STUDIES ON THE ACCUMULATION AND DEGRADATION
OF CYTOCHROME P-450
FROM THE YEAST SACCHAROMYCES CEREVISIAE

Being a Thesis presented in accordance with the Regulations governing the Award of the Degree of Doctor of Philosophy in the University of Surrey

by

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To my wife and parents
I would like to express my sincere thanks to Dr. A. Wiseman for guiding me through the work presented in this thesis. I especially thank him for the generous allocation of his time and ability to inspire me when the experimental results seemed fruitless.

I would also like to thank the following people;

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The Biochemical Society in granting me the Teaching Fellowship which allowed full-time study on this thesis.
"The interest in cytochrome P-450 has been especially stimulated as a consequence of the ongoing shift of emphasis within basic cancer research towards chemical carcinogenesis, where cytochrome P-450 mediated metabolic activation of precarcinogens is an important issue."

Jan-Ake Gustaffson
(1980)
SUMMARY

The work described in this thesis attempts to analyse the accumulation and degradation of cytochrome P-450 in *Saccharomyces cerevisiae*. The effect of environmental parameters such as oxygen and constituents of the growth medium have been examined here in an attempt to understand the mechanism underlying the accumulation and degradation of this enzyme.

The highest level of *S. cerevisiae* cytochrome P-450 accumulation was recorded with a new strain NCYC 754 obtained from NCYC 240 and first investigated here. Cytochrome P-450 was found to accumulate during growth of *S. cerevisiae* only at high glucose concentrations under conditions of mitochondrial repression. It was found that in non-growing yeast a 100 ml 8% glucose (w/v) solution would enhance cytochrome P-450 accumulation. Scale-up of this effect in a 5 l bioreactor was attempted.

In experiments on the removal of oxygen during the exponential growth of *S. cerevisiae* there was found to be a decline in cytochrome P-450 accumulation in which case it is suggested that oxygen may be acting as a substrate inducer of yeast cytochrome P-450.

Culture shake speed was also used to control oxygen availability. An optimum shake speed was found which allowed the greatest rate of cytochrome P-450 accumulation, it was also found that the same shake speed caused the greatest rate of degradation of the enzyme during stationary phase. It was also discovered that semi-anaerobic conditions caused less degradation than aerobic conditions. The agents chloramphenicol, dinitrophenol and cycloheximide offered less protection against degradation than semi-anaerobic conditions. Ethanol was found to induce cytochrome P-450 in *S. cerevisiae* under conditions where cytochrome P-450 is not normally detectable. Added alkanols, other than ethanol, cause rapid degradation of cytochrome P-450 in non-growing yeast.
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INTRODUCTION
1.1 INTRODUCTION

Oxygenases are important because of their ability to oxygenate unactivated carbon-hydrogen bonds. No other enzymes have this function and given the organic nature of life on this planet it is only to be expected that monooxygenases play a major role in biosynthetic and degradative pathways.

Aerobic microorganisms are capable of degrading and utilizing, for growth, inert organic compounds which originate continuously as final products of animal and plant metabolism and of microbial fermentative reactions. The compounds included methane, other aliphatic, dicyclic, aromatic and polycyclic hydrocarbons and heterocyclics. Oxygenation is usually the first step in the metabolism of these compounds.

In the twentieth century the number of xenobiotics increases daily in number, volume and distribution. These xenobiotics may be degraded by biological systems, involving monooxygenase reactions, hence their study is also increasing in importance.

Oxygenases function to incorporate molecular oxygen into organic substrates. There are two kinds of oxygenase enzymes, dioxygenases and monooxygenases. Dioxygenases catalyse the addition of both atoms of the oxygen molecule into a substrate. Monooxygenases catalyse the addition of only one atom of the oxygen molecule into the organic substrate, the other atom being reduced to water. A reducing agent is thus required and is normally a reduced pyridine nucleotide. The following equation summarises the monooxygenation reaction,

\[ S + O_2 + H_2R \rightarrow SO + H_2O + R \]

where \( S \) represents a substrate and \( H_2R \) represents the reducing agent.
Since oxidation and oxygenation reactions occur in the above equation the monooxygenase enzymes may be termed mixed function oxidases, of which cytochrome P-450 is one type.

Cytochromes were first discovered in baker's yeast by Keilin in 1925 who called them respiratory pigments due to their presence in respiratory chains.

Klingenberg (1958) and Garfinkel (1958) independently reported the occurrence of a carbon monoxide binding pigment in rodent liver microsomes. In 1964 Omura and Sato characterized this pigment and suggested the name cytochrome P-450 which is now generally accepted as the cellular term for this group of protohaem-containing proteins which exhibit a Soret absorption band between 446 and 454 nm in the dithionite reduced carbon monoxide difference spectrum.

Estabrook et al. (1963) were the first to give evidence for the involvement of cytochrome P-450 in monooxygenase reactions. By 1970 cytochrome P-450 was appreciated as being very important in the metabolism of many xenobiotics such as drugs, insecticides, carcinogens and endogenous compounds such as steroids and fatty acids.

1.2 CYTOCHROME P-450 REACTIONS
Cytochrome P-450 enzymes are the terminal oxidases of a variety of biotransformations employed by many organisms. Cytochrome P-450 uses molecular oxygen and electrons supplied by NADPH via a flavoprotein, cytochrome P-450 reductase, to catalyse the general reaction,
Figure 1.1: Hydroxylation of anisole-$4-^2$H by cytochrome P-450 showing an NIH shift.
SH + NADPH + H^+ + O_2 \rightarrow SOH + NADP^+ + H_2O

where SH represents the substrate and SOH the monoxygenated product.

