressures) and lyticase treatment.**

| Pressure setting in KPSI | Specific P450 content in pmol.mg$^{-1}$ of total protein |
|---|---|
| 15 | 29.2 |
| 20.25 | 37.2 |
| 21.75 | 51.9 |
| 25.5 | 43.4 |
| 30 | 31.7 |
| Lyticase | 89.6 |

Using mechanical cell disruption of YH50 cells that harbour an episomal plasmid bearing a *GAL1* promoter-driven *CYP1B1* expression cassette, it was observed that there was an increase in the specific content of the P450 up to the point of 21.75 KPSI in the cell disruptor; extra pressure was not helpful. The mechanical cell disruption was not as successful as the lyticase treatment. However, lyticase treatment cannot be used for large-scale culture because of extraordinary high costs.

Cells were grown in shake flasks at 30°C, 220 rpm in YP media overnight and the contents were combined to give a final $OD_{600}$ of ~20. The cell culture was portioned off for lyticase treatment. 15 g of wet weight cells were re-suspended in 9 ml of sorbitol buffer B (containing sorbitol, Tris and EDTA); protease inhibitors and a reducing agent were added and then cells were incubated with 85 mg of lyticase. The cells were then sonicated. The remaining part of the culture was pelleted (31.8 g) and re-suspended in sorbitol buffer A (sorbitol, Tris and EDTA); 1 ml of buffer was used to re-suspend 1 g of cell pellet; to the re-suspended cells, protease inhibitors and a reducing agent were added before cell disruption. The cells were divided into 5 lots of 7.5 ml volumes and subjected to a range of different pressure settings in the cell disruptor: 15, 20.25, 21.75, 25.5 and 30 KPSI. Microsomes were prepared after all cell disruptions.

Lyticase treatment gave the best specific content. However, the lyticase process is impossible to scale-up due to costs. So the best mechanical disruption pressure setting was considered to be an acceptable compromise with the view that the process could be scaled up to hundreds of litres. After mechanical cell disruption, a lot of undisrupted cells are unfortunately wasted. With the sonication technique, all undisrupted cells are collected for further sonication (i.e. cells that survive lyticase treatment are still challenged by further sonication).

## 5.4.2 Results from testing an alternative to ultracentrifugation

After mechanical disruption of cells, microsomal P450s were precipitated using $CaCl_2$ and PEG-3350. Amounts of microsomal P450s, obtained from the two precipitations, were compared; see Table 5.2.

**Table 5.2     Amounts of P450 produced from two precipitation methods.**

| Concentration method | Specific P450 content in pmol.mg$^{-1}$ of total protein |
|---|---|
| Calcium Chloride | 7.3 |
| PEG-3350 | 102.6 |

Using two different reagents, microsomes were precipitated from YH50 cells that harbour an episomal plasmid bearing a *GAL1* promoter-driven *CYP1B1* expression cassette. It is observed that there is over ten-fold increase in the P450 specific content when PEG-3350 is used for precipitation.

Cells were grown in shake flasks at 30°C, 220 rpm in YP media overnight and the contents of flasks were combined to give a final $OD_{600}$ of ~21. The culture was pelleted and re-suspended sorbitol buffer A (sorbitol, Tris and EDTA buffer) which contained protease inhibitors and reducing agent; 1 ml of buffer was used to re-suspend 1 g of cell pellet; The re-suspended cells were used for cell disruption. The S9 fraction was produced and split into two: (1) for calcium chloride precipitation and (2) for PEG precipitation.

Microsomes were prepared from the S9 fraction as follows. 2 M $CaCl_2$ stock was added to the S9 fraction for a final concentration of 18 mM and centrifuged at 16,000g for 15 min. The concentrations used were obtained from published sources. The $CaCl_2$ concentration range which had been used before was 15 to 20 mM. The centrifugation speed which was published was 14,000g for 10 min to 20,000g for 20 min (Peyronneau *et al*., 1992; Scheller *et al*., 1996).

For the PEG precipitation, ice cold PEG-3350 50% (w/v) was added to a cold Tris, EDTA, sorbitol and sodium chloride buffer suspended S9 fraction. After 20 min, the viscous precipitate was centrifuged at 12,000g at +4°C for 10 min; microsomal pellets were produced and gently re-suspended using glass homogenisers. The microsomal pellets were homogenised in buffer C (containing glycerol, Tris and EDTA).

It was observed from the experiment that precipitation with calcium chloride yielded very little P450. Hence, the process was deemed unsuccessful.

However, PEG precipitation gave very good P450 content and a classically shaped CO-difference P450 spectrum; see Graph 5.1.

**Graph 5.1    CO spectra of CYP1B1-bearing microsomes, after PEG precipitation.**
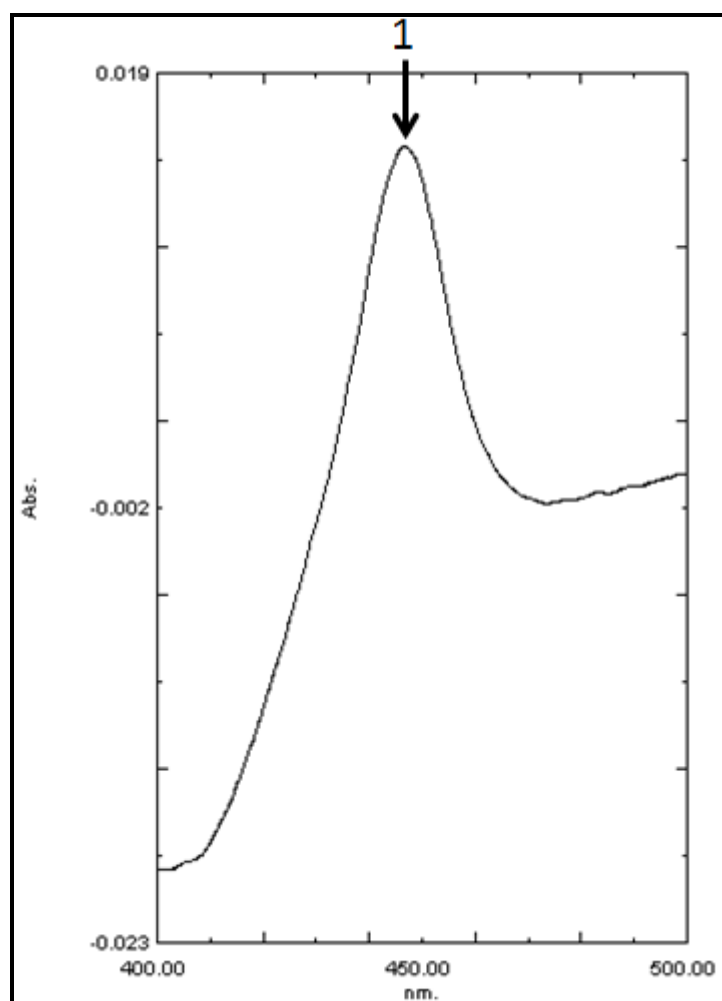


The formation of a CO-P450 peak, at position 1 of the spectra, of CYP1B1 obtained from YH50 cells that harbour an episomal plasmid bearing a *GAL1* promoter-driven *CYP1B1* expression cassette. The spectral analysis was performed in a cuvette; 1.5 mg of microsomes were topped up to 1 ml with 100 mM potassium phosphate buffer (pH 7.4) which contained 20% glycerol; 5 mg of sodium hydrosulphite was added and a blank scan was run from 400 to 500 nm. After bubbling carbon monoxide (CO) at 1 bubble per second for 1 min, the sample was rescanned from 400 to 500 nm. There was minimal disturbance of the line at 420 to 430 nm which implies that minimal misfolded P450 was formed during preparation of the microsomes.

## 5.5    Discussion

It was expected that the mechanical cell disruption, at a certain pressure, would produce a large amount of cells that still remained undisrupted. But this was acceptable, since

the process could conceivably deal with hundreds of litres in several hours. The process involving lyticase and sonication is definitely not an alternative because of its extremely high costs and also being so labour-intensive.

The fact that the $CaCl_2$ precipitation did not work was unexpected. It was disappointing as it would have been a very simple and cheap procedure.

PEG precipitation is more involved than the $CaCl_2$ procedure but it has proved to be very successful. Compared to the process that used ultracentrifugation, the PEG precipitation process has greatly reduced the overall time for making microsomes. It should be pointed out that maintenance of an ultracentrifuge (or multiple ultracentrifuges) would have been extremely costly. The ability to precipitate microsomes with PEG offers the potential of running multiple precipitations per day to process large amounts of S9 fractions obtained after mechanical cell disruption. With a continuous-flow centrifuge this process could even further be streamlined.

# Chapter 6     Stabilisation of P450s via lyophilisation

## 6.0 Introduction

During the processes of production and using microsomes for assaying, it was apparent that there are many ways of causing damage to the microsomes or the P450 enzymes themselves. This could be due to mechanical, thermal or chemical stresses. Different P450s would be expected to react similarly to these stresses. Despite their great genetic variation, they are essentially proteins with overall similar tertiary structures (Denisov *et al.*, 2011).

It has been demonstrated that there are two critical features that are essential for P450 enzyme activity:

    (1) Association with membranes and

    (2) Retention of the haem group.

P450s require interactions with lipids to place them in proximity with their typical exogenous and endogenous substrates. P450s also require access to water. It is intriguing that the lipids do not, in any way, inhibit movement of water towards the active site of P450s (Denisov *et al.*, 2011).

The lipids allow correct orientation of the P450 reductase and the P450 proteins, see Figure 6.1. Clearly, membrane-association of P450s and the co-activator protein P450 reductase has both a functional as well as a structural role.

**Figure 6.1    A P450 Microsomal structure (Reed & Backes, 2012).**
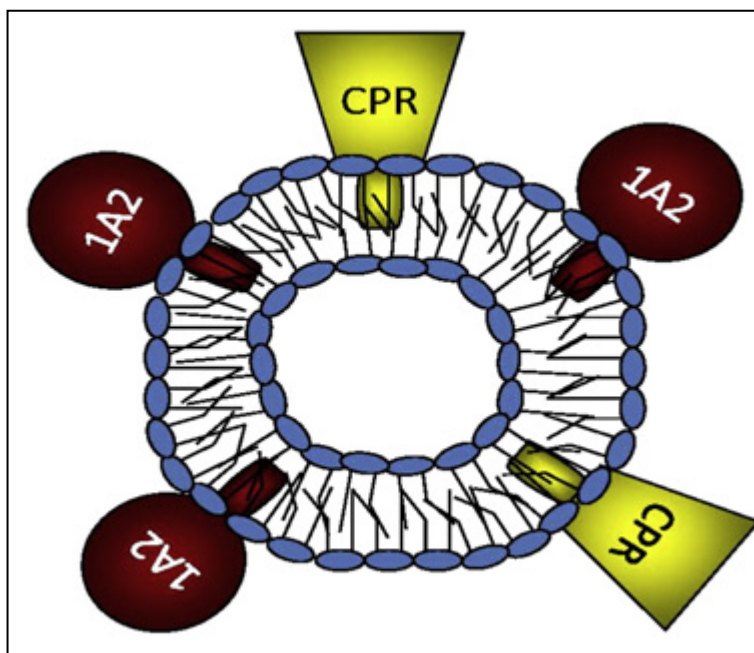


The blue ovals represent the hydrophilic phospholipid heads that have arranged themselves into a spherical lipid bi-layer with their hydrophobic hydrocarbon black tails pointing inwards. The P450s, in this case 1A2, have an N-terminal lipid binding region. The rest of the P450 protein has access to the water soluble buffer partition. The cytochrome P450 reductase (CPR) is attached in the same way. Evidence points to the fact that the P450 and its reductase should be on the same microsomal membrane for proper P450 activity. It is important to think of a microsomal sample as a collection of these structures in solution. Each of these is likely to have a variable size and slightly different ratios of lipids, general microsomal proteins, P450, P450 reductase and b5 proteins (not shown). During an assay, the use of a representative sample size of this population would reduce sample to sample variation. The yeast microsomes provide the best representative state of a human P450, as if it was in its native environment bound to the smooth endoplasmic reticulum membranes.

When considering stability of P450 activity the relationship between membrane, P450 and P450 reductase (and/or $b_5$) needs to be considered. The relationship with water in the active site, where the haem resides, is also important to form the inter-linked structure with a P450 as described in the Introduction (see Section 1.8). Water is required in the enzyme's active site and also to allow formation of the three-dimensional bilayer structure.

Matejtschuk (2007) states that membrane-bound proteins have special problems during lyophilisation He also mentions that "intracellular membranes are prone to disruption during dehydration and structures will be at risk".

Heikal *et al*., (2009) researched P-glycoprotein, a cell membrane pump, overexpression of which causes resistance to anticancer drugs. The authors were interested in establishing a high-throughput screen. They noted that the protein is only stable for short periods at -80°C and required stabilisation to allow design of a high-throughput assay. The P-glycoprotein was purified and reconstituted onto artificial membranes. The paper states that the instability of membrane-bound proteins is well known. The authors also add that freeze-drying of liposomes is well documented but it is not applicable to membrane-bound microsomal proteins. Moreover, there has been no reported success in freeze-drying protein-embedded liposomes (i.e. proteo-liposomes).

Like P450s, P-glycoprotein has a complex relationship with its lipid host. Heikal *et al*., (2009) found that the presence of either 20% trehalose or maltose allowed 60% of activity to be retained after 150 days, at 30 to 37°C. They also found glycerol to be very poor, allowing retention of less than 5% of the original activity. This is in contrast to commercial microsomes that are frozen in buffer containing glycerol for storage at minus 80°C.

P450-bearing microsomes are shipped on dry ice and the product data sheets encourage minimal freeze-thawing events. One way to avoid the use of the cold chain is to lyophilise proteins. However, it was expected that this process, for the reasons detailed above, would destroy P450 activity. The use of sugars has been known to thermo-

stabilise P450s to prevent formation of apoprotein at up to 48°C. This was encouraging but only shown to preserve structure, activity had not been tested (Kuhm-Velten, 1997).

## 6.1 Comparison of CYP2D6 activities, co-expressed with two different reductases, and lyophilised in the presence of two different cryopreservants

It was investigated if there would be any differences in CYP2D6 activity when microsomal CYP2D6 (derived from both insect and yeast cells) was lyophilised in the presence of glycerol and sucrose (two different cryopreservants). Two different preparations of CYP2D6 were used for lyophilisation, one enzyme co-expressed with the yeast reductase (hRD) and the other co-expressed with ΔhRDM, a relatively low-toxic variant of wild-type human P450 reductase (hRD). The aim was to see if relative reductase activities (hRD being slightly better in activity than ΔhRDM) would lead to differential loss of stability; see Graphs 6.1 to 6.2.