During catalysis, cytochrome P-450 overcomes the low kinetic reactivity of molecular oxygen and oxidises carbon-hydrogen bonds with variable stereochemical selectivity. Cytochrome P-450 also catalyses the NADPH-dependent reduction of molecular oxygen to H_2O_2 (Gillette et al., 1957; Hildebrandt and Roos, 1973) in the following reaction,

H^+ + NADPH + O_2 \rightarrow NADP^+ + H_2O_2

Cytochrome P-450 also catalyses the lysis of the oxygen-oxygen bond of certain organic hydroperoxides (O'Brien and Rahimtula, 1980) in the following reaction,

X\_1OOH + XH \rightarrow X\_1OH + XOH

The cytochrome P-450 reaction depends on the chemistry of the substrate, with aromatic compounds the first step is considered to be a hydroxylation reaction (Gillette, 1966) involving the incorporation of one oxygen atom into the substrate to form an epoxide which can then proceed to any one of three transformations:

(a) spontaneous isomerisation to phenolic products

(b) enzymic hydration to form trans-dihydrodiols (by the enzyme epoxide hydrase)

(c) the formation of conjugates, for example by reaction with glutathione (Jerina and Daly, 1974).

The above rearrangements of epoxide intermediates results in the 'NIH shift', as shown for p-hydroxylation of anisole in Figure 1.1.

The microsomal monoxygenase system has various components; cytochrome P-450 and NADPH : cytochrome P-450 (c) reductase.
Figure 1.2: Electron transport chain from microsomal cytochrome P-450. SH represents substrate. Transfer of second electron not shown.
e$^1$ is transferred from NADPH via NADPH:cytochrome P-450 reductase

e$^2$ is transferred from either NADPH via NADPH:cytochrome P-450 reductase or from NADH via NADH:cytochrome b$_5$ reductase and cytochrome b$_5$.

**Figure 1.3:** The cytochrome P-450 reaction cycle
Cytochrome P-450 contains the substrate and oxygen binding sites, whilst the reductase serves to transport electrons from NADPH to the cytochrome P-450 component. Together these components form an electron transport chain (see Figure 1.2).

1.2.1 Cytochrome P-450 Monooxygenase Reaction Mechanism

Figure 1.3 shows the most important of the series of events in the mechanism of the cytochrome P-450 monooxygenase reaction, not all of which is fully understood.

The substrate is first bound to the ferric iron form of the cytochrome P-450 enzyme, closely followed by one electron transfer from NADPH via NADPH: cytochrome P-450 reductase. To this complex, oxygen is then bound and one more electron transfers from either NADPH via NADPH: cytochrome P-450 reductase or NADH via NADH: cytochrome b5 reductase and cytochrome b5. The oxygen would now be activated enabling a reaction with the substrate; after which the product would then dissociate from the complex, allowing regeneration of the ferric haemoprotein.

It is possible that some cytochrome P-450 isoenzymes are involved in monooxygenase reactions through free active oxygen species generated by the cytochrome P-450 enzymes in the absence of bound substrate. This may be the case for the cytochrome P-450 isozyme induced by ethanol in mammalian liver (Ingelman-Sundberg and Hagbjork, 1982).

The estimated stoichiometry ratio of cytochrome P-450 molecules to NADPH: cytochrome P-450 reductase molecules in the endoplasmic reticulum ranges between 10 : 1 and 100 : 1 (Estbrook et al., 1971; Sato...
and Omura, 1978). It has been suggested that one cytochrome P-450 molecule may donate electrons to another form of cytochrome P-450 as in mitochondrial electron transport (Nebert, 1979). There have been two theories put forward on the membrane arrangement of cytochrome P-450 and its reductase. The first suggests that 8-12 molecules of cytochrome P-450 are arranged in a rigid cluster around the reductase molecule (Peterson et al., 1976). The second theory considers the possibility of both the cytochrome P-450 and the reductase enzymes diffusing freely in the lateral plane of the membrane (Yang, 1975). There is no direct evidence against either theory, however Schwarz et al. (1982) using studies with saturation transfer EPR spectroscopy demonstrated evidence for a cluster-like organization cytochrome P-450 molecules around the reductase in the microsomal membrane.

Active cytochrome P-450 molecules contain a haem group with iron in the ferric form, so the iron atom has five electrons in the outer d-orbital and can exist in two forms, high spin and low-spin, depending on the extent of the spin-pairing. The low spin form occurs when four of the five d-electrons are paired and corresponds to a six-coordinated haem iron. The high spin form occurs when the five electrons are in separate energy levels and are not paired, this corresponding to a five-coordinated haem iron. When microsomal membranes are intact the ratio of the two spin states is approximately 1:1 but this changes with temperature (Cinti et al., 1979).

Difference spectrophotometry has been used to study the binding of cytochrome P-450 to substrates and other compounds resulting in the classification of three different types of spectrum (Schenkman et al., 1967) which was not yet fully understood. Type I has a spectral
maximum at 385-390 nm and a minimum of 420 nm. Type II has a spectral maximum 425-430 nm, and a minimum 390-410 nm. The third is termed a reverse type I which is a lateral inversion of the type I spectrum. The type I spectrum is considered to result from the binding of a substrate to the binding site in the cytochrome P-450 molecule. The type II spectrum is thought to result from the binding of a compound to a site near the haem group, possibly at the site of the fifth ligand to the haem iron, occupied during catalysis (Schenkman et al., 1981).

Type I substrates of cytochrome P-450 cause a change in the spin state in vivo from low to high spin (Kumaki et al., 1978; Ristau et al., 1979). The spin state of cytochrome P-450 controls the redox potential of the molecule, with the high spin form having a lower redox potential (Sligar, 1976). The binding to a substrate may thus also change the redox potential making it less negative and allowing the electrons to flow to the cytochrome P-450 molecule more easily (Sligar et al., 1979). This infers that the substrate induced spin state change can result in an acceleration of the rate of reduction of the Fe^{3+} of the cytochrome (Misselwitz et al., 1980). A mechanism may thus exist where the substrate facilitates the electron flow to cytochrome P-450 enabling the reaction to proceed.

Cytochrome b_{5} and NADPH : cytochrome P-450 reductase can also modulate the cytochrome P-450 spin state (Tamurini and Gibson, 1983) as can membrane phospholipids (Gibson et al., 1980) each factor possibly being capable of controlling cytochrome P-450 enzyme mechanism (Gibson, 1985).
Figure 1.4: Heme prosthetic groups of haemoproteins (from Jones & Poole, 1985)
Electron spin resonance spectroscopy has been employed to detect unpaired electrons and the technique has demonstrated that cytochrome P-450 can be found in high spin state with 5 unpaired electrons and low spin states with 2 paired electrons and one unpaired electron (Jefcoate and Gaylor, 1969; Hill et al., 1970).

1.3 STRUCTURE

Cytochrome P-450 is a b-type cytochrome and contains an iron protoporphyrin IX prosthetic group. The enzyme has an absorption peak at 450 nm when reduced and complexed with carbon monoxide but degradative agents may cause a conversion to cytochrome P-420 (Mason et al., 1965).

The iron atom at the centre of cytochrome P-450 has four bonds with the nitrogen atoms of the protoporphyrin ring system all in one plane; in an axial plane there are two further axial ligand positions for additional bonding. Figure 1.4 shows the haem prosthetic groups of haemoproteins.

Typically, cytochrome P-450 enzymes are single polypeptide chains of 50-60,000 molecular weight. Yoshida et al. (1977) reported a molecular weight of 51,000 for cytochrome P-450 from S. cerevisiae grown semi-anaerobically but more recently have found that the figure of 58,000 is more accurate (Yoshida and Aoyama, 1984). For cytochrome P-450 from S. cerevisiae grown at high glucose concentration, a molecular weight of 55,000 has been reported (Azari and Wiseman, 1982; King et al., 1984).
THE ACTIVATION OF CARCINOGENS WITH CYTOCHROME P-450

Certain carcinogens may express their carcinogenicity only after metabolism by cytochrome P-450 to reactive intermediates (Nelson, 1982). Indeed protein pyrolysis products become more highly mutagenic when activated by mouse liver microsomal fractions containing a high level of cytochrome P-450 accumulation (Nebert et al., 1979).

Oxygenation by cytochrome P-450 in hindered positions (such as the "bay-region" of polycyclic aromatic hydrocarbons) may convert xenobiotic chemicals into mutagens (Parke and Ioannides, 1982). The oxygenation of carcinogenic polycyclic aromatic hydrocarbons (e.g. benzo(a)pyrene) at the "bay-regions" may form epoxides which are not detoxified by epoxide hydrolase and other enzymes (Levin et al., 1977). Consequently these "bay-region" epoxide compounds react readily with DNA and other cellular molecules and are now known to be the ultimate carcinogen molecular species (Sims, 1974). Cytochrome P-448 isoenzymes are known to be involved in this pathway in mammalian systems (Parke and Ioannides, 1982).

FUNCTION OF YEAST CYTOCHROME P-450 ISOENZYMES IN THE METABOLISM OF XENOBIOTICS

Wiseman and Woods (1979) established the role of cytochrome P-450 from S. cerevisiae in the metabolism of xenobiotics such as benzo(a)pyrene. These workers demonstrated the hydroxylation of benzo(a)pyrene to 3hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene. The cytochrome P-450 isoenzyme involved has a peak at 448 nm in the reduced carbon monoxide difference spectrum (Wiseman and Woods, 1979). These workers found that on binding to benzo(a)pyrene the spin state equilibrium shifted to
higher spin, a change considered important in the cytochrome P-450 mechanism of catalysis.

Yoshida and Kumaoka (1975) reported the hydroxylation of aniline and the demethylation of aminopyrine by yeast cytochrome P-450 isoenzymes Sauer et al. (1982) confirmed the demethylation of aminopyrine and also reported the demethylation of caffeine and p-nitroanisole, but because the activity was measured by formaldehyde production they may be artefactual (Kappeli, 1986).

Wiseman et al. (1975) reported biphenyl hydroxylase activity in yeast cytochrome P-450 microsomal fraction. King (1982) in the same laboratory did find biphenyl hydroxylase activity in yeast spheroplasts but not in the microsomal fraction. Karelampi and Hynninen (1981) showed that S. cerevisiae produced benzoic acid from biphenyl and discussed the difficulties of detecting hydroxylated biphenyl intermediates which might decompose during performance of the assay.

Callen and Philpot (1977) showed that promutagens could be activated by S. cerevisiae. The promutagens dimethylnitrosamine, β-naphthylamine, ethyl carbamate, cyclophosphamide and dimethylsulphoxide were activated to genetically active molecules only in the presence of yeast containing demonstrable cytochrome P-450 isoenzymes.

Callen et al. (1980) also showed that cytochrome P-450 isoenzymes were involved in the metabolism of the halogenated aliphatic hydrocarbons including, methylene chloride, halothane, chloroform, carbon tetrachloride, trichloroethylene, tetrachloroethylene, and S-tetrachloroethane, to active mutagenic species.
Cytochrome P-450 is detected in microsomes from the endoplasmic reticulum, and other organelles such as mitochondria and the nuclear membrane. Cytochrome P-450 is most abundant in microsomes obtained from the endoplasmic reticulum of liver (Klingenberg, 1958; Garfinkel, 1958; Omura and Sato, 1964) but the enzyme had also been found in adrenal cortex (Estabrook et al., 1963), small intestine (Takesue and Sato, 1968), placenta (Meigs and Ryan, 1968), lung (Matusubara and Tochino, 1971), kidney (Ellin et al., 1972), skin (Poland et al., 1974), testis (Betz et al., 1976) and other tissues. Microsomes from mammalian organs such as the brain, thyroid gland and muscle seem to lack cytochrome P-450. In non-mammalian vertebrates cytochrome P-450 has been found in the liver of fish, amphibians, reptiles and birds (Garfinkel, 1963; Strittmatter and Underger, 1969).