**Graph 6.1    Comparison of insect cell-derived CYP2D6 activity co-expressed with hRD (prepared in-house), lyophilised in the presence of two different cryopreservants, and after keeping lyophilised samples for 40h at +21°C.**
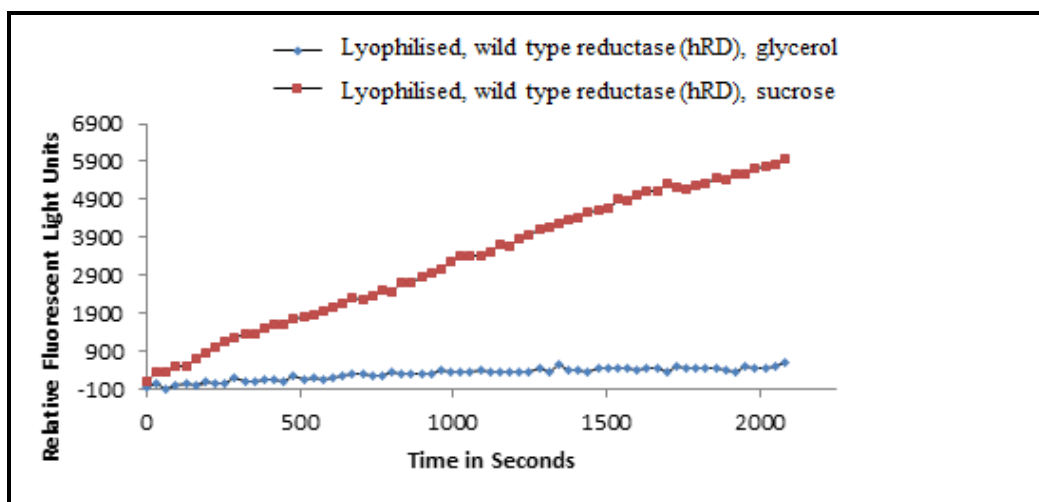


2.5 pmol of insect cell-derived CYP2D6 (prepared in-house), co-expressed with wild type P450 reductase (hRD), was lyophilised after re-suspension in 100 mM potassium phosphate buffer (pH 7.6), in the presence of either 20% (w/v) sucrose or glycerol (two different cryopreservants), in wells of a microtitre plate. These were stored at +21°C for 40h before rehydration and testing. Activity was measured by kinetic analysis of AMMC turnover at Excitation wavelength 400 nm/Emission 460 nm in a 100 mM potassium phosphate pH 7.6 at 37°C. The regenerating system was the non-standard A* for use with AMMC. The kinetics demonstrates that glycerol was not effective as a P450 cryopreservant when the P450 was co-expressed with wild-type P450 reductase, hRD. The sucrose samples had 9.3 times more activity than the glycerol samples.

**Graph 6.2    Comparison of insect cell-derived CYP2D6 activity co-expressed with ΔhRDM (prepared in-house), lyophilised in the presence of two different cryopreservants, and after keeping lyophilised samples for 40h at +21°C.**



2.5 pmol of insect cell-derived CYP2D6 (prepared in-house), co-expressed with the P450 reductase variant (ΔhRDM), was lyophilised after re-suspension in 100 mM potassium phosphate buffer (pH 7.6), in the presence of either 20% (w/v) sucrose or glycerol (two different cryopreservants), in wells of a microtitre plate. These were stored at +21°C for 40h before rehydration and testing. Activity was measured by kinetic analysis of AMMC turnover at Excitation wavelength 400 nm/Emission 460 nm in a 100 mM potassium phosphate pH 7.6 at 37°C. The regenerating system was the non-standard A* for use with AMMC. The kinetics demonstrates, once again, that glycerol was not effective as a P450 cryopreservant when the P450 was co-expressed with the P450 reductase variant, ΔhRDM. The sucrose samples had 3.25 times more activity than the glycerol samples. The glycerol samples from both Graphs 6.1 and 6.2 are nearly identical in rates of reaction. However, the sucrose samples of the wild type P450 reductase (Graph 6.1) had a 2.9 times greater rate of reaction of the ΔhRDM (Graph 6.2).

It was concluded that the glycerol buffer was much poorer than the sucrose buffer at retaining CYP2D6 activity after 40h at +21°C, post-lyophilisation. This was regardless of the P450 reductase co-expressed with CYP2D6, hRD (wild-type human P450 reductase) or ΔhRM (the P450 reductase variant).

After establishing that lyophilisation in the presence of sucrose was far better than in the presence of glycerol, lyophilized CYP2D6 enzyme, individually co-expressed with the

two P450 reductases, were tested against the untreated samples which had been stored at

-80°C; see Graphs 6.3 and 6.4.

**Graph 6.3    Comparison of activity of insect cell-derived CYP2D6 enzyme, co-expressed with hRD (prepared in-house), before and after lyophilisation (in the presence of sucrose), after having kept the lyophilised samples for 40h at +21°C.**



2.5 pmol of insect cell-derived CYP2D6 (prepared in-house), co-expressed with the P450 reductase variant (hRD), was lyophilised after re-suspension in 100 mM potassium phosphate buffer (pH 7.6), in the presence of 20% (w/v) sucrose, in wells of a microtitre plate. These were stored at +21°C for 40h before rehydration and testing. The Control was the enzyme stored at -80°C which was also assayed for activity, in the presence of sucrose. Activity was measured by kinetic analysis of AMMC turnover at Excitation wavelength 400 nm/Emission 460 nm in a 100 mM potassium phosphate pH 7.6 at 37°C. The regenerating system was the non-standard A* for use with AMMC. The kinetics demonstrates that that sucrose was effective as a P450 cryopreservant when the P450 was co-expressed with the wild-type P450 reductase, hRD.

**Graph 6.4    Comparison of activity of insect cell-derived CYP2D6 enzyme, co-expressed with ΔhRDM (prepared in-house), before and after lyophilisation (in the presence of sucrose), after having kept the lyophilised samples for 40h at +21°C.**
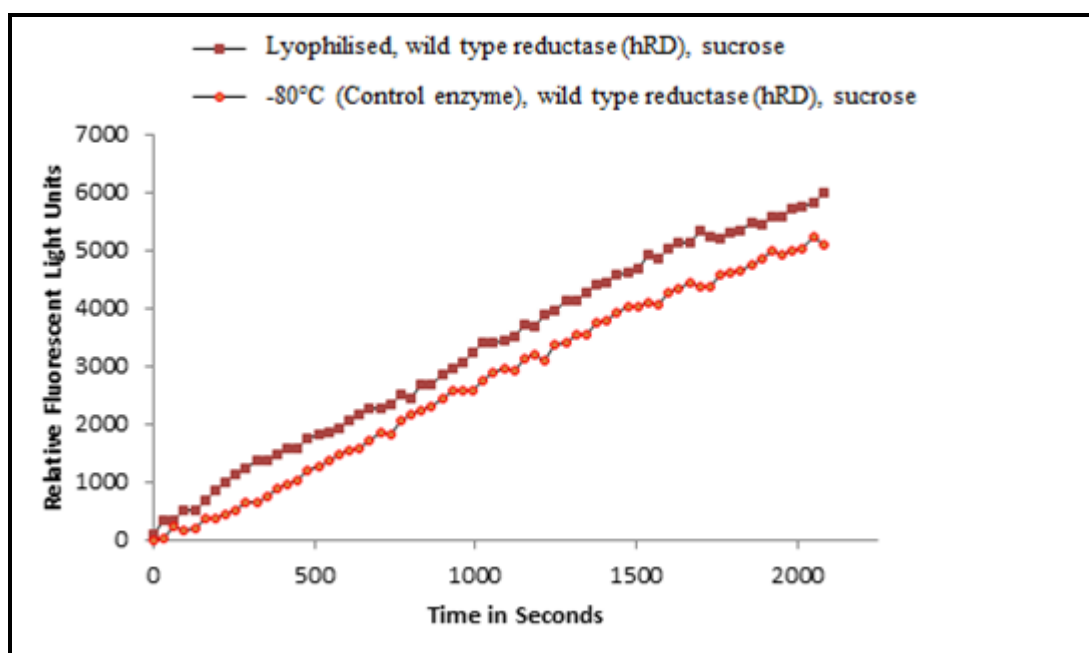


2.5 pmol of insect cell-derived CYP2D6 (prepared in-house), co-expressed with the P450 reductase variant (ΔhRDM), was lyophilised after re-suspension in 100 mM potassium phosphate buffer (pH 7.6), in the presence of 20% (w/v) sucrose, in wells of a microtitre plate. These were stored at +21°C for 40h before rehydration and testing. The Control was the enzyme stored at -80°C which was also assayed for activity, in the presence of sucrose. Activity was measured by kinetic analysis of AMMC turnover at Excitation wavelength 400 nm/Emission 460 nm in a 100 mM potassium phosphate pH 7.6 at 37°C. The regenerating system was the non-standard A* for use with AMMC. The kinetics demonstrates that that sucrose was effective as a P450 cryopreservant when the P450 was co-expressed with the P450 reductase variant, ΔhRDM.

The activity of CYP2D6, co-expressed with the wild type human P450 reductase (hRD) or the human P450 reductase variant (ΔhRDM), 40h after keeping at +21°C post-lyophilisation, compared with untreated –80°C stock samples (i.e. enzymes that had been stored at –80°C) show that lyophilsation does not have any effect on CYP2D6 microsomal activity.

## 6.2 Conclusions from experiments that compared the impact of two different cryopreservants on CYPs, co-expressed with two different reductases

The experiments in Section 6.1 show that the product obtained after lyophilisation in the presence of 20% (v/v) concentration of glycerol was clearly much inferior to that obtained after lyophilisation in the presence of 20% (w/v) sucrose. Neither P450 reductase had an advantage over the other regarding post-lyophilisation activities. The human P450 reductase variant, ΔhRDM, had been shown earlier in our group to be far less toxic than the wild type reductase, hRD during yeast expression. However, in these insect cell microsomes the reverse seemed to occur.

The ratios of CYP2D6 activity loss between sucrose and glycerol were not equal for both types of reductase. The difference was that the sucrose was 9.3 times more CYP2D6 active than the glycerol samples with the wild type P450 reductase. For the ΔhRDM P450 reductase the sucrose samples were 3.25 times more active with CYP2D6 than the glycerol samples. The glycerol samples for both P450s reductases had nearly identical CYP2D6 activities.

The results would indicate that:

(a) Reductase type plays no role in the stability of P450 enzyme activity during the process of lyophilisation;

(b) More importantly, the lyophilised samples retained the activities seen in untreated enzymes that had been stored at -80°C;

(c) As the wild type P450 reductase and the ΔhRDM were both reduced to nearly identical CYP2D6 activities (and in light of point (a) above), the loss of CYP2D6 activity to a basal level was due to the use of glycerol rather than specific to P450 reductase type. It was just the case that the wild type P450 reductase produced 2.9 times more CYP2D6 activity that the ΔhRDM.

(d) The process works with Insect cell expressed P450s.

## 6.3 Experiments to determine if the 'stabilisation of microsomes' was specific to the P450-bearing microsomes, produced in yeast and insect cells at De Montfort University

There are many different ways of preparing recombinant microsomal P450 enzymes. Depending on the organism in which the enzymes are produced, the downstream production techniques vary. Often the reagents used during the process of isolation of microsomal enzymes are different. Moreover, each commercial producer has their unique buffers to re-suspend their microsomes. Hence, it would be apt to consider if the outcome of lyophilisation would be altered if lyophilisation was performed with microsomal P450s obtained from different sources, for example, with the enzymes obtained commercially from the leading P450 manufacturers,

(a) BD-Gentest,

(b) Invitrogen, and

(c) Cypex.

The comparative analysis was performed on enzymes that had been kept for 24h at 21°C, after lyophilisation.

After 24h at +21°C, the activity of the lyophilised Gentest CYP2C8 enzyme was preserved, implying that the activity of the lyophilised enzyme remained unchanged from the enzyme that had continuously been stored at -80°C; see Graph 6.5.

**Graph 6.5   Comparison of lyophilised BD-Gentest's CYP2C8 enzyme, kept for 24h at +21˚C, with enzyme stored at -80°C.**
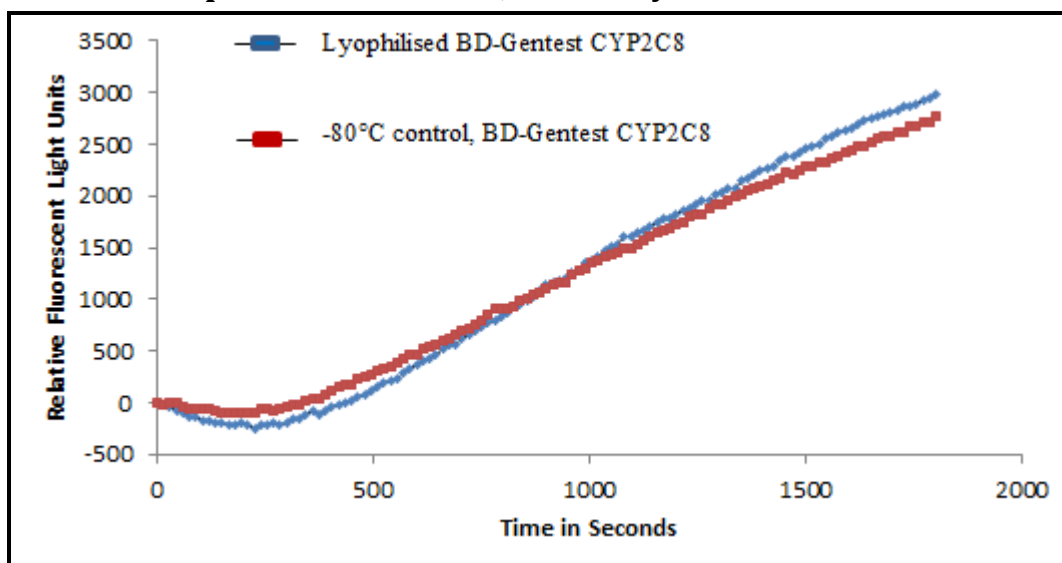


4 pmol of BD-Gentest's CYP2C8 microsomal enzyme (produced from insect cells; also referred to as 'Supersomes') was first diluted in a 100mM potassium phosphate buffer (pH 7.6) which contained 20% (w/v) sucrose, and then aliquoted into wells of a 96-well microtitre plate. The contents of the plate were lyophilised. After lyophilisation, the plate was stored at +21°C for 24h before rehydration and testing against Gentest's CYP2C8 microsomal sample that had been stored at -80°C. Activity was measured by kinetic analysis of DBF turnover at Excitation wavelength 530 nm/Emission wavelength 590 nm in a 100mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the standard A.

The kinetics in Graph 6.5 demonstrates that sucrose was effective in preserving activity post-lyophilisation of a BD Gentest's microsomal P450 which had been isolated from insect cells.

Graph 6.6 shows that, similar to the preservation of BD Gentest's microsomal P450 enzyme after lyophilisation, Cypex's CYP2C8 activity could also be preserved after keeping the lyophilised plate for 24h at +21°C.

**Graph 6.6   Comparison of lyophilised Cypex's CYP2C8 enzyme, kept for 24h at +21°C, with enzyme stored at -80°C.**



4 pmol of Cypex's CYP2C8 microsomal enzyme (produced from bacterial cells; also referred to as 'Bactosomes') was first diluted in a 100mM potassium phosphate buffer (pH 7.6) which contained 20% (w/v) sucrose, and then aliquoted into wells of a 96-well microtitre plate. The contents of the plate were lyophilised. After lyophilisation, the plate was stored at +21°C for 24h before rehydration and testing against Cypex's CYP2C8 microsomal sample that had been stored at -80°C. Activity was measured by kinetic analysis of DBF turnover at Excitation wavelength 530 nm/Emission wavelength 590 nm in a 100 mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the standard A.

Graph 6.7 shows that, after keeping 24h at +21°C, the activity of lyophilised CYP2C8-bearing microsomes (prepared in-house) surprisingly increased when compared with the control which is the CYP2C8 enzyme that had been stored at -80°C.