Cytochrome P-450 has also certainly been shown to be present in the mitochondria of mammalian endocrine glands which are involved in the synthesis and excretion of steroid hormones. The endocrine glands include the adrenal cortex (Harding et al., 1964), corpus luteum (Yohroe and Horie, 1967) and testis (Purvis et al., 1973). The mitochondria of rat liver (Taniguchi et al., 1973) and chicken kidney (Henry and Norman, 1974) have been found to contain cytochrome P-450.

The nuclear membrane of rat liver contains cytochrome P-450 (Khaudwala and Kaster, 1973) it is considered that the enzyme may be important in the metabolism of carcinogens (Bresnick, 1978). The cell membrane has been demonstrated to contain cytochrome P-450 and there too the enzyme may play a role in the metabolism of carcinogens (Stasiecki et al., 1980). In invertebrates fewer studies have been carried
out, but in insects microsomal fractions from whole insects such as the housefly and the fruit-fly contain cytochrome P-450 (Morello et al., 1971; Capdevila et al., 1975).

In plants the cytochrome P-450 enzyme has been found only in the microsomal fraction, in sorghum seedlings (Potts et al., 1974) cauliflower apical meristems (Rich and Bendall, 1975) castor bean endosperm (Young and Beavers, 1976) and avocado mesocarp (Markham, 1976). Cytochrome P-450 enzymes have also been found in many microorganisms such as bacteria, filamentous fungi and yeasts. Bacterial cytochrome P-450 seems to be localised in the soluble cytoplasmic fraction, whereas those in eukaryotic microorganisms such as filamentous fungi and yeasts are microsomal.

As can be seen, there is a widespread distribution of cytochrome P-450 enzymes in nature. More recently, genes coding for cytochrome P-450 enzymes have been cloned from different organisms and the DNA sequences compared. Considerable sequence homology was demonstrated and it was suggested that all cytochrome P-450 enzymes had a common ancestor millions of years ago (Kimura et al., 1984; Nebert et al., 1984; Nebert and Gonzalez, 1985; Dus, 1985). In cytochrome P-450 enzymes taken from human liver or from Pseudomonas putida, several identical regions can be seen, especially in the alignment of sequences surrounding conserved cysteines which are considered to be important in chelation of the haem iron of cytochrome P-450, suggesting that the structural integrity of the catalytic active site must not change (Kappeli, 1986).
<table>
<thead>
<tr>
<th>Inducer</th>
<th>Description</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>Sedative</td>
<td><img src="image" alt="Phenobarbital" /></td>
</tr>
<tr>
<td>3-methylcholanthrene</td>
<td>Carcinogen</td>
<td><img src="image" alt="3-Methylcholanthrene" /></td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>Carcinogen</td>
<td><img src="image" alt="Benzo(a)pyrene" /></td>
</tr>
<tr>
<td>β-naphthoflavone</td>
<td>Hydrocarbon analog</td>
<td><img src="image" alt="β-Naphthoflavone" /></td>
</tr>
<tr>
<td>Polychlorinated biphenyls (aroclors)</td>
<td>Insulators, lubricants, heat exchange fluids</td>
<td><img src="image" alt="Polychlorinated Biphenyls" /></td>
</tr>
<tr>
<td>Safrole</td>
<td>Carcinogen (formerly a flavouring agent)</td>
<td><img src="image" alt="Safrole" /></td>
</tr>
<tr>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)</td>
<td>Herbicide impurity</td>
<td><img src="image" alt="2,3,7,8-Tetrachlorodibenzo-p-Dioxin" /></td>
</tr>
<tr>
<td>Pregnenolone 16α-carbonitrile</td>
<td>Steroidal derivative</td>
<td><img src="image" alt="Pregnenolone 16α-Carbonitrile" /></td>
</tr>
</tbody>
</table>

Table 1.1: Mammalian cytochrome P-450 inducing agents
The sequencing of several structural genes of different cytochrome P-450 isoenzymes has been determined (Fujii-Kuriyama et al., 1982; Sogawa et al., 1984) and amino acid sequencing of several of the cytochrome P-450 proteins (Haniu et al., 1984; Ozds, 1986). The structural homologies indicate that cytochrome P-450 isoenzymes can be grouped into families.

MAMMALIAN CYTOCHROME P-450

Cytochrome P-450 enzymes from the rodent liver are of two kinds. The first kind are of broad specificity and function to detoxify drugs and xenobiotics enabling a conversion to more polar compounds such as to be dispelled from the organism. The second kind are the narrow specificity (Wiseman and King, 1982) cytochrome P-450 enzymes. These enzymes are involved in the biosynthesis of biologically active agents such as steroid hormones and prostaglandins.

The induction of rodent cytochrome P-450 enzymes can be changed by the treatment of the animal with a wide variety of drugs and carcinogens (see Table 1.1). Induction occurs by de novo synthesis of the induced enzymes, which results from the induction of specific mRNA species (Kumar and Padmanaban, 1980).

After rodent ingestion with phenobarbital the major cytochrome P-450 isoenzyme present has a wide substrate specificity and functions in the metabolism and detoxication of many xenobiotic compounds. There are seven forms of this kind of enzyme each with some level of overlap with regard to substrate specificity (Nebert et al., 1981). The major rodent liver cytochrome P-450 isozyme present after ingestion of 3-methylcholanthrene or benzo(a)pyrene or 2,3,7,8-tetrachlorodibenzo-p-
Table 1.2: Major rabbit liver cytochrome P-450 enzymes

<table>
<thead>
<tr>
<th>Cytochrome P-450</th>
<th>Molecular weight</th>
<th>Soret peak (carbon monoxide bound)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM 2</td>
<td>48,000</td>
<td>451</td>
<td>Haugen &amp; Coon (1976)</td>
</tr>
<tr>
<td>LM 3a</td>
<td>51,000</td>
<td>452</td>
<td>Koop et al. (1982)</td>
</tr>
<tr>
<td>LM 3b</td>
<td>52,000</td>
<td>450</td>
<td>Koop et al. (1981)</td>
</tr>
<tr>
<td>LM 3c</td>
<td>53,000</td>
<td>449</td>
<td>Koop et al. (1981)</td>
</tr>
<tr>
<td>LM 4</td>
<td>54,000</td>
<td>448</td>
<td>Coon et al. (1978; 1980)</td>
</tr>
<tr>
<td>LM 6</td>
<td>57,000</td>
<td>448</td>
<td>Norman et al. (1978)</td>
</tr>
<tr>
<td>Organism</td>
<td>Role</td>
<td>References</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>camphor hydroxylation</td>
<td>Gunsalus et al., 1975</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>O'Keefe et al., 1978</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>steroid 158-hydroxylation</td>
<td>Berg et al., 1979</td>
<td></td>
</tr>
<tr>
<td>ATCC 13368</td>
<td></td>
<td>Berg &amp; Rafter, 1981</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>hydroxylation of fatty acids, alcohols and amides</td>
<td>Matson et al., 1977, 1980</td>
<td></td>
</tr>
<tr>
<td>ATCC 14581</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp. 7E1C</td>
<td>hydroxylation of alkanes</td>
<td>Cardini &amp; Jurtshuk, 1970</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter</em> sp.</td>
<td>hydroxylation of alkanes</td>
<td>Asperger et al., 1981</td>
<td></td>
</tr>
<tr>
<td><em>Nocardia</em> NHI</td>
<td>p-O-dealkylation</td>
<td>Broadbent &amp; Cartwright, 1974</td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium japonicum</em></td>
<td>Role in $N_2$ fixation?</td>
<td>Appleby, 1978</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>p-nitroanisole dealkylation</td>
<td>Edelson &amp; McMullen, 1977</td>
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<tr>
<td><em>Photobacterium fischeri</em></td>
<td>aliphatic hydroxylation (in bacterial luminescence)</td>
<td>Ismailova et al., 1981</td>
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<td></td>
<td></td>
<td>Danilov et al., 1982</td>
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</tbody>
</table>

Table 1.3: Bacterial cytochrome P-450 systems
Figure 1.5: Electron transport chain for cytochrome P-450cam from *Pseudomonas putida*
Dioxin is an enzyme with narrow specificity with a Soret peak in the reduced carbon monoxide difference spectrum at 448 nm. This type of cytochrome P-450 may be referred to as cytochrome P-448 isozymes. The exact number of cytochrome P-450 isozymes is not known since there are several hundred known inducers. Recent estimates suggest about twelve narrow specificity isozymes (Coon et al., 1985; Levin et al., 1985). It is possible that many more cytochrome P-450 isoenzymes exist perhaps being induced in a manner analogous to antibody synthesis (Nebert et al., 1981) (see Table 1.2 for major rabbit hepatic cytochrome P-450 enzymes). There is no agreed nomenclature on cytochrome P-450 isozymes and there may be several different names adopted by different authors for any isoenzymes. Several isoenzymes have been purified and characterized immunologically (Levin et al., 1985; Ozols, 1986; Sesardic et al., 1986). Examples of narrow specificity cytochrome P-450 isozymes are the mitochondrial enzyme cytochrome P-450SCC which functions to cleave the cholesterol side chain (Takemori et al., 1978) also microsomal cytochrome P-450c21 which is specific for steroid 21-hydroxylation (White et al., 1984).

1.8 CYTOCHROME P-450 IN PROKARYOTIC MICROORGANISMS

Table 1.3 shows the presence of cytochrome P-450 in bacteria. All are soluble enzymes with narrow specificity. The best known is Pseudomonas putida which produces a cytochrome P-450 enzyme when grown on D(+)-camphor, which metabolizes camphor, enabling growth of the organism on this compound as sole carbon and energy source (Yu et al., 1974) (see Figure 1.5 for electron transport chain in cytochrome P-450CAM). The enzyme from P. putida (termed cytochrome P-450CAM) hydroxylates the 5-methylene carbon of camphor to form the exo-5-alcohol (Gunsalus et al., 1975). Because cytochrome
P-450\textsubscript{CAM} is soluble (not bound on membrane) it has been easier to purify and crystallise. It has been found to contain 414 amino acid residues, have a molecular weight of 45,000 and a Soret peak in the reduced carbon monoxide difference spectrum at 446 nm (O'Keefe et al., 1978). X-ray analysis has shown that the substrate molecule is buried in an internal pocket above the haem distal surface adjacent to the oxygen binding site (Poulos et al., 1985).

Cytochrome P-450\textsubscript{CAM} has its own associated electron transport system (see Figure 1.5) which carries an additional protein component known as putidaredoxin, a small iron-sulphur protein. Cytochrome P-450\textsubscript{CAM} thus differs from mammalian mitochondrial cytochrome P-450 which also contains an additional iron-sulphur protein (Suhura et al., 1978).

From \textit{Bacillus megaterium} strain ATCC 14581 a cytochrome P-450 isoenzyme has been found with \textit{w}-2 hydroxylase activity towards fatty acids, which can also hydroxylate corresponding amides and alcohols (Matson et al., 1977) and catalyse the epoxidation of unsaturated fatty acids (Reuttinger and Fulco, 1981).

From \textit{Bacillus megaterium} strain ATCC 13368 another cytochrome P-450 isoenzyme P-450 isoenzyme has been found with 15\textbeta-hydroxylase activity for 3-oxo-\Delta\textsuperscript{4}-steroids such as progesterone (Berg et al., 1977).