**Graph 6.7    Comparison of lyophilised yeast-derived CYP2C8 enzyme (prepared in-house), kept for 24h at +21°C, with enzyme stored at -80°C.**
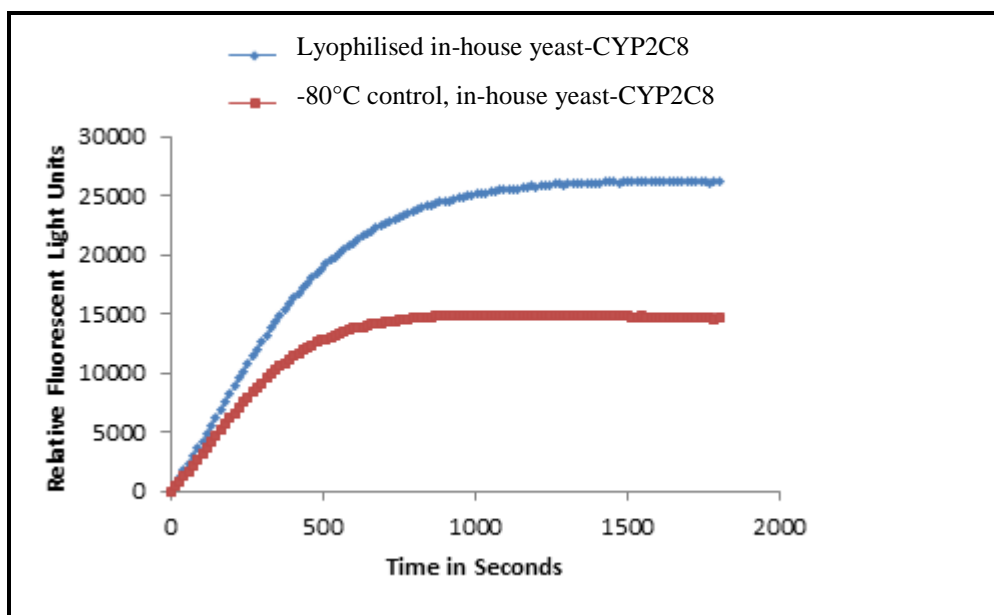


4 pmol of in-house CYP2C8 microsomal enzyme (produced from yeast cells; also referred to as 'Sacchrosomes') was first diluted in a 100 mM potassium phosphate buffer (pH 7.6) which contained 20% (w/v) sucrose, and then aliquoted into wells of a 96-well microtitre plate. The contents of the plate were lyophilised. After lyophilisation, the plate was stored at +21°C for 24h before rehydration and testing against yeast-CYP2C8 microsomal sample that had been stored at -80°C. Activity was measured by kinetic analysis of DBF turnover at Excitation wavelength 530 nm/Emission wavelength 590 nm in a 100 mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the standard A.

Graph 6.8 shows that, after 24h at +21°C, the activity of the sucrose-treated and

lyophilised CYP2D6, obtained from Invitrogen, had similar activity to that of the

untreated -80°C control.

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**Graph 6.8    Comparison of lyophilised Invitrogen's CYP2D6 enzyme, kept for 24h at +21°C, with enzyme stored at -80°C.**
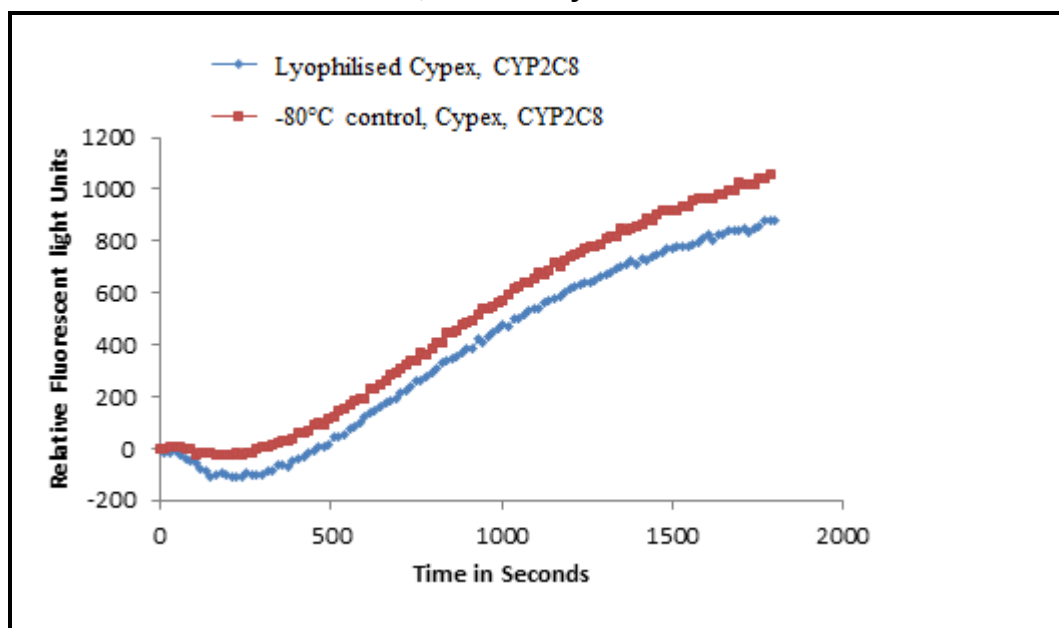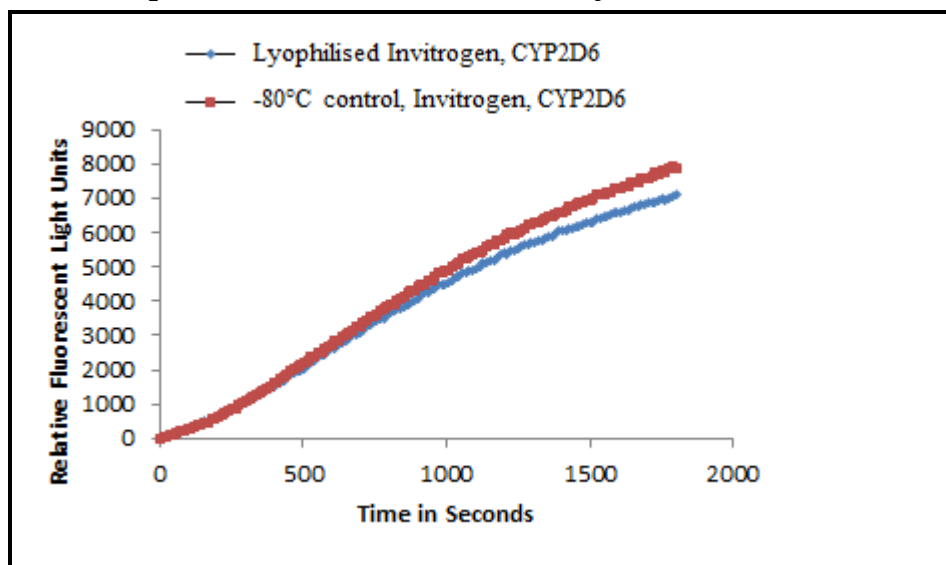


2.5 pmol of Invitrogen CYP2D6 microsomal enzyme (produced from insect cells; also referred to as 'Baculosomes') was first diluted in a 100mM potassium phosphate buffer (pH 7.6) which contained 20% (w/v) sucrose, and then aliquoted into wells of a 96-well microtitre plate. The contents of the plate were lyophilised. After lyophilisation, the plate was stored at +21°C for 24h before rehydration and testing against Invitrogen's CYP2D6 microsomal sample that had been stored at -80°C. Activity was measured by kinetic analysis of AMMC turnover at Excitation wavelength 400 nm/Emission wavelength 460 nm in a 100mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the non-standard A* for use with AMMC.

The kinetics in Graph 6.8 demonstrated once again that sucrose was as effective as a

P450 cryopreservant with Invitrogen's CYP2D6 as it had been with CYP2C8

microsomal samples, obtained from three different sources.

## 6.4    Conclusions from experiments that determined if the 'stabilisation of microsomes' was specific to yeast and insect cell-produced microsomes prepared at De Montfort University

The most obvious feature of the data obtained above was that the in-house yeast and

insect derived CYP-bearing microsomes were not unique. Activity could be maintained

after lyophilisation of all microsomal samples, whatever their source (bacteria, insect or yeast cells). Different expression systems could have yielded different levels of recovery. This would be because of:

(1) The tensile strength of intra- and outer-cellular membranes in different organisms, very likely, is different.

(2) The proteins that constitute the microsomes in different organisms are definitely different.

(3) Moreover, the physico-chemical properties of different reagents used in the isolation of microsomes from different organisms are also different.

All these could have affected recovery of P450 activity after lyophilisation.

Two of the commercial microsomes (from BD-Gentest and Invitrogen) are produced from insect cells whereas the Cypex enzymes are derived from a bacterial expression system. Insect cell microsomes are similar to yeast cell microsomes in that they both belong to the intracellular environment of eukaryotic cells. It would be reasonable to expect that the architecture of microsomes derived from all eukaryotic cells would be similar although the numbers and the functions of the proteins contained in the microsomes could be very diverse.

Bacterial cells, being prokaryotic, are quite different from eukaryotes in the sense that bacteria do not contain intra-cellular compartments. Since they do not contain the endoplasmic reticular membranes, the P450s (with reductases and $b_5$) are usually purified from the cell and reconstituted as membrane-bound proteins using artificial membranes.

A difference between yeast and insect cells is that membrane fluidity in the two types of cells is modified by ergosterol and cholesterol, respectively. Further research should be able to determine if alteration of these sterols influence membrane fluidity and whether these alterations affect the process of lyophilisation (Chen, *et al*., 2010). Normally, the endoplasmic reticulum does not contain these sterols. They are mostly outer cell membrane agents. However, cholesterol is often found in the preparation of microsomes (Brenner, 1990). There seems to be no clear cut difference between retention and loss of activities between the in-house yeast and commercial insect cell-derived microsomes post-lyophilisation. This is probably because the endogenous levels of sterols in the microsomes are too low.

The storage buffers, used in different commercial preparations, are all variations on a similar theme. However, there are some differences in the concentrations of the components of the buffers (see Table 6.1):

- Three of the four microsome producers use glycerol at 20% (v/v). The other uses sucrose in its place at 250 mM which is 8.6 % (w/v).

- All use Tris buffer but there is a five-fold difference between concentrations used, 10 mM to 50 mM, with little pH variance (usually between 7.4 and 7.6).

- The buffers have a range of EDTA concentrations of between 0.1 mM and 1 mM, a ten-fold difference.

It was also a surprise that various suppliers recommend very specific buffers for individual CYP-specific activity assays.

**Table 6.1.** **Components of buffers used by 3 commercial suppliers for re-suspension of their microsomes compared to that used in the preparation of in-house yeast microsomes.**

| Reagent | Invitrogen | Cypex | BD Gentest | In-house yeast |
|---|---|---|---|---|
| Glycerol | 20% | 0 | 20% | 20% |
| Sucrose | 0 | 8.6% (250 mM) | 0 | 0 |
| Potassium Phosphate | 100 mM (pH 7.5) | 0 | 0 | 0 |
| Tris | 0 | 50 mM (pH 7.6) | 10 mM (pH 7.4) | 100 mM (pH 7.5) |
| EDTA | 0.1 mM | 0.25 mM | 1 mM | 0 |
| DTT | 1 mM | 0 | 0 | 0 |

A range of similar buffers are used by different manufacturers for re-suspension of their microsomes before supplied as frozen samples, on dry ice, before their use. This has been sourced from supplier-provided product data sheets.

In order to obtain optimal levels of activities for each P450, a manufacturer re-suspends a P450 in buffers where the volumes of different components differ, sometimes greatly, depending on a particular P450. Hence, there are differences in the buffer contents used for re-suspension of P450s by the three suppliers which may get carried over into the final lyophilised product.

Some P450s received less added cryopreservants and contained more of the formulation buffer, respectively, in the following order CYP3A4, CYP2D6 and CYP2C8. The specific numbers for this may be seen in Table 6.2 which depicts the "differences in the assay well contents due to the nature of the microsome samples and differences in assay optimisation".

It could be argued that certain samples may recover activity better than others. Since there are only subtle variances in the buffer types, mixtures and concentrations, it is difficult to envisage that there would be a sudden disastrous loss of activity. This may suggest three things:

(1) The formulation buffers that the microsomes are supplied in may contribute little to lyophilisation.

(2) CYP2C8 data implies that the ratios of P450 to sugar are more or less appropriate.

(3) Since the Invitrogen microsomes and the yeast-derived microsomes, prepared in-house, use the same buffers it cannot clearly be stated that there are endogenous factors that provide advantages towards lyophilisation of either the insect or yeast microsomes.

**Table 6.2.   Differences in the assay well content due to the nature of the microsome samples and differences in assay optimisations.**

| Sample CYPs | Ratio of microsome volume to sucrose buffer | % microsome to sucrose buffer | µg of sucrose per well | [Protein] in mg.ml$^{-1}$ | P450 specific content in nmol.mg$^{-1}$ | Ratio of protein to sucrose | Ratio of sucrose to specific content | Ratio of sucrose to pmol of P450 |
|---|---|---|---|---|---|---|---|---|
| 2D6 Invitrogen | 0.4 | 27.8 | 1440 | 4.2 | 0.22 | 345.3 | 6666.7 | 576 |
| 2C8 Gentest | 0.7 | 40 | 1200 | 3.5 | 0.29 | 342.9 | 4200.0 | 300 |
| 2C8 Cypex | 0.8 | 44 | 1100 | 6.0 | 0.15 | 186.7 | 7383.0 | 280 |
| In-house 2C8−yeast | 0.7 | 40 | 1200 | 4.1 | 0.24 | 292.7 | 4926.1 | 300 |

These values represent the final formulation of the named reagents in the microplate well for the P450 activity assays. There was a lot of variability in the different components of these microplates and this was because of the nature of the product formulations.

## 6.5 Experiments to analyse P450 activities in lyophilised yeast microsomal samples, prepared in-house, after keeping the lyophilised products over longer periods of time at +21°C

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP1A1 demonstrated

25% loss of activity compared to the untreated -80°C control; Graph 6.9.

## Graph 6.9 Analysis of sucrose-treated, lyophilised CYP1A1, after 24 days at +21°C.
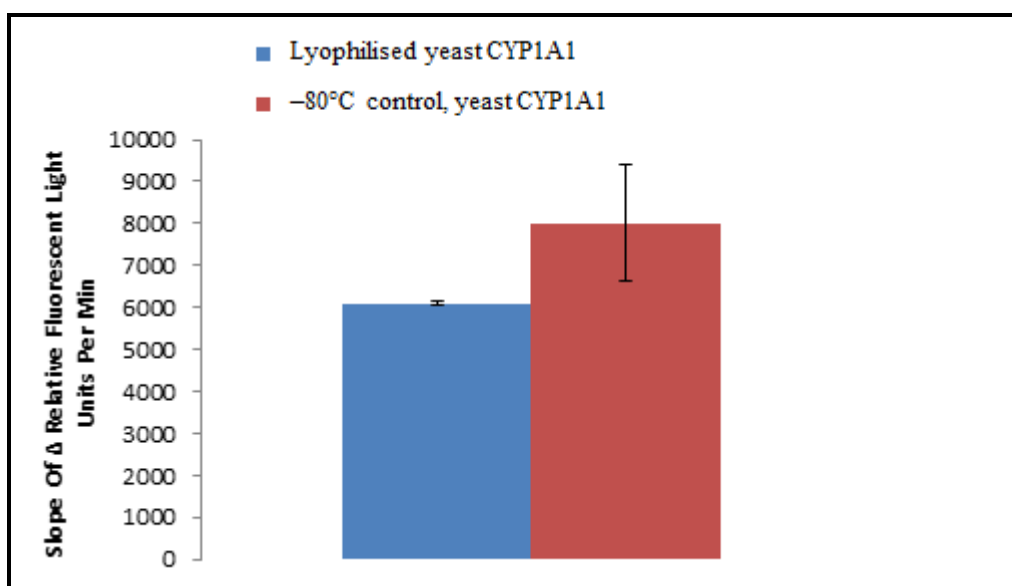


1.5 pmol of yeast CYP1A1 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of EROD turnover at Ex 530 nm/Em 590 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and demonstrate that sucrose was quite effective as a P450 cryopreservant when compared with the untreated -80°C control, taking into account the error bars, +/- 1x standard deviations. However, the mean loss in activity was approximately 25%.

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP1A2 demonstrated

40% loss of activity compared to the untreated -80°C control; Graph 6.10.