A cytochrome P-450 isoenzyme has been found in \textit{Corynebacterium} when grown on n-octane as the only carbon and energy source (Cardini and Jurtshuk, 1970). The isoenzyme can oxygenate several other hydrocarbon substrates such as benzene, toluene and other aliphatic hydrocarbons.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claviceps purpurea</td>
<td>alkaloid biosynthesis</td>
<td>Ambike &amp; Baxter, 1970</td>
</tr>
<tr>
<td>Rhizopus nigricans</td>
<td>steroid hydroxylation</td>
<td>Breskvar &amp; Hudnik-Plevnik, 1977, 1981</td>
</tr>
<tr>
<td>Cunninghamella bainieri</td>
<td>aromatic hydroxylation</td>
<td>Ferris et al., 1973, 1976</td>
</tr>
<tr>
<td></td>
<td>N- and O-demethylation</td>
<td></td>
</tr>
<tr>
<td>Cunninghamella elegans</td>
<td>aromatic hydroxylation</td>
<td>Cerniglia &amp; Gibson, 1978, 1979</td>
</tr>
</tbody>
</table>

Table 1.4: Fungal cytochrome P-450 systems
Several strains of *Acinetobacter* accumulate cytochrome P-450 when grown on n-alkanes (Asperger et al., 1981). The cytochrome P-450 is considered to hydroxylate the alkane (Asperger et al., 1981) much as a similar hydrocarbon inducible cytochrome P-450 dependent alkane hydroxylase found in some yeast species (see on).

Three cytochrome P-450 isoenzymes have been purified from *Rhizobium japonicum* grown symbiotically on soybean root nodules. The three enzymes had Soret peaks in the reduced carbon monoxide difference spectrum at 449 nm, 449 nm and 447 nm (Appleby, 1978). The function of the enzymes is not known. It is possible they may remove oxygen in the anaerobic environment or be involved in electron transport at very low oxygen tension to generate ATP required for nitrogenase activity (Bergersen and Turner, 1975).

1.9 CYTOCHROME P-450 FROM EUKARYOTIC MICROORGANISMS

1.9.1 Fungi

The electron transport chain in eukaryotic microorganisms is similar to the microsomal system in mammals and contains only two protein components, a reductase (with two flavin groups, one FAD and one FMN) and a cytochrome P-450. Eukaryotic microorganisms cytochrome P-450 systems have all been found to be membrane-bound to the endoplasmic reticulum just as in mammalian systems and in having broader cytochrome P-450 substrate specificities than bacteria but not as broad as in mammals. Table 1.4 shows the fungal cytochrome P-450 systems in species known to produce the enzyme.

*Claviceps purpurea* possesses a microsomal cytochrome P-450 which is induced 100% by phenobarbital (Ambike and Baxter, 1970). When treated
with 3-methylcholanthrene a much smaller induction occurs with a shift in the Soret peak of the reduced carbon monoxide difference spectrum from 450 to 448 nm, with the use of protein synthesis inhibitors it was shown that this shift in the wavelength is from de novo protein synthesis (Ambike and Baxter, 1970). These workers also showed a direct correlation between alkaloid production and cytochrome P-450 monooxygenase levels, which suggested that a cytochrome P-450 enzyme is involved in alkaloid biosynthesis, although it is not yet known how.

*Rhizopus nigricans* has been demonstrated to possess a cytochrome P-450 enzyme which is capable of catalysing the 11α-hydroxylation of progesterone (Breskvar and Hudnik-Plevik, 1977). *Cunninghamella bainieri* has been shown to possess a cytochrome P-450 enzyme capable of N-demethylation of aminophenazone, O-demethylation of p-nitroanisole and the hydroxylation of anisole, aniline and naphthalene (Ferris et al., 1973; 1976). *Cunninghamella elegans* has been shown to possess cytochrome P-450 which is induced to five times its original level by naphthalene (Cerniglia and Gibson, 1978). The enzyme is capable of hydroxylating naphthalene and benzo(a)pyrene (Cerniglia and Gibson, 1979). Hydroxylation of benzo(a)pyrene is also well known in the cytochrome P-450 system of *S. cerevisiae* (see on).

### 1.9.2 Occurrence of Cytochrome P-450 in Yeasts

In 1964 Lindenmayer and Smith showed that *Saccharomyces cerevisiae* contained cytochrome P-450. In 1971 cytochrome P-450 was found to accumulate in the n-alkane degrading yeasts of the genus *Candida* (Lebeault et al., 1971). Here the enzyme was induced by the n-alkane substrate, since in glucose, as the sole carbon source, even under oxygen limitation no cytochrome P-450 could be detected. Thus it was not
<table>
<thead>
<tr>
<th>Organism</th>
<th>Preferred Carbon Source</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Candida tropicalis</em></td>
<td>n-tetradecane</td>
<td>Lebeault et al., 1971</td>
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<tr>
<td><em>Candida quilliermondii</em></td>
<td>hexadecane-</td>
<td>Muller et al., 1979</td>
</tr>
<tr>
<td></td>
<td>octadecane</td>
<td></td>
</tr>
<tr>
<td><em>Candida lipolytica</em></td>
<td>hexadecane</td>
<td>Mauersberger et al., 1980</td>
</tr>
<tr>
<td><em>Torulopsis candida</em></td>
<td>hexadecane</td>
<td>Il chenko et al., 1980</td>
</tr>
<tr>
<td><em>Loddermyces elongisporus</em></td>
<td>tetradecane</td>
<td>Mauersberger et al., 1980</td>
</tr>
<tr>
<td><em>Saccharomyces lipolytica</em></td>
<td>hexadecane</td>
<td>Marchal et al., 1982</td>
</tr>
</tbody>
</table>

Table 1.5: Yeasts utilizing n-alkanes
understood whether the presence of the cytochrome P-450 was related to specific function or whether it was generally present in these eukaryotes. Table 1.5 shows the yeasts utilising n-alkanes.