**Graph 6.10  Analysis of sucrose-treated, lyophilised CYP1A2, after 24 days at +21°C.**
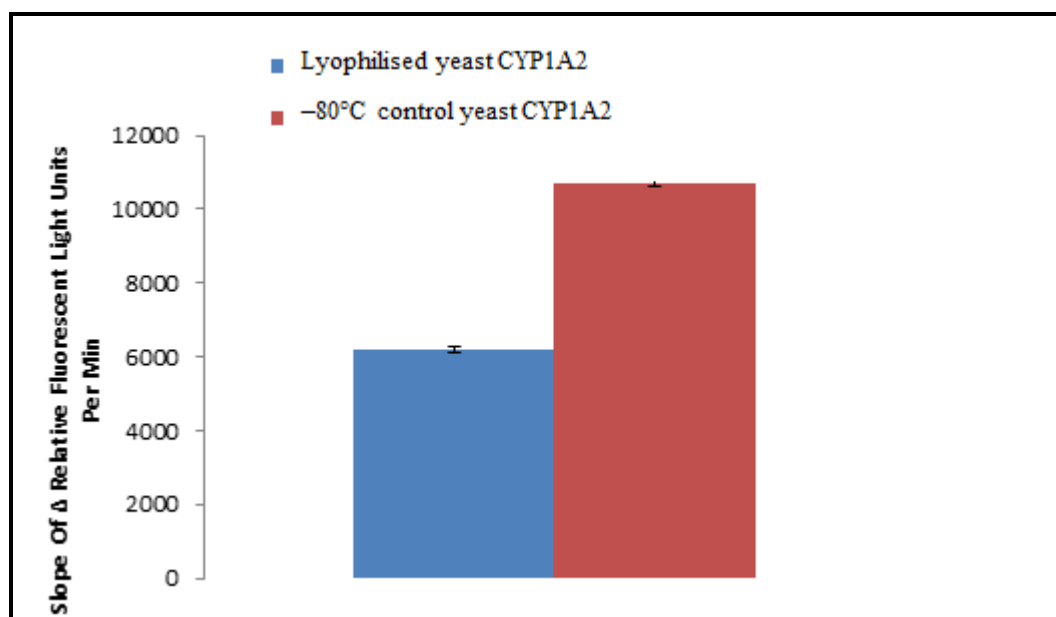


1 pmol of yeast CYP1A2 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of CEC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and demonstrate that lyophilisation in the presence of sucrose, as a P450 cryopreservant, lost about 40% activity compared to untreated -80°C control. Error bars are +/- 1x standard deviation.

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP1B1 demonstrated

30% loss of activity compared to the untreated -80°C control; Graph 6.11.

**Graph 6.11  Analysis of sucrose-treated, lyophilised CYP1B1, after 24 days at +21°C.**
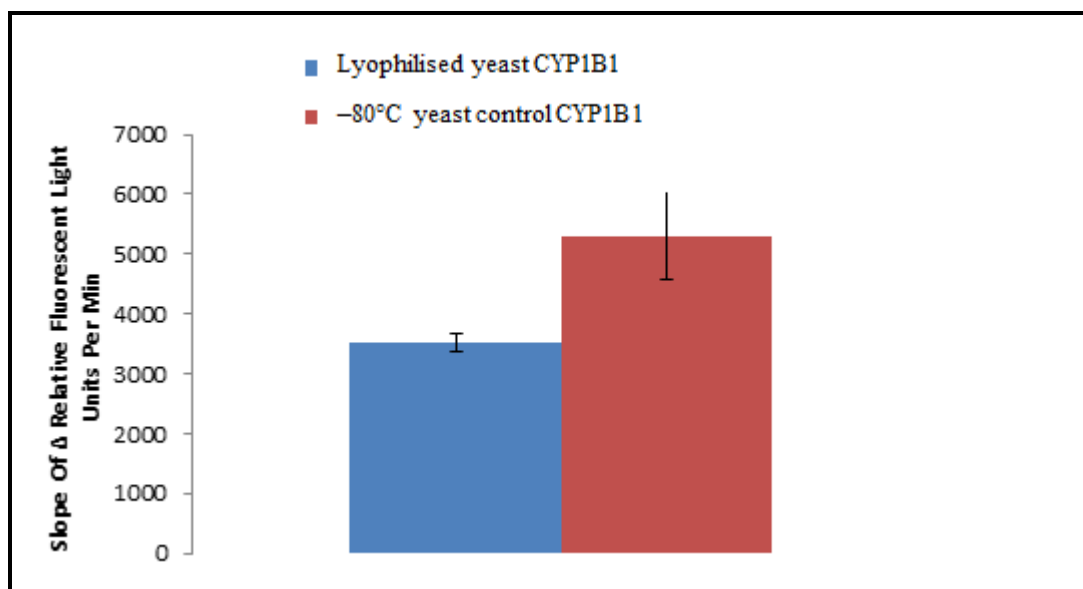


1.5 pmol of yeast CYP1B1 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of EROD turnover at Ex 530 nm/Em 590 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and demonstrate that sucrose was mostly effective as a P450 cryopreservant when compared with the untreated -80°C control, taking into account the error bars are +/- 1x standard deviations. However, the mean loss in activity was approximately 30%.

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP2A6 demonstrated

30% 'increase' of activity compared to the untreated -80°C control; Graph 6.12.

**Graph 6.12.** **Analysis of sucrose-treated, lyophilised CYP2A6, after 24 days at +21°C.**
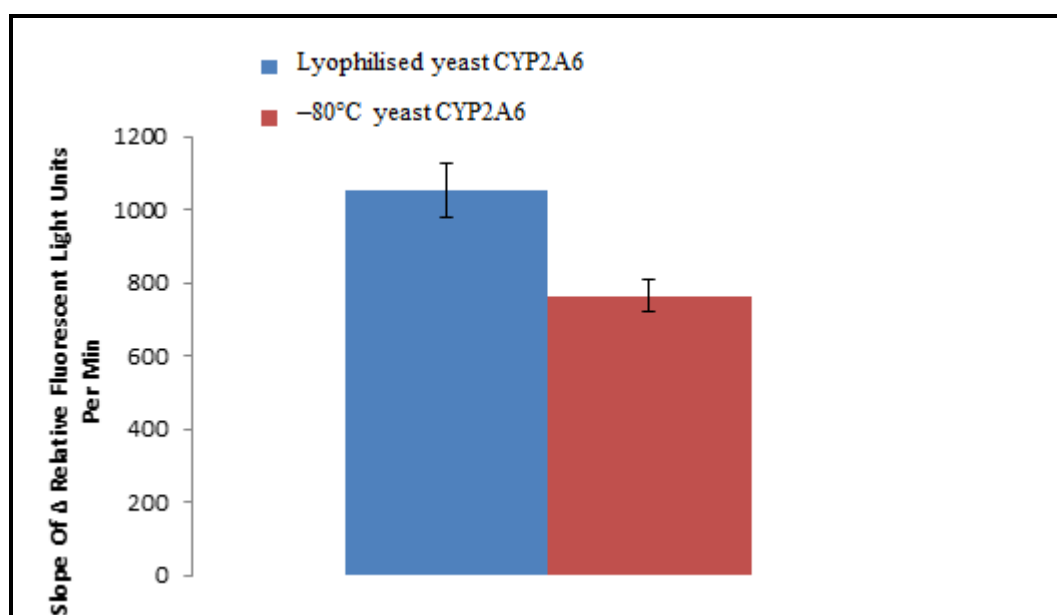


1 pmol of yeast CYP2A6 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of Coumarin turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the non-standard A*. The rates of reactions are shown and demonstrate that sucrose was very effective as a P450 cryopreservant when compared with the untreated -80°C control, error bars are +/- 1x standard deviations. The mean *gain in activity* was approximately 30%.

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP2C8 a poor retention

of activity, losing approximately 75% of activity compared to the untreated -80°C

control; Graph 6.13.

**Graph 6.13.     Analysis of sucrose-treated, lyophilised CYP2C8, after 24 days at +21°C.**



4 pmol of yeast CYP2C8 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of DBF turnover at Ex 485 nm/Em 530 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and demonstrate that sucrose was not effective as a P450 cryopreservant when compared with the untreated -80°C control, error bars are +/- 1x standard deviations. The mean value loss in activity was approximately 75%.

After 24 days at +21°C, the sucrose-treated, lyophilised yeast CYP2D6 demonstrated

minimal loss of activity against the untreated -80°C control; Graph 6.14.

**Graph 6.14  Analysis of sucrose-treated, lyophilised CYP2D6, after 24 days at +21°C.**



2.5 pmol of yeast CYP2D6 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of AMMC turnover at Ex 485 nm/Em 530 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the non-standard A*. The rates of reactions are shown and demonstrate that sucrose was effective as a P450 cryopreservant when compared with the untreated -80°C control, error bars are +/- 1x standard deviations. The mean loss in activity was approximately 10%.

After 24 days at +21°C, the treated and lyophilised yeast CYP2E1 demonstrated a loss

of about one third the activity compared to the untreated -80°C control; Graph 6.15.

**Graph 6.15  Analysis of sucrose-treated, lyophilised CYP2E1, after 24
days at +21°C.**
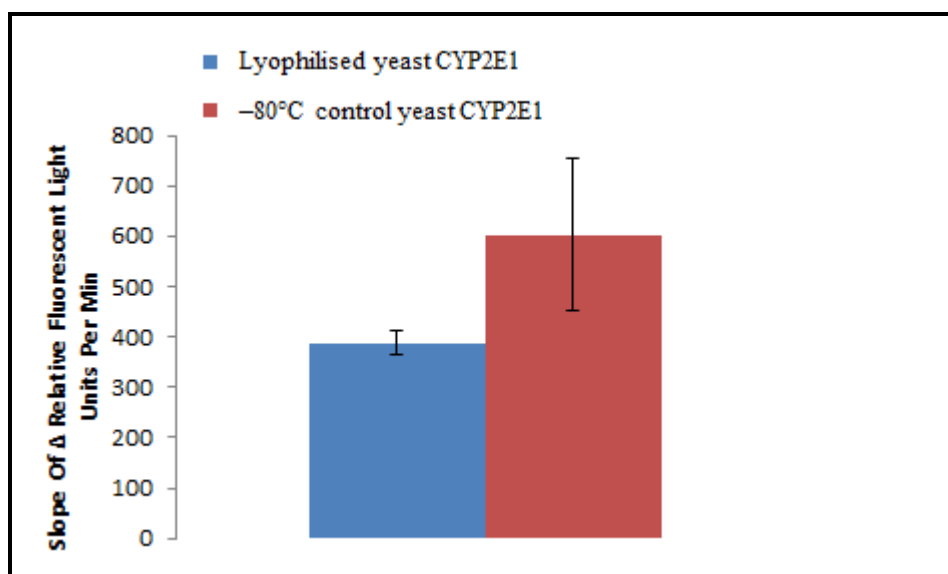


2.5 pmol of yeast CYP2E1 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which
contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before
rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was
measured by kinetic analysis of EOMCC turnover at Ex 400 nm/Em 460 in 100 mM potassium phosphate
(pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and
demonstrated that sucrose was not effective as a P450 cryopreservant when compared with the untreated -
80°C control, error bars are +/- 1x standard deviations. The mean value loss in activity was approximately
35%.

After 24 days at +21°C, the treated and lyophilised yeast CYP3A4 demonstrated a loss

of about one sixth of activity against the untreated -80°C control, Graph 6.16.
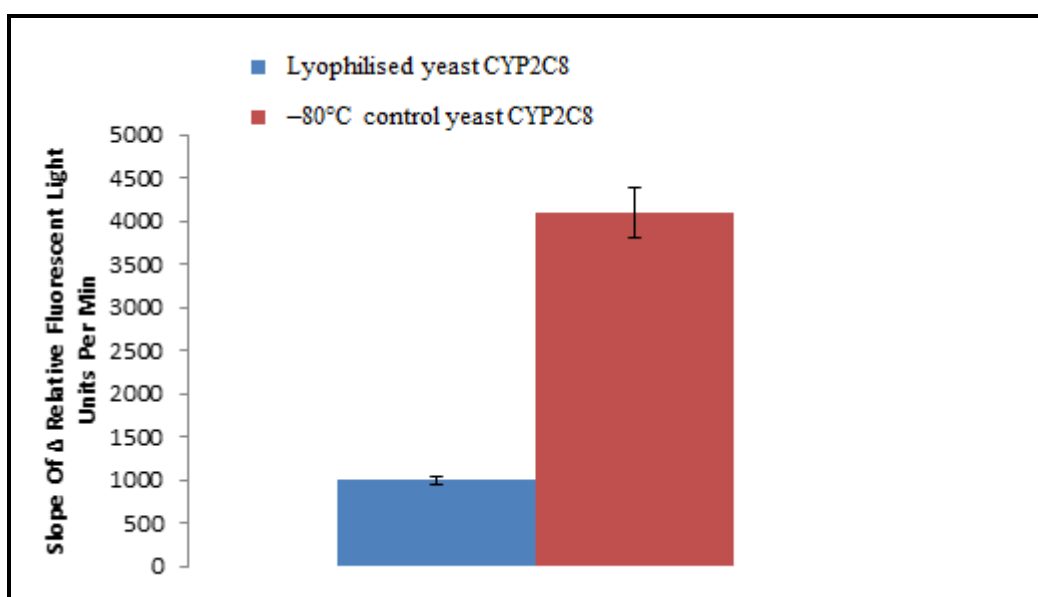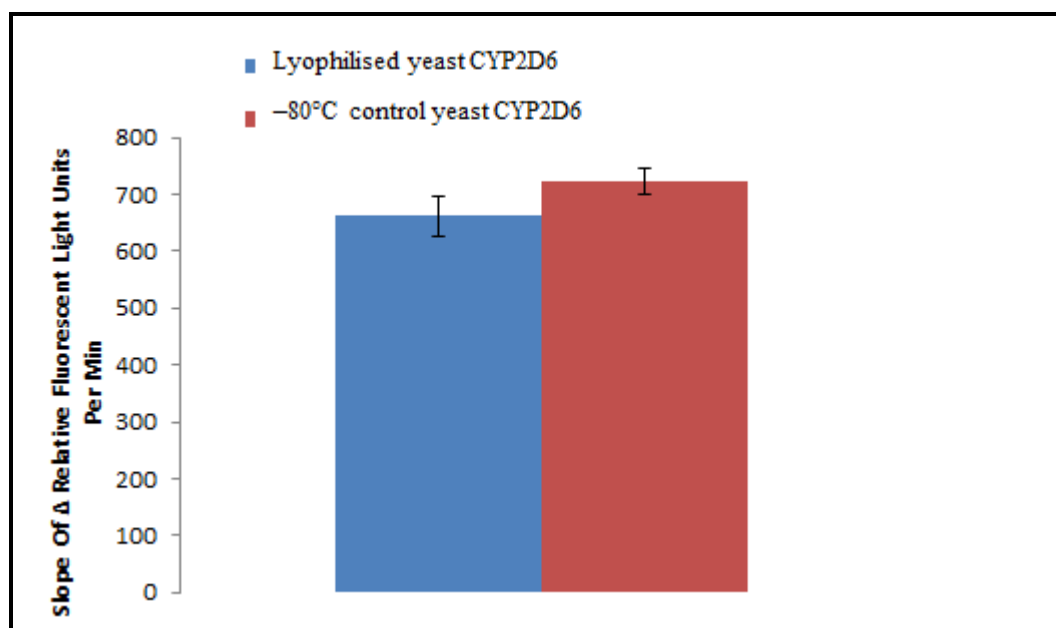
**Graph 6.16  Analysis of sucrose-treated, lyophilised CYP3A4, after 24 days at +21°C.**



1 pmol of yeast CYP3A4 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), which contained 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 24 days before rehydration and testing against the same untreated samples which had been stored at -80°C. Activity was measured by kinetic analysis of DBOMF turnover at Ex 400 nm/Em 460 in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the non-standard A. The rates of reactions are shown and demonstrated that sucrose was effective as a P450 cryopreservant when compared with the untreated -80°C control, error bars are +/- 1x standard deviations. The mean loss in activity was approximately 15%.

## 6.6    Experimental Conclusions: analysis of in-house yeast-produced P450-bearing microsomes, lyophilised, and kept for 24 days +21°C

It was noticed that certain P450s had retained activities better than others. Between each

sample there was a great variance in the protein compositions; Table 6.3.

**Table 6.3.    Measurable parameters of the in-house yeast P450s.**

| Sample | Total Microsomal Protein content, mg.ml$^{-1}$ | Specific P450 content, nmol.mg$^{-1}$ |
|--------|------------------------------------------------|---------------------------------------|
| 1A1 | 10.5 | 95 |
| 1A2 | 8.62 | 116 |
| 1B1 | 15.2 | 65.7 |
| 2A6 | 16.1 | 44 |
| 2C8 | 4.1 | 243.6 |
| 2D6 | 8.4 | 119 |
| 2E1 | 9.1 | 109.8 |
| 3A4 | 21.8 | 42 |

The total microsomal protein content of the different P450s had a range of over 4-fold. The specific P450 content is a measure of the amount of P450 relative to the total protein content had a range of over 5-fold. It is possible that these parameters assist the stability after lyophilisation.

CYP3A4 had an unusually high protein content, more than double the mean of all the other samples used. It also had a very low specific content and it may be the case that extra levels of proteins assist the sucrose in stabilising the P450s after lyophilisation. CYP2C8, which showed poorest stability, had the lowest protein content and the highest P450-specific content.

CYP2A6 had an unusual ability to greatly increase its activity after lyophilisation by approximately a quarter. Looking at its parameters, it also had the second highest protein content and the second lowest P450-specific content. During production, the last step of the process creates a gelatinous mass that must manually be homogenised. This step is temperature and perhaps time-sensitive. The quality of homogenisation is a trade-off against denaturation due to physical stresses. It may be the case that

lyophilisation, in the case of CYP2A6, gave rise to an advantageous reorganisation of the microsomes.

## 6.7 Use of nine cryopreservants, mostly simple carbohydrates, in the stabilisation of Cypex's CYP1A2

The agents used were selected on the basis that they were sugars or that they had been used previously in protocols for manufacture of microsomes and, hence, not known to be harmful to microsomes.

All prospective cryopreservants were added to either warm phosphate or Tris buffer both at 100 mM, and filter sterilised. The use of phosphate buffer in lyophilisation potentially could be a problem. Tris was therefore tested, as Matejtschuk (2007) states that for lyophilisation, "phosphate buffers are a no because of pH change and so Tris is preferred". The trial of Tris was considered important as BD-Gentest recommends its use with certain P450s. Tris inhibits certain enzymes but there is documented use of P450s with Tris (Ghalanbor *et al*., 2008). Using EDTA as an alternative was not considered to be worthwhile since the P450 regeneration system contained the enzyme glucose-6-phosphate dehydrogenase, which requires magnesium cations for improved activity (Lendzian 1978).

Cypex's microsomes from bacteria-expressed P450s were used to investigate the use of reconstituted microsomes, so there were no (non-P450 related) proteins upon these microsomes. These microsomes should have been far more uniform in production so that any effect of the sugar should be more apparent.

CYP1A2 obtained from Cypex was lyophilised in the presence of different sugars, stored for 12 days at +21°C; see Graph 6.17. There was a large range of activities recovered but it was clear that the samples that contained Tris and also glycerol performed badly.

**Graph 6.17  Cypex's CYP1A2 lyophilised in the presence of different sugars, stored at room temperature (+21°C) for 12 days.**
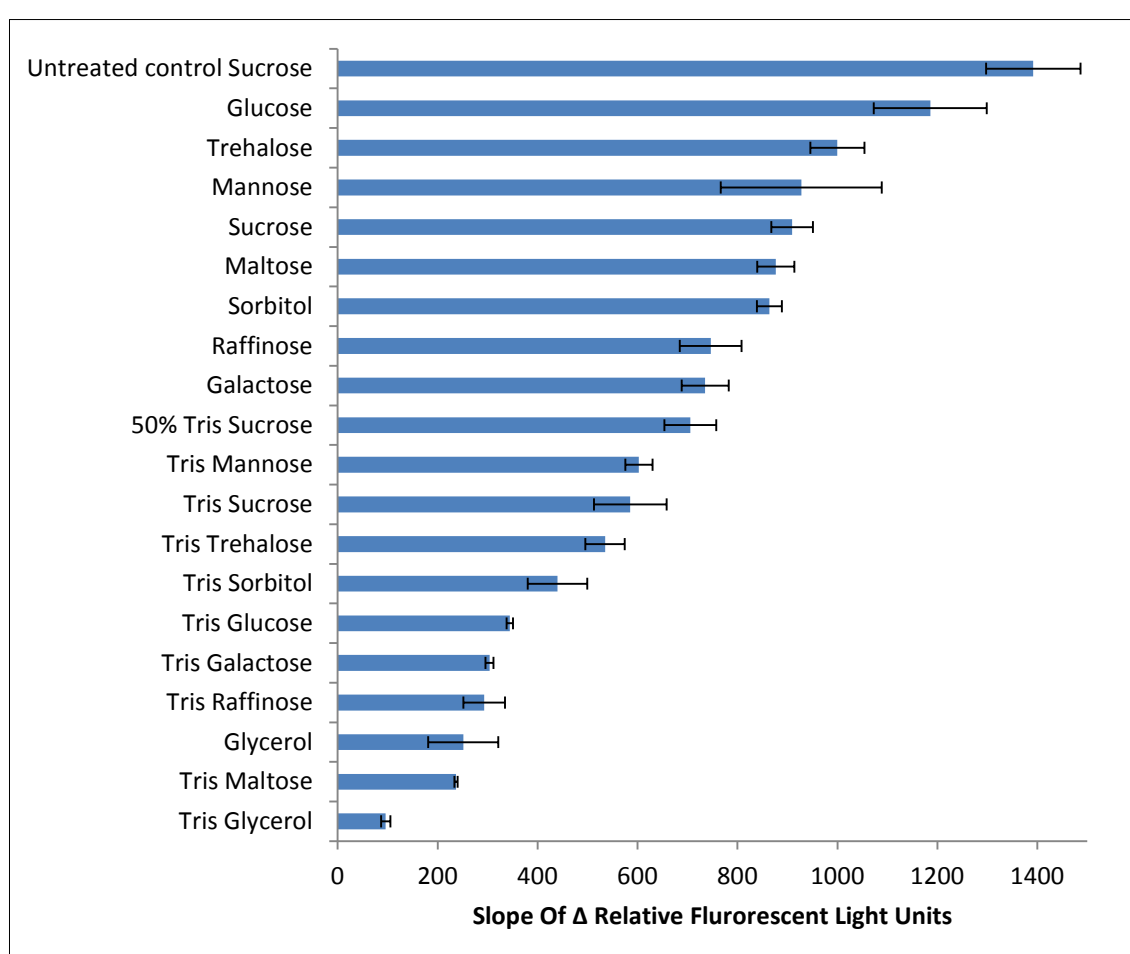


1 pmol of Cypex CYP1A2 was lyophilised in either 100 mM potassium phosphate (pH 7.6) or 100 mM Tris. The 50% Tris sample is 50 mM Tris together with 50 mM phosphate buffer. A range of sugars and other molecules was used, at 20% (w/v). The microsomes, together with different sugars, were lyophilised in microplate wells. These were stored at +21°C for 12 days before rehydration and testing against the same untreated samples stored at -80°C. Activity was measured by kinetic analysis of CEC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the standard A. The rates of reactions are shown and demonstrate that glucose, trehalose and mannose were better than sucrose. It was also the case that Tris was not better than potassium phosphate. Error bars are +/- 1x standard.

## 6.8 Experimental Conclusions from the use of nine cryopreservants, mostly simple carbohydrates, for the lyophilisation of Cypex's CYP1A2

The major observation was that Tris did not allow as much recovery of P450 activity as the phosphate buffer regardless of the sugar used. Also, glycerol functioned poorly regardless as to whether Tris or phosphate buffer was used.

The use of Cypex's artificial membrane microsomes meant that it was less likely that unusual background reactions may have occurred that may have interfered with the outcome.

In optical assays, sugars can also scatter light (Dreux and Lafosse, 1995). It is feasible that some of the lyophilisation agents used interact with the P450s under assay conditions. This is not documented in the scientific literature but as water resides in the active site of P450s (Section 1.8), it is possible that solutes may interfere with the way water binds and releases to the active site.

The carbohydrates used were 20% (w/v) but upon rehydration usually formed a maximum of 9 µl per 100 µl reaction. Therefore, the final maximum reaction concentration was just under 2% (w/v) but was still quite high for a component that really has no function after rehydration. As percentages were used for comparisons it should be noted that, due to their different molecular weights, the reagents used for lyophilisation are not all equal in the numbers of molecules per reaction.

Trehalose was interesting as it is the main sugar in the fluids of many insects and protects them from freezing and gives rise to protein stabilisation from thermal and osmotic stresses (Nelson-Thompson, 2003). *Saccharomyces cerevisiae* is an industrial source of trehalose. It can be forced to produce up to 20% of dry cell weight (Schiraldi *et al.*, 2002). It may be logical then to suspect that yeast proteins may be tolerant to high levels of trehalose than perhaps other lyophilisation agents. In *S. cerevisiae*, trehalose regulation is so important that trehalose is present at all stages of growth (Souza and Panek, 1967).

## 6.9 To determine if lyophilised P450s could be used for drug metabolism studies

Thanks to Dr Jacob Biboy for mass spectrometry guidance.

While fluorescent P450 assays are very useful for cheap and rapid screening of P450 inhibitors, they are limited in their ability to detect what may be occurring at a molecular level when a P450 reacts with a chemical substrate (i.e. when a P450 reacts with a chemical compound, acting as a substrate, to form a metabolite).

For drug metabolism studies, mass spectrometry is preferred. Therefore, it was important to consider if the lyophilisation process would interfere in any way with the use of P450s in HPLC/Mass Spectrometric analysis.

The experiment was performed using in-house CYP2D6-bearing yeast microsomes. The lyophilised CYP2D6 samples were produced by lyophilisation in the presence of 20% trehalose or mannose. The lyophilised samples were left at 21°C for 72h before

rehydration and testing against the untreated –80ºC CYP2D6 control. The substrate used was dextromethorphan hydrobromide which converts to dextromethorphan via a dextromethorphan O-demethylase reaction, mediated by CYP2D6.

Reactions were carried out for 10, 20 and 30 min, and then stopped.

The CYP2D6 mass spectrometry data, using the enzymes lyophilised in the presence of sugars, trehalose and mannose, was formed from the individual time-points of the reactions, stopped at 10, 20 and 30 min. The reaction measured conversion of dextromethorphan hydrobromide to dextromethorphan by a dextromethorphan O-demethylase reaction (see Sections 2.22, 2.23). Graph 6.18 depicts the results obtained.

**Graph 6.18  A discontinuous composition from data run over three time periods; mass spectrometry using in-house yeast CYP2D6, lyophilised with two different sugars, and having stored at +21°C for 72h. The substrate for CYP2D6-mediated reaction was dextromethorphan.**
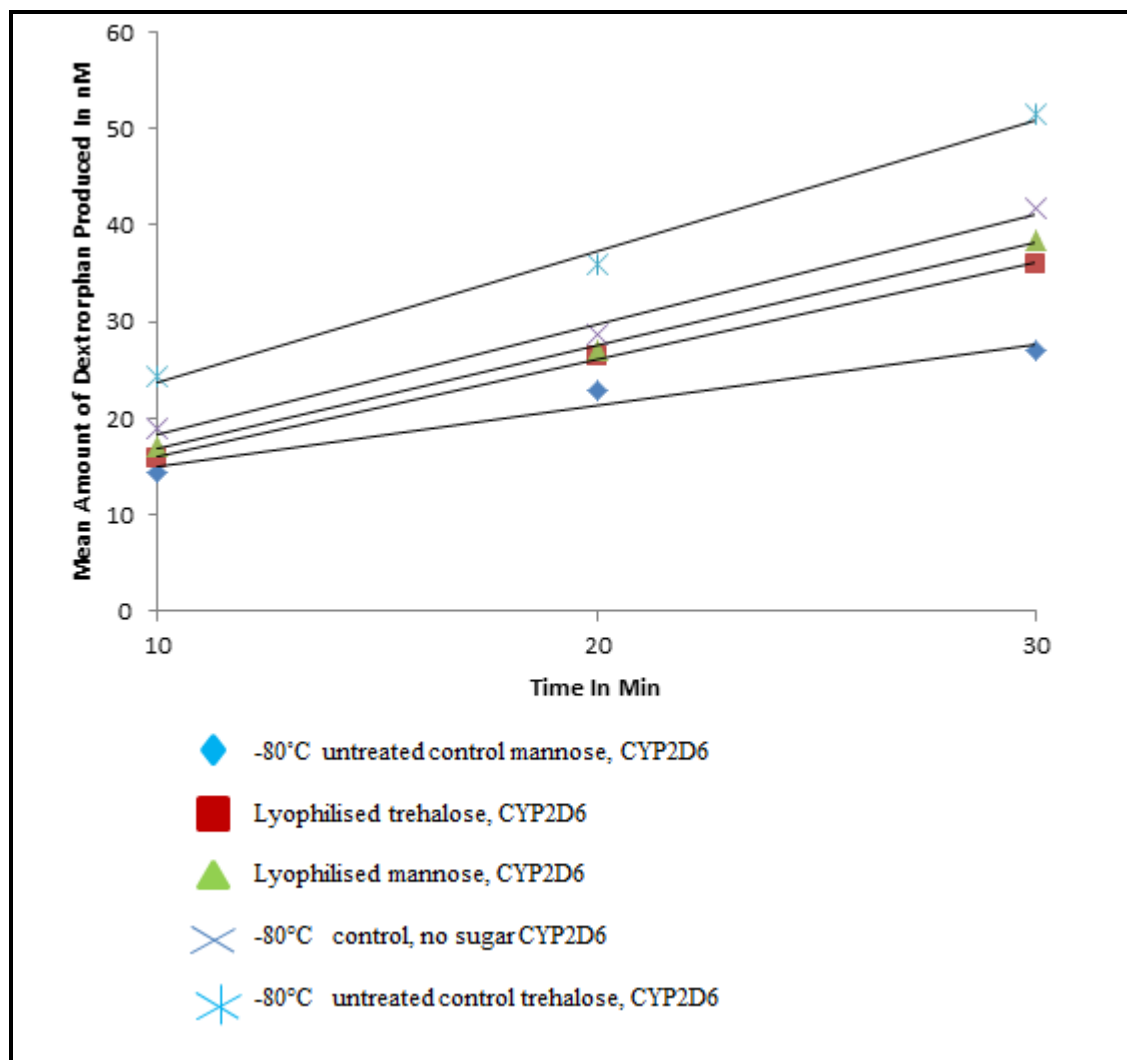


3 pmol of yeast CYP2D6 was lyophilised in 100mM potassium phosphate buffer (pH 7.6), containing either 20% (w/v) trehalose or mannose in microplate wells. These were stored at +21°C for 72h before rehydration and testing against the same untreated samples stored at -80°C. Activity was measured, by mass spectrometry, via kinetic turnover of dextromethorphan (MW 370.3) to dextrorphan (MW 356.3) through a dextromethorphan O-demethylase reaction (removal of –CH$_2$). Dextromethorphan elutes from the column with a retention time of ~8.5 min, whereas the product's retention time is ~7 min. The indivdual reactions were stopped at defined time points after incubation for 10, 20, 30min at 37°C. The data was compiled to form a discontinous kinetic curve. The kinetics showed formation of product, in nM, and demonstrated that the P450 cryopreservants trehalose and mannose had similar turnovers. There was a slight loss of activity in the lyophilised samples against the untreated -80°C controls, except in the case of the mannose control. The results were indicative rather than absolute as small amounts of substrate and product binding were a concern. Each mass spectrometry sample run took about 20 min and it was a concern that the samples may have either precipitated, bound to the vial or that the product was unstable or degraded. This was exacerbated by producing samples in triplicate.