The yeast species growing on n-alkanes as the sole carbon source employ cytochrome P-450 as an alkane hydroxylase to catalyse the degradation of n-alkanes for growth similar to the prokaryotes Corynebacterium and Acinetobacter (q.v. previous section). In n-alkane degrading yeasts, the primary hydroxylation of the substrate by cytochrome P-450, (no other hydroxylation system has been reported) may be the rate-limiting step in the alkane degradation (Gmunder et al., 1981).

Muller et al. (1979) describe the accumulation of cytochrome P-450 in Candida guilliermondii grown on n-alkanes, which hydroxylate long chain alkanes to their primary alkanols. In this yeast glucose was found to act as a repressor of cytochrome P-450 (Muller et al., 1979). The level of alkane-induced cytochrome P-450 in this yeast is higher under conditions of low oxygen concentration, suggesting that oxygen limitation causes a decrease in alkane hydroxylation rate which in turn affects an increase in the production of cytochrome P-450 (Mauersberger et al., 1980).

More recently, Sanglard et al. (1984) have postulated that a different cytochrome P-450 isoenzyme can be detected in Candida tropicalis under glucose growth conditions, which gives a peak at 448 nm in the reduced carbon monoxide difference spectrum, this is contrary to Muller et al. (1979) but more sensitive spectrophotometric detection may have been employed by Sanglard et al., since the values of cytochrome P-450 were very low. Also spectra of cytochrome P-450 and mitochondrial cytochrome oxidase overlap in the reduced carbon monoxide difference
<table>
<thead>
<tr>
<th>Yeast Organism</th>
<th>Physiological Function or Catalyzed Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>-----</td>
<td>Lindenmayer and Smith, 1964</td>
</tr>
<tr>
<td></td>
<td>14α-demethylation of lanosterol (a)</td>
<td>Aoyama and Yoshida, 1978a,b</td>
</tr>
<tr>
<td></td>
<td>hydroxylation of biphenyl (c)</td>
<td>Wiseman <em>et al.</em>, 1975</td>
</tr>
<tr>
<td></td>
<td>activation of promutagens (c)</td>
<td>Callen and Philpot, 1977</td>
</tr>
<tr>
<td></td>
<td>metabolism of halogenated hydrocarbons (c)</td>
<td>Callen <em>et al.</em>, 1980</td>
</tr>
<tr>
<td><em>Saccharomyces carlsbergensis</em></td>
<td>unknown</td>
<td>Cartledge <em>et al.</em>, 1972</td>
</tr>
<tr>
<td><em>Saccharomyces bayanus</em></td>
<td>unknown</td>
<td>Karenlampi <em>et al.</em>, 1980b</td>
</tr>
<tr>
<td><em>Saccharomyces chevalieri</em></td>
<td>unknown</td>
<td>Karenlampi <em>et al.</em>, 1980b</td>
</tr>
<tr>
<td><em>Saccharomyces italicus</em></td>
<td>unknown</td>
<td>Karenlampi <em>et al.</em>, 1980b</td>
</tr>
<tr>
<td><em>Saccharomyces uvarum AH15</em></td>
<td>unknown</td>
<td>Karenlampi <em>et al.</em>, 1980b</td>
</tr>
<tr>
<td><em>Saccharomyces lipolytica</em></td>
<td>hydroxylation of n-alkanes (b)</td>
<td>Delaisse and Nyns, 1984</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>unknown</td>
<td>Poole <em>et al.</em>, 1974</td>
</tr>
<tr>
<td><em>Schizosaccharomyces japonicus</em></td>
<td>unknown</td>
<td>Karenlampi <em>et al.</em>, 1980b</td>
</tr>
</tbody>
</table>

Table 1.6: *Saccharomyces* and *Schizosaccharomyces* cytochrome P-450 systems (from Muller, *et al.*, 1984)
spectrum, cytochrome oxidase has a trough at 441 445 nm and hence cytochrome P-450 may not be detected in cells with high mitochondrial content, one answer may be to employ high glucose concentration in the growth medium to decrease synthesis of mitochondrial cytochromes (see on). Table 1.6 shows the level of cytochrome P-450 accumulation in some yeasts.

1.9.3 Cytochrome P-450 from Saccharomyces cerevisiae

The formation of cytochrome P-450 in \textit{S. cerevisiae} depends on the cultivation conditions. Lindenmayer and Smith (1964) found that high concentrations of cytochrome P-450 accumulated in \textit{S. cerevisiae} grown semianaerobically in a growth medium containing 4% (w/v) glucose, much lower concentrations accumulating when the yeast was grown aerobically in the same glucose concentration growth medium. Ishidate \textit{et al.} (1969) showed that cytochrome P-450 was to be found in the microsomal fraction of the cell and that the spectral properties were similar to those from mammalian microsomal fractions.

In batch cultures Wiseman and King (1982) outlined the conditions which give rise to high cytochrome P-450 accumulations in \textit{S. cerevisiae}, suggesting that one of the following conditions is required:

(i) high glucose concentration (4%-20%, w/v) in the growth medium.
(ii) semi-anaerobic growth conditions.
(iii) the addition to the medium of inhibitors of mitochondrial protein synthesis such as chloramphenicol.
(iv) the use of respiratory-deficient mutants.

The prime effect of such conditions is a decreased synthesis of mitochondrial cytochromes. It has been noted that there is an inverse relationship between mitochondrial cytochrome content, especially
cytochrome a, and cytochrome P-450 accumulation in cells. High cytochrome P-450 accumulation is obtained when the mitochondrial cytochrome concentration decreases (Trinne et al., 1982). Trinne et al. reported that by oxygen limitation alone it was not possible to accumulate cytochrome P-450, both glucose repression and oxygen limitation were required.