## 6.10 Conclusions from the experiment that determined if lyophilised P450s could be used for drug metabolism studies

The most interesting phenomenon from the calculated rates of activity was that the lyophilised mannose sample gained about 60% more activity than the –80ºC sample. However, in the case of trehalose, the –80ºC control with trehalose had an increase of 25% over the lyophilised sample. Superficially, mannose seemed to be the better cryopreservant. However, the –80ºC control with mannose had the least activity and the –80ºC control with trehalose seemed to have the best activity.

Since the –80ºC control with trehalose had better activity than the –80ºC control with no sugars, perhaps trehalose may have somehow interfered with the assay itself. This would indicate that any increase in activity may not be due to the physical optical properties of light being altered by the sugar. It may be intrinsic to the biochemistry of the microsomes/P450s which appeared to be influenced by a specific sugar.

## 6.11 Does the presence of sugar in lyophilised CYP samples alter IC$_{50}$ values?

P450s are widely used for screening chemical libraries for CYP inhibition properties. It was important to not just to show stability of the lyophilised P450s with their substrates but also that they were not altered in their ability to be inhibited.

The effect of sucrose on P450 active sites, after lyophilisation, was tested in $IC_{50}$ assays, using known inhibitors. Three P450s used for these studies were CYP1A2, CYP2D6 and CYP3A4 since they are the most important CYP enzymes for drug developmental studies. In-house yeast P450s were lyophilised and stored at 21°C for 21 days. The lyophilised enzymes were compared, in $IC_{50}$ determination assays, with the untreated -80°C in-house yeast samples. Gentest untreated -80°C P450s were used as another control.

**Graphs 6.19**  **$IC_{50}$ values of CYP2D6 inhibition by quinidine: comparison of yeast-lyophilised, untreated yeast and Gentest enzymes**
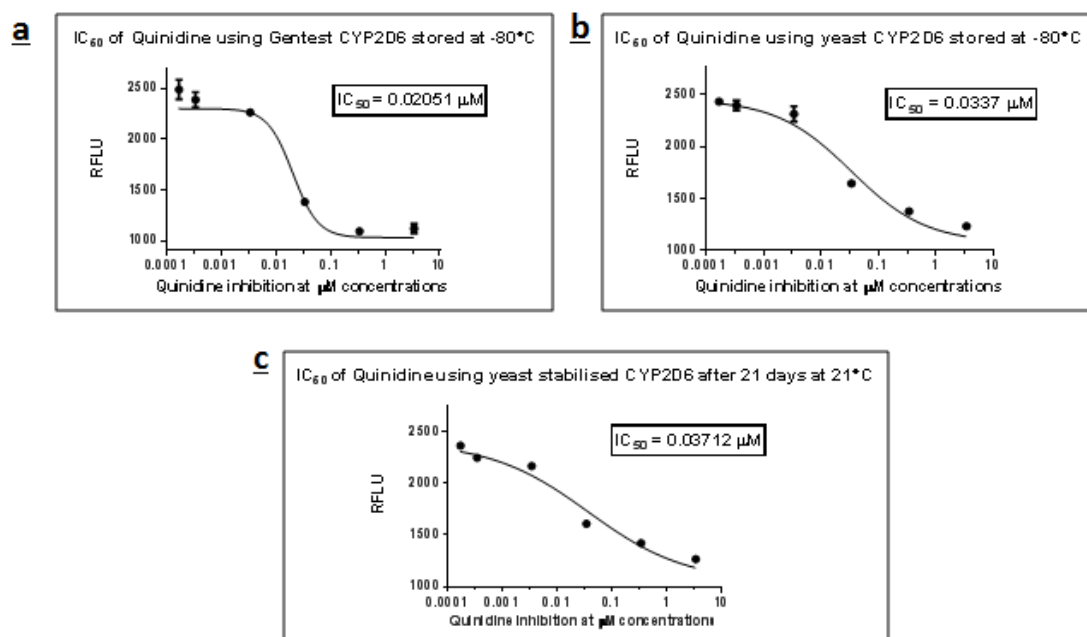


2 pmol of yeast-CYP2D6 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.4), containing 20% (w/v) sucrose in microplate wells. These were stored at +21°C for 21 days before rehydration and testing against the same yeast and untreated Gentest samples which had been stored at -80°C. Activity was measured by an endpoint reaction after 10 min of EOMCC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.4) at 30°C. The enzymes were tested in the presence of the inhibitor quinidine at concentrations ranging from 3.4 to 0.00017 µm. The regenerating system was the standard A. The endpoint values were scaled against positive and negative control samples. The data was analysed using GraphPad Prism which calculated the $IC_{50}$ values. The $IC_{50}$ values demonstrated that not only that yeast lyophilised P450s (c) had fidelity with the -80°C yeast samples (b) but also with the -80°C Gentest P450 (a). The published $IC_{50}$ of quinidine is < 0.3 µM in human liver microsomes (Donato *et al*., 2004).

**Graphs 6.20**  **$IC_{50}$ values of CYP3A4 inhibition by ketoconazole: comparison of yeast-lyophilised, untreated yeast and Gentest enzymes**
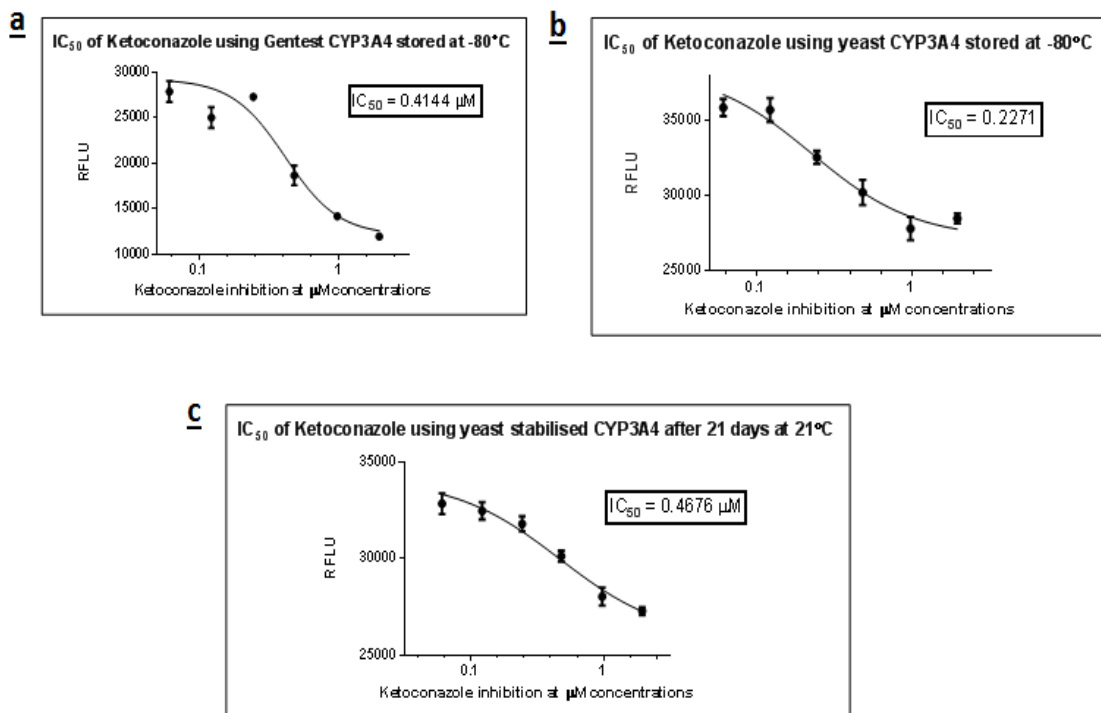
**a** $IC_{50}$ of Ketoconazole using Gentest CYP3A4 stored at -80°C

$IC_{50} = 0.4144$ µM

**b** $IC_{50}$ of Ketoconazole using yeast CYP3A4 stored at -80°C

$IC_{50} = 0.2271$

**c** $IC_{50}$ of Ketoconazole using yeast stabilised CYP3A4 after 21 days at 21°C

$IC_{50} = 0.4676$ µM

1 pmol of yeast-CYP3A4 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.4), containing 20% (w/v) sucrose in microplate wells. These were stored at +21°C for 21 days before rehydration and testing against the same yeast and untreated Gentest samples which had been stored at -80°C. Activity was measured by an endpoint reaction after 10 min of DBF turnover at Ex 485 nm/Em 538 nm in 100 mM potassium phosphate (pH 7.4) at 30°C. The enzymes were tested in the presence of the inhibitor ketoconazole at concentrations ranging from 1.95 to 0.061 µm. The regenerating system was the standard A. The endpoint values were scaled against positive and negative control samples. The data was analysed using GraphPad Prism which calculated the $IC_{50}$ values. The $IC_{50}$ values demonstrated that not only yeast lyophilised P450s (c) had fidelity with the -80°C yeast samples (b) but also with the -80°C Gentest P450 (a). The published $IC_{50}$ value of ketoconazole is < 0.3 µM in human liver microsomes (Donato *et al*., 2004).

**Graphs 6.21**    **IC$_{50}$ values of CYP1A2 inhibition by furafylline: comparison of yeast-lyophilised, untreated yeast and Gentest enzymes**



a — IC$_{50}$ of Furafylline using Gentest CYP1A2 stored at -80°C; IC$_{50}$ = 7.605 µM

b — IC$_{50}$ of Furafylline using yeast CYP1A2 stored at -80°C; IC$_{50}$ = 6.507 µM

c — IC$_{50}$ of Furafylline using yeast stabilised CYP1A2 after 21 days at 21°C; IC$_{50}$ = 7.513 µM
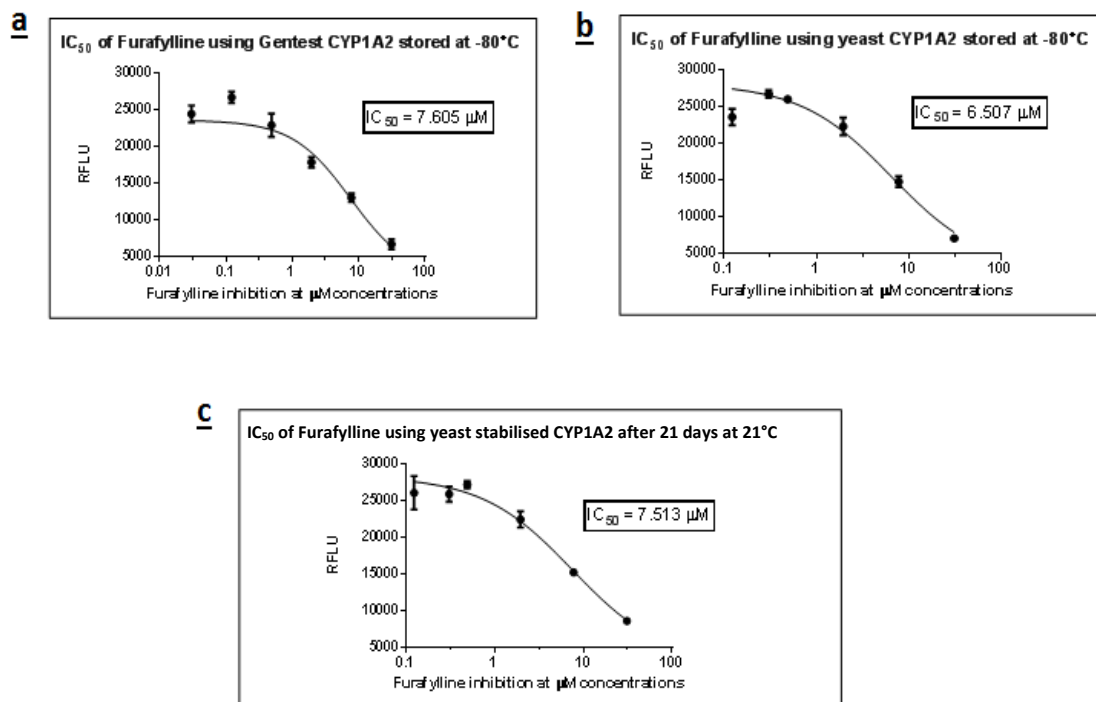
1 pmol of yeast-CYP1A2 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.4), containing 20% (w/v) sucrose buffer in microplate wells. These were stored at +21°C for 21 days before rehydration and testing against the same yeast and untreated Gentest samples which had been stored at -80°C. Activity was measured by an endpoint reaction after 10 min of CEC turnover at Ex 409 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.4) at 30°C. The enzymes were tested in the presence of the inhibitor furafylline at concentrations ranging from 31.25 to 0.0305 µm. The regenerating system was the standard A. The endpoint values were scaled against positive and negative control samples. The data was analysed using GraphPad Prism which calculated the IC$_{50}$ values. The IC$_{50}$ values demonstrated not only that yeast lyophilised P450s (c) had fidelity with their -80°C yeast samples (b) but also with the -80°C Gentest P450 (a). The published IC$_{50}$ of Furafylline is 6.2 µM in human liver microsomes (Donato *et al*., 2004).

## 6.12 Conclusions from the experiment that attempted to answer the question, does the presence of sugar in lyophilised CYP samples alter IC$_{50}$ values?

With the CYP2D6, CYP3A4 and CYP1A2, the IC$_{50}$ values in all the untreated yeast minus 80°C enzymes were very close to the yeast P450 enzymes that had been lyophilised and stored at 21°C for 21 days. The IC$_{50}$ values in untreated minus 80°C Gentest CYPs 2D6, 3A4 and 1A2 enzymes were close to the values obtained using both treated and untreated yeast P450 enzymes. This experiment was very important to demonstrate that the nature of the lyophilised P450s was not altered in a way that would change their relationship with their inhibitors.

## 6.13 Does lyophilisation affect the use of different substrates for enzyme activity studies?

Up to this point, the enzyme CYP2D6 has been used together with BD-Gentest's substrate, AMMC. Invitrogen's EOMCC substrate may also be used for CYP2D6 fluorescent microplate assays. Invitrogen do not offer structural details for EOMCC. AMMC is a coumarin derivative, 3-[2-(N,N-diethyl-N-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin (Chauret, 2001). EOMCC may not be a coumarin-based molecule. This experiment was to test if lyophilisation had an effect on the ability of a P450 to interact with different substrates.

There were two important observations in the experiment where CYP2D6 activity was tested, using AMMC as a substrate.

(1) For both the in-house yeast and BD-Gentest CYP2D6 the presence of sucrose did not seem to alter the activities between the untreated control –80˚C and the lyophilised sample; see Graph 6.22.

(2) Gentest's CYP2D6 had much more activity than the in-house yeast CYP2D6.

**Graph 6.22  CYP2D6 enzyme activities using AMMC as a substrate.**
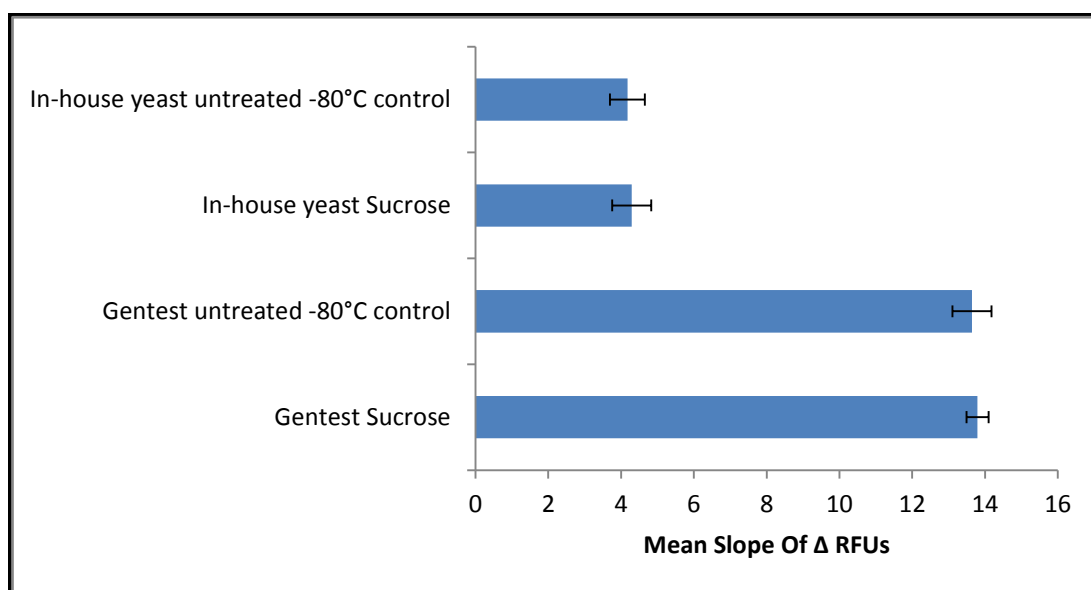


2.5 pmol of yeast and Gentest CYP2D6 was lyophilised in 100 mM potassium phosphate buffer (pH 7.6), containing 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 7 days before rehydration and testing against the same untreated samples stored at -80°C. Activity was measured by kinetic analysis of AMMC turnover at Ex 485 nm/Em 530 nm in a100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the non-standard A*. The rates of reactions are shown and they demonstrate that sucrose was effective as a P450 cryopreservant when compared with the untreated control (i.e. enzymes which had been stored at -80°C), error bars are +/- 1x standard deviations.

Using EOMCC, the above trend holds implying that the untreated –80˚C control and lyophilised samples do not differ much in their activities. Also there was much less variance between the in-house yeast and BD Gentest samples; see Graph 6.23.

**Graph 6.23  CYP2D6 enzyme acitivities using EOMCC as a substrate.**
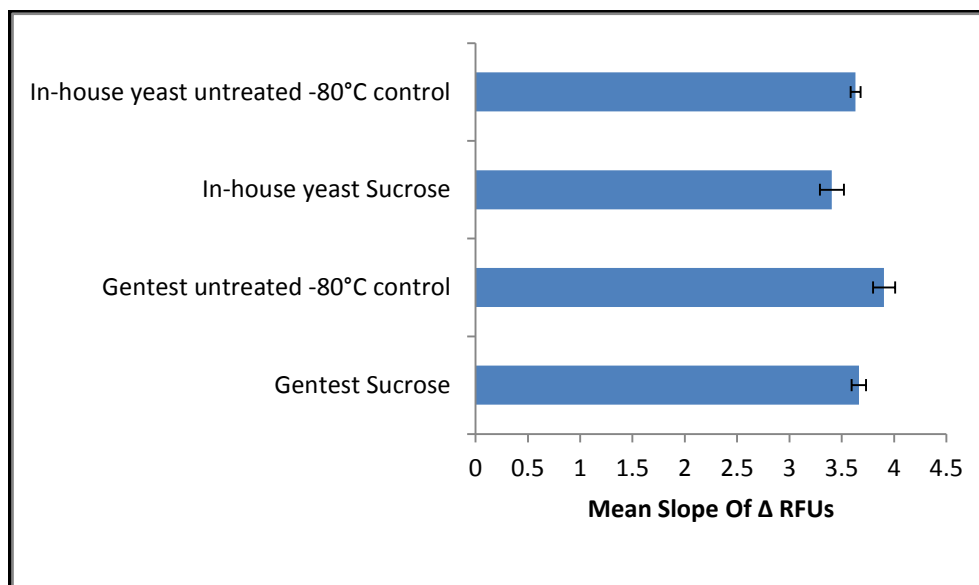


2.5 pmol of yeast and Gentest CYP2D6 was lyophilised in a 100 mM potassium phosphate buffer (pH 7.6), containing 20% (w/v) sucrose, in microplate wells. These were stored at +21°C for 7 days before rehydration and testing against the same untreated samples stored at -80°C. Activity was measured by kinetic analysis of EOMCC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The rates of reactions are shown and again demonstrate that sucrose was effective as a P450 cryopreservant against the untreated -80°C control, error bars are +/- 1x standard deviations. Like AMMC, EOMCC did not demonstrate any diffrences between the untreated -80˚C controls and the lyophilised samples. This was the case for both sets of microsomes, one originating from yeast and the other from insect cells.

## 6.14  Conclusions from the experiment that attempted to answer the question, does lyophilisation affect the use of different substrates during specific enzyme activity studies?

The use of two substrates AMMC (chemical structure known) and EOMCC (chemical structure known) in assessing the activities of CYP2D6 enzyme, lyophilised in the presence of a sugar, indicate that there is no obvious augmentation or reduction of activity specifically towards one substrate.

The differing activities between in-house yeast and BD Gentest using AMMC may possibly be explained by the fact that BD Gentest had a patent for AMMC (WO/2000/004008) and published a paper on its use (Chauret, 2001). It may be that

BD-Gentest used chemical-biological techniques to optimise for the best activity of CYP2D6 using the specific substrate, AMMC.

## 6.15 Determination of the effects of escalating concentrations of sugars on the activities of lyophilised CYP3A4

20% (w/v) of sugars had been successful in preserving P450-bearing microsomes after lyophilsation for some periods of time. But the validity of this amount of sugar that was used for lyophilisation required further testing.

Escalating amounts of cryopreservants were tested to check if enhancement of the amounts of sugars used for the process of lyophilisation would provide greater stability of P450s. For this experiment, samples of CYP3A4 (produced in-house) were chosen. They were lyophilised in the presence of increasing doses of sucrose and mannose, followed by storage for 72h at ambient temperature (+21°C).

Graph 6.24 shows that:

(a) Increasing concentrations of sucrose did not seem to cause much variation in activity in untreated –80ºC control CYP3A4 enzymes, and

(b) A concentration of sucrose between 4 and 9% (w/v) was sufficient to maintain activity in lyophilised CYP3A4 sample; adding a higher concentration of sucrose was not of great benefit.

**Graph 6.24  Measurement of activity of CYP3A4 samples, lyophilised in the presence of escalating concentrations of sucrose, and then keeping for 72h at 21°C.**
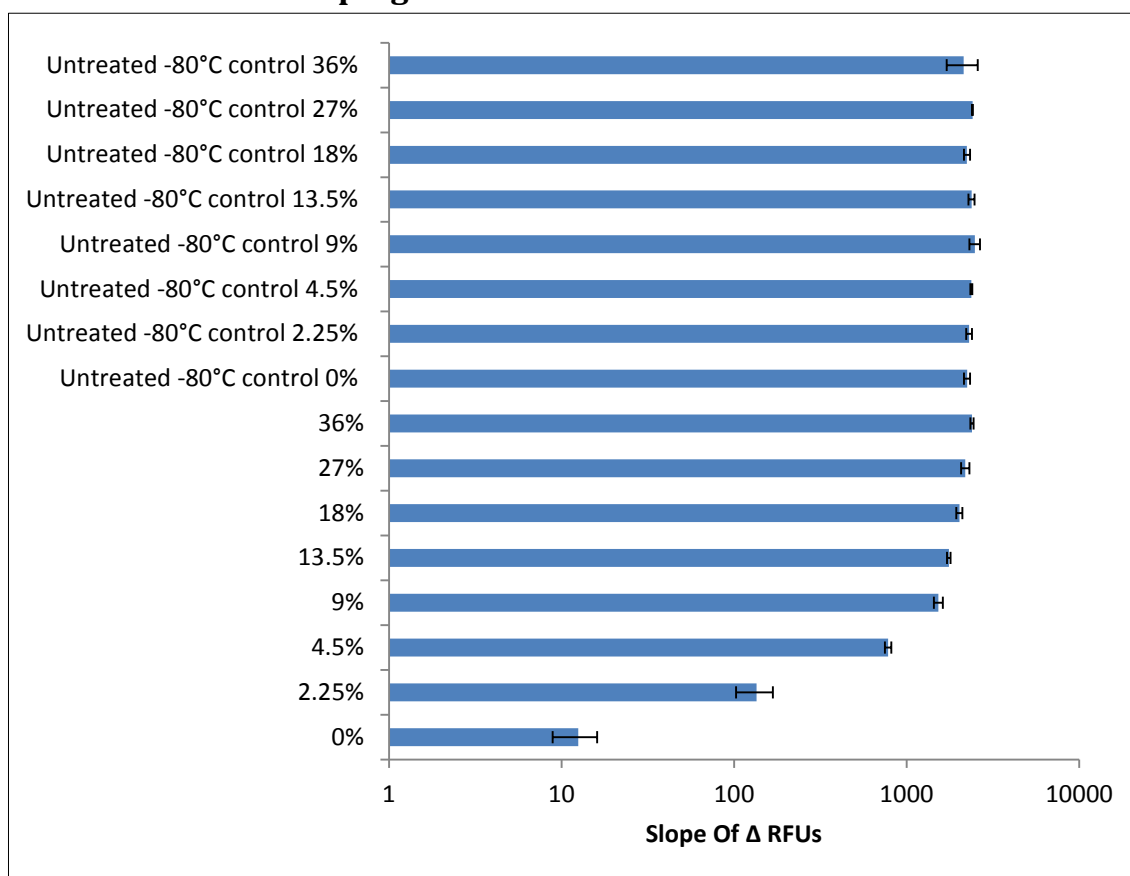


1 pmol of yeast CYP3A4 was lyophilised in 100 mM potassium phosphate buffers (pH 7.6), containing increasing concentrations of sucrose, in microplate wells. The lyophilised samples were stored at +21°C for 72h before rehydration and testing against the same untreated CYP3A4 samples stored at -80°C. Activity was measured by kinetic analysis of DBOMF turnover at Ex 485 nm/Em 530 nm in 100 mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the non-standard A. The rates of reactions are shown and demonstrate that sucrose was effective as a P450 cryopreservant when compared with the untreated -80°C controls and sucrose levels above 9% did not add much value. Error bars are +/-1x standard deviations.

Graph 6.25 shows that:

(a) Although different concentrations of mannose have very little effect on untreated –80°C CYP3A4 control enzymes,

(b) Lyophilised samples lost a significant amount of activity in the presence of mannose; however, it appeared that activities increased with increasing concentrations of mannose.

**Graph 6.25. Measurement of activity of CYP3A4 samples, lyophilised in the presence of escalating concentrations of mannose, and then keeping for 72h at 21°C.**
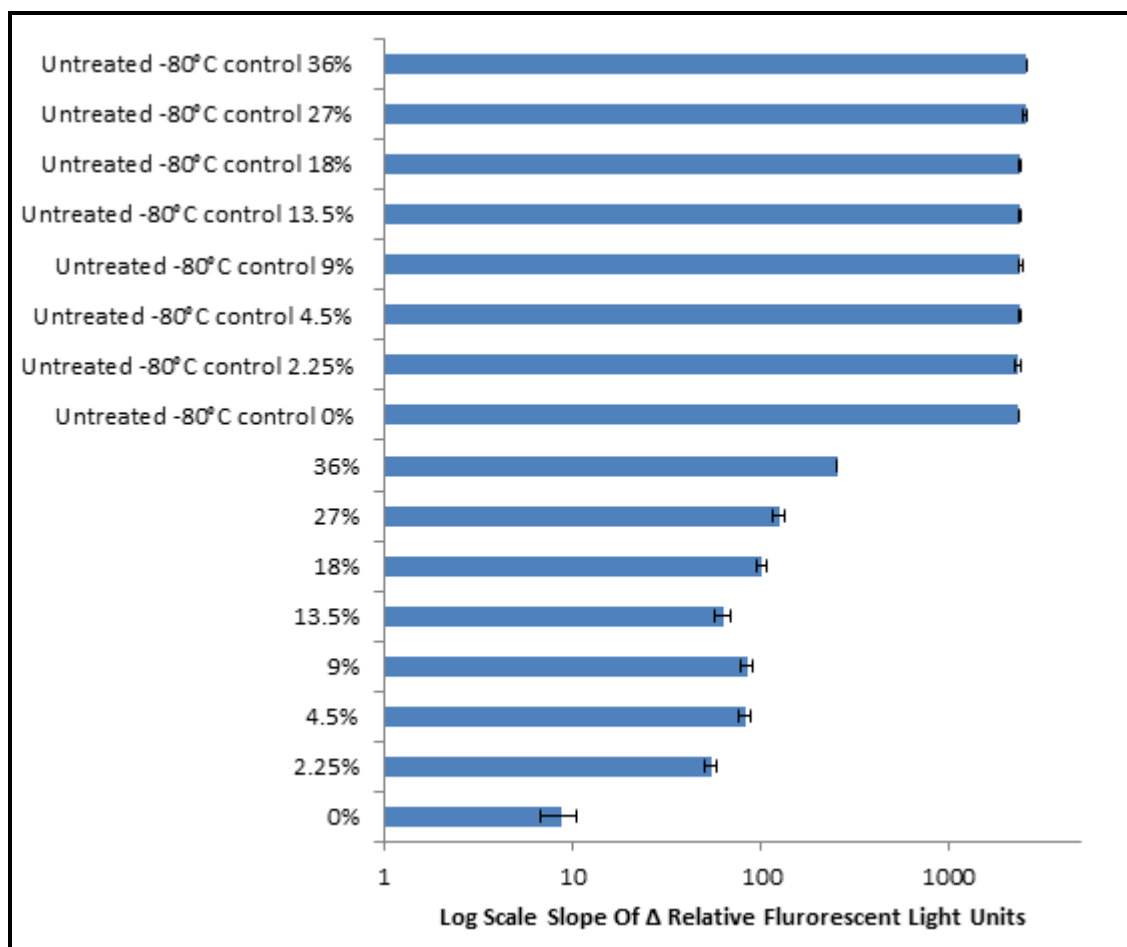


1 pmol of yeast CYP3A4 was lyophilised in 100 mM potassium phosphate buffers (pH 7.6), containing increasing concentrations of mannose, in microplate wells. These were stored at +21°C for 72h before rehydration and testing against the same untreated CYP3A4 samples stored at -80°C. Activity was measured by kinetic analysis of DBOMF turnover at Ex 485 nm/Em 530 nm in 100 mM potassium phosphate (pH 7.6) at 37°C. The regenerating system was the non-standard A. The rates of reactions are shown and demonstrate that extra mannose only caused rates to decrease compares to the untreated -80°C control CYP3A4 samples, irrespective of the concentrations of mannose. Error bars are +/- 1x standard deviations.

## 6.16 Conclusions from the experiment that determined the effects of escalating concentrations of sugars (sucrose and mannose) on the activities of lyophilised CYP3A4

(1) CYP3A4 enzyme samples required 36% or higher mannose levels to recover a

useful level of activity.

(2) Levels of sucrose of around 9% appeared to be enough to preserve most

CYP3A4activity after lyophilisation.

It was concluded that the sugar, sucrose, consistently performed as a cryopreservant and that it would not be worthwhile going forward with mannose, as a cryopreservant, any further.

## 6.17  Long-term storage at +21°C and shipping of lyophilised P450s, without dry ice

CYP1A2 was tested for long-term stability at ambient laboratory temperature, 21°C, in-house. For comparison's sake, a lyophilised CYP1A2-containing microplate was sent to a large biopharmaceutical company who had experience in using P450 assays. Alongside, a frozen sample of un-lyophilised CYP1A2 was shipped on dry ice. Both packages were sent by aeroplane to the USA and then from the airport by road, for the remainder of the journey. Both CYP1A2 samples were in phosphate buffer that contained 20% (w/v) sucrose.

It was found that in the lyophilised sample, CYP1A2 activity was maintained even after 95 days of storage. This was considered a very successful outcome. Graph 6.26 shows the results.

**Graph 6.26  Activity of CYP1A2, lyophilised in the presence of sucrose, and kept for 95 days at ambient temperature +21°C; tested in-house.**
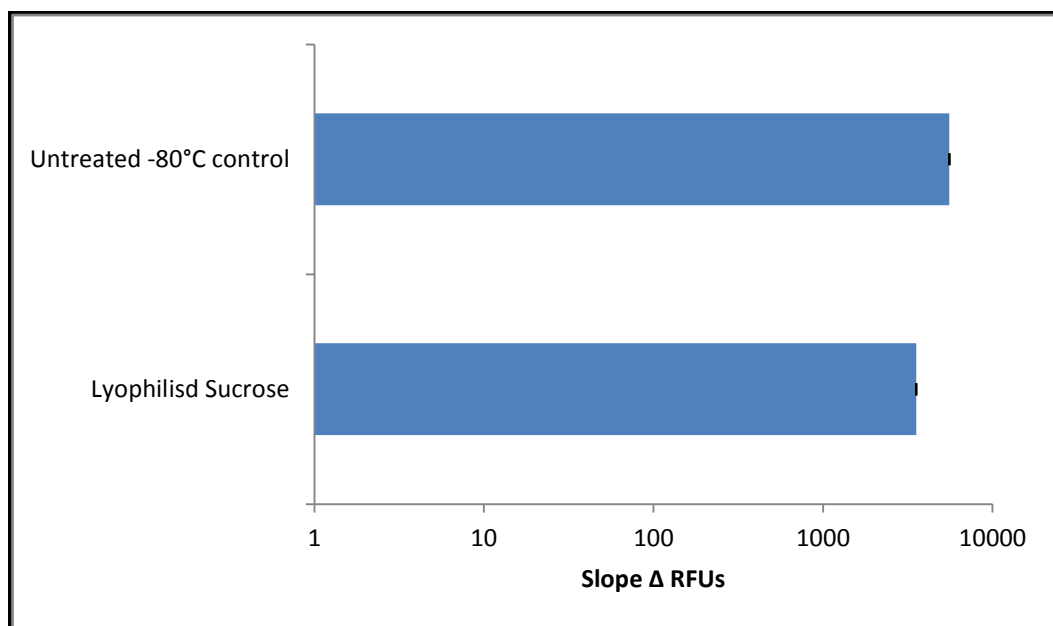


1 pmol of yeast CYP1A2 was lyophilised in 100 mM potassium phosphate buffer (pH 7.6), containing 20% (w/v) sucrose, in microplate wells. The plate was stored at +21°C for 95 days before rehydration and testing against the same untreated CYP1A2 samples, stored at -80°C. Activity was measured by kinetic analysis of CEC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The lyophilised CYP1A2 samples retained most of their activity over 95 days when compared with the untreated CYP1A2 control enzyme which had been stored at-80°C. Error bars are +/- 1x standard deviation.

For the transit test, CYP1A2 sucrose microplates were made and sent to the third party by air frieght and tested in their laboratory against untreated control CYP1A2 sent on dry ice. Both samples showed similar activities, with standard deviations, Graph 6.27. The third party's report stated that the P450 activity of the lyophilised CYP1A1 was "slightly less" than the untreated –80°C control after international shipment at ambient temperature and several weeks in their laboratory at ambient temperature.

**Graph 6.27  Activity of CYP1A2, lyophilised in the presence of sucrose, and kept for 95 days at ambient temperature +21°C; tested by third-party (a multi-national biopharma).**



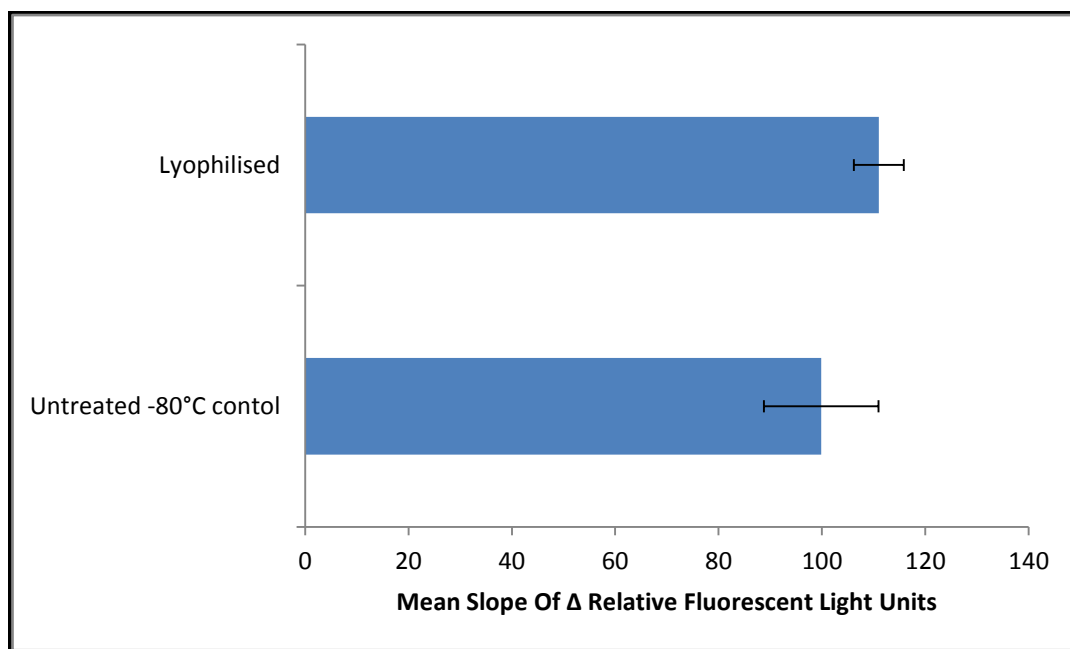1 pmol of yeast CYP1A2 was lyophilised in 100 mM potassium phosphate (pH 7.6), containing 20% (w/v) sucrose, in microplate wells. After 4 weeks the plates were shipped by air and road to the USA at varying ambient temperatures and stored at +21°C for 2 months before rehydration and tested against the same untreated CYP1A2 samples that had been stored at -80°C. Activity was measured by kinetic analysis of CEC turnover at Ex 400 nm/Em 460 nm in 100 mM potassium phosphate (pH 7.6), at 37°C. The regenerating system was the standard A. The lyophilised CYP1A2 samples retained most of their mean activities when compared with control untreated CYP1A2 enzyme that had been stored at -80°C over the same period. Using the error bars (+/- 1x standard deviation) the activities look similar.

## 6.18  Conclusions from the long-term storage experiment where CYP1A2 samples had been stored at +21C or kept at varying ambient temperatures, over a period of 95 days, after lyophilsation

The fact that the activity was maintained after 95 days at an ambient laboratory temperature (+21°C) confirms that lyophilisation is a pivotal step towards the wider global use of P450 microsomal enzymes which are highly temperature labile. The results emphasised that lyophilised P450 enzymes:

(1) Can be kept at varying ambient temperatures during inter-continental shipment, and

(2) Could be used by different people and with pieces of equipment remote from the ones that were used for development of the P450 assay at De Montfort University.

## 6.19  Chapter Summary: Conclusions

The work described above forms a solid proof of principle or feasibility study towards establishing a process that would allow fragile P450-bearing microsomal enzymes to be routinely lyophilised in a way that enzyme activity could be maintained even after keeping the enzymes at ambient temperatures for long periods of time (i.e. up to 95 days). This work also demonstrates that there is scope for further studies that may allow the formulation of a product that has significantly longer lifespan, at sensible ambient temperatures. Since

(a) The P450 enzymes are structurally quite complex and

(b) There are varieties of lyophilised P450 products that could be envisaged for different uses,

there are still a lot of possible variables to test.

While the concept of P450 lyophilisation may be there in the literature, all published work refer to lyophilisation of 'inactive' purified P450 proteins (i.e. shorn of membranes). The work done in this thesis is unique, since it is the first to lyophilise fragile P450 microsomal complexes as viable, active enzymes.

Some relevant papers, which have previously described P450 lyophilisation, are individually discussed below. The last few examples are noted briefly as they are less relevant.

Lotlikar *et al*., (1976) have described the need for phospholipids for correct function of the P450s. Lyophilisation was used to remove water from membrane-less P450s before solvents were used to extract purified P450 proteins; see Table 6.4.

**Table 6.4.    Differences between current work and Lotlikar *et al* (1976)**

| Parameter | Lotlikarr's work | Current work | Important Difference(s) |
|---|---|---|---|
| Material used | Hamster liver microsomes. | Recombinant yeast P450 microsomes (also from other expression sources, but to a lesser degree). | The large amount of junk protein content in hamster liver microsomes perhaps protects them? |
| Cryopreservant | None; used only water. | Used various agents mostly sugars. | Self-explanatory. |
| Freeze drying | Dried to powder. | Dried to thick gel (film) | No real detail of method in the paper. |
| Sample storage | -15°C | Ambient lab temp, 21°C. | Self-explanatory. |
| Stability | 1 to 2 weeks, for purified inactive protein. | Many months. | Self-explanatory. |

Mulligan (2008) published a paper with a description of products offered by Codexis Inc; see Table 6.5.

**Table 6.5.      Differences between current work and Mulligan (2008).**

| Parameter | Mulligan's work | Current work | Important difference(s) |
|---|---|---|---|
| Material | Recombinant lyophilised P450 powder. Only mentions bacterial enzymes, not microsomes.\n\nUse of bacterial fusion enzyme of P450 & P450 reductase. | Recombinant yeast P450 microsomes (also from other expression sources, but to a lesser degree).\n\nUse of separate proteins. | Microsomes contain a sugar for proper P450 function.\n\nFusion proteins, produced in yeast, may provide the same data as separate P450 and P450 reductase. |
| Use | No mention of storage or stability. | Characterised storage & stability. | Self-explanatory. |

There is a patent that describes plant P450s in the context of lyophilisation [International patent application WO2008/047112 A1 (Casey & Hughes, 2008)]; main relevant pages are: 15, 16, 43, 44, 45, 53 and 54; comparative results described in Table 6.6.

**Table 6.6.      Differences between current work and patent application WO2008/047112 A1.**

| Parameter | Patent's claims | Current work | Difference |
|---|---|---|---|
| Material | Plant P450s enzymes, specifically CYP74, includes purification of protein as His-tags, not as microsomes | Recombinant yeast P450 microsomes (also from other expression sources, but to a lesser degree). | Plants have very different membrane structures from yeast cells; examples in patent does not use microsomes |
| Detergents | Requirement of detergents for hydration, to obtain activity | No detergents used | Self-explanatory |

Aikawa *et al* (1976) used lyophilised S9 fractions which are intermediates in the process

of producing microsomes; see Table 6.7.

**Table 6.7.      Differences between current work and Akawa *et al* (1976)**

| Parameter | Aikawa's work | Current work | Important Difference(s) |
|---|---|---|---|
| Material | Liver S9 fractions from rat, mouse, guinea pig, rabbit and dog; not microsomes. | Recombinant yeast P450 microsomes (also from other expression sources, but to a lesser degree). | The massive amount of junk protein content in animal liver microsomes perhaps have a protective effect. |
| Stability | No activity change after lyophilisation for one month at -20°C. | Stored over one month at ambient lab temperature, ~21°C. | Self-explanatory. |
| Cryopreservation | No mention of cryopreservation agents. | Use of cryopreservation agents. | Self-explanatory. |

Kamataki and Kitagawa (1974) published their paper on storage of rat liver microsomes; see Table 6.8.

**Table 6.8.     Differences between current work and Kamataki and Kitagawa, (1974).**

| Parameter | Kamataki and Kitagawa's work | Current work | Important Difference (s) |
|---|---|---|---|
| Material | Rat liver microsomes (but also S9 fractions and microsome supernatants). | Recombinant yeast P450 microsomes (also from other expression sources, but to a lesser degree). | The massive amount of junk protein content in animal liver microsomes perhaps has a protective effect. |
| Storage | Storage at –20ºC. | Stored at ambient lab temperature, ~+21°C. | Self-explanatory. |
| Stability | Quote from paper  "S9 fractions were more stably stored than microsomes". | Used microsomes. | It is a common perception that soluble proteins are easy to lyophilise but microsomes are not easy. |

Leibman (1983) worked on storage of liver and lung preparations, Table 6.9.

**Table 6.9.     Differences between current work and Leibman, (1983).**

| Parameter | Leibman's work | Current work | Important difference(s) |
|---|---|---|---|
| Material | Rat liver and lungs. | Recombinant yeast P450 microsomes (Also other expression sources to a lesser degree). | The massive amount of junk protein content in animal microsomes perhaps protects them. |
| Storage | –15°C. | Stored at ambient lab temperature approx +21°C. | Self-explanatory. |
| Stability | Only recommend several weeks' storage before activity loss becomes a problem. | Characterised longer than several weeks. | Self-explanatory. |

Iwata *et al* (1996) freeze-dried isolated mouse liver microsomes for purification of proteins from the microsomes for non-active protein studies. Oddly enough, the paper describes large-scale preparation of microsomes with a low speed centrifuge. It does not discuss the use of cryopreservants and does not mention any activity of microsomal enzymes.

Chefson *et a*l (2007) described in their paper ("His-select P450 with a nickel affinity gel") purification of histidine-tagged P450 proteins, not microsomes.

A paper by Nakhgevany *et al* (1996) is about P450 proteins purified from rabbit microsomes.

On the basis of a lack of publications in the specific area of research that has been described in this Chapter of the thesis, it was decided to file for a patent application so that the work could be protected to explore further new applications of lyophilised microsomal P450 enzymes [Stabilisation, WO 2010010343 A1, Chaudhuri, *et al*., (2008)].

## 6.20  ABSTRACT of the filed Patent Application from De Montfort University

A dried composition comprising a membrane-associated recombinant cytochrome P450 (CYP) enzyme, wherein the CYP enzyme is stable at 21°C for a period of at least 10 days, and preferably for at least 3 months. A method of storing a recombinant membrane-associated CYP enzyme, the method comprising providing a recombinant

membrane-associated CYP enzyme, freeze-drying the CYP enzyme, and storing the dried CYP enzyme at room temperature.

## 6.21  Future work

Development of lyophilised products also involves experimenting simultaneously with many different variables. The main problem faced was that some procedures could be quite involved, costly and required a lot of set-up and waiting time periods.

The lyophilisation work was done with a very basic machine that had no changeable settings. As the machine did not have cooled shelves, the risk of the product thawing before lyophilisation was a worry. With a more sophisticated machine the shelves can keep the samples frozen before lyophilisation, the temperature ramping and vacuum strength could be changed, and a secondary drying process could be performed. More sophisticated lyophilisation equipment, than what has been used here, should be able to optimise the procedure and produce more consistent results.

The use of electron microscopy may be useful for testing different sugars and methods, providing an insight to the specific structural changes that occurs in the presence of sugars which, in turn, could be correlated to more successful long-term stability. This may also allow prediction of the outcome with the use of new sugars without waiting for months for results.

All new applications of stabilised P450s would implicitly involve ease of use. A simple application, which has been considered for drug metabolism studies, is providing a P450 or multiple P450s on a microtitre plate which could directly be used for assaying

just by the addition of water and test candidates. This would greatly benefit a laboratory user with limited experience and equipment.

With the proven ability to produce thermostable P450s, diverse P450s could be stabilised for use as kits that could be used in the field by persons testing samples in adverse conditions. For example, kits could be created for testing of:

(a) The quality of water following chemical spills,

(b) Chemical weapon agents in war zones, and

(c) Toxic agents in developing countries for routine toxicological testing.

These would require developing colorimetric metabolites and inhibition profiles of key enzymes. Toxic compounds, as either competitive or non-competitive inhibitors, would prevent formation of a colour. Smart phone camera applications would be able to assess colours and then could be used to quantify the outcome, for example, results obtained by Choodum *et al* (2013).

The work done by Maves and Sligar (2001) which attempted to understand what makes the P450s function in hotter climates could be applied to create fully thermostable lyophilised P450s for use in very hot places.

Ideally, the formulation of insoluble lyophilisation agents could be used to encapsulate P450s. This would allow P450s to be used for bioremediation and biotransformations. These encapsulated P450s could be packed into columns and used to perform cleaning operations or chemical transformations and allow the products to be quickly and easily separated. Finally, P450s stabilised onto microarray chips could be used to form highly sophisticated screens of drug candidates and medical toxicology samples.

# Chapter 7    References

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