The amount of cytochrome P-450 produced during growth in high glucose concentration growth media by S. cerevisiae depends, substantially, on the strain employed. King et al. (1983a) found wide variations in the level of cytochrome P-450 accumulation in 18 haploid strains of S. cerevisiae.

Cytochrome P-450 accumulates rapidly during the exponential phase of growth (Woods, 1979; Karenlampi et al., 1981). During the stationary phase accumulation ceases and cytochrome P-450 degradation ensues enhanced by oxygen and mitochondrial protein biosynthesis (Biaiak et al., 1980).

Salihon et al. (1983) optimized the yield of cytochrome P-450 from S. cerevisiae grown in glucose growth medium in batch culture using shake flasks and the 5 litre bioreactor (Salihon et al., 1985). These workers found that impeller speed and air flow rate are important factors in the maintenance of most optimal dissolved oxygen availability.

Karenlampi et al. (1981) found cytochrome P-450 accumulated during growth of yeast at high concentrations of fructose or sucrose as well as glucose; less enzyme accumulated with galactose or maltose and no enzyme was detected on glycerate, lactate or ethanol. The workers
concluded that cytochrome P-450 accumulation was dependent on conditions of rapid growth and fermentation (in agreement with Wiseman and King, 1982) though perhaps the mitochondria need not be repressed.

King et al. (1983a) made a study of the genetic regulation of cytochrome P-450 production in S. cerevisiae. Figure 1.6 summarises the results the workers obtained. King et al. concluded that a single nuclear gene appears to control the production of cytochrome P-450 but since the diploid produced no cytochrome P-450 the single nuclear gene may well be a regulatory gene rather than a structural gene. From the tetrad, one strain produced a cytochrome P-450 level similar to the parent producer strain and the other producer strain gave twice as much cytochrome P-450. Therefore a second nuclear gene may be modifying the regulatory cytochrome P-450 production.

1.10 PHYSIOLOGICAL FUNCTIONS OF CYTOCHROME P-450 IN S. CEREVISIAE

There was considerable uncertainty concerning the physiological function of cytochrome P-450 from S. cerevisiae. Alexander et al. (1974) suggested an involvement in ergosterol biosynthesis. In 1978, Ohba et al., showed that the oxygen and NADPH-dependent conversion of lanosterol to 4,4-dimethylzymosterol is inhibited by cytochrome P-450 antibodies. In the same year Aoyama and Yoshida used a reconstituted system of cytochrome P-450 and NADPH-cytochrome P-450 reductase to show the oxidative removal of the 14α-methyl group of lanosterol. In both mammalian and yeast sterol biosynthesis, lanosterol is a key intermediate and the lanosterol 14α-demethylation reaction, which involves removal of the C-32 methyl group (see Figure 1.7), is a rate-limiting step: cytochrome P-450 probably catalyses this step in mammals (Gibbons et al., 1979) and yeast (Aoyama et al., 1984).
**Haploid Parent**

<table>
<thead>
<tr>
<th>cyt⁺ rep⁻ mod⁻</th>
<th>cyt⁺ rep⁺ mod⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>(cytochrome P-450 producer)</td>
<td>(non-producer)</td>
</tr>
</tbody>
</table>

**Diploid**

<table>
<thead>
<tr>
<th>cyt⁺ rep⁻ mod⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>(non-producer)</td>
</tr>
</tbody>
</table>

- meiotic tetrad
  - cyt⁺ rep⁻ mod⁻ (parental type producer)
  - cyt⁺ rep⁺ mod⁺ (parental type non-producer)
  - cyt⁺ rep⁻ mod⁺ (modified producer)
  - cyt⁺ rep⁺ mod⁻ (non-producer)

**cyt:** structural gene for cytochrome P-450

**rep⁻/rep⁺:** non-repressing and permanently repressing allelomorphs, respectively at a regulatory gene.

rep⁺ is dominant in the heterozygote.

**mod⁻/mod⁺:** allelomorphs of a modifying gene active in rep⁻ cells. The presence of mod⁺ enhances the amount of cytochrome P-450 accumulated.

---

Figure 1.6: Model for the genetic control of P-450 accumulation in *Saccharomyces cerevisiae*
Figure 1.7: The 14α-demethylation of Lanosterol
There was further uncertainty concerning the synthesis of ergosterol in yeast grown under growth conditions such that cytochrome P-450 was not detectable spectrally. Aoyama et al. (1981), however, showed that cytochrome P-450 catalysed lanosterol 14α-demethylation did occur since antibodies to purified cytochrome P-450 inhibited the reaction. Thus, a low level of cytochrome P-450 not detectable spectrally must be sufficient for lanosterol 14α-demethylation. Also inhibiting the same reaction are medical and agricultural antifungal agents which belong to the group ofazole-containing compounds such as imidazole and triazole derivatives and nonazole nitrogen heterocycles (van den Bossche, 1985), which lead to a build-up of lanosterol and disrupts the membrane structure causing permeability to protons (Thomas et al., 1983). Inhibition of cytochrome P-450 catalysis of lanosterol 14α-demethylation causing lanosterol build-up can decrease the presence of membrane bound enzymes such as fatty acid desaturase (van den Bossche, 1985).

Hata et al. (1981) suggest the involvement of cytochrome P-450 in the \( \Delta^{22} \)-desaturation of ergosta-5,7-dien-3\( \beta \)-ol to form ergosterol. By using mutant strains which lacked the 14α-demethylase, \( \Delta^{22} \)-desaturation was still possible, this may indicate that two different cytochromes P-450 isoenzymes may be involved in ergosterol biosynthesis (Hata et al., 1983). Cytochrome P-450 may be involved in an alternative regeneration of reducing equivalents, referred to as cyanide insensitive respiration (Ainsworth et al., 1980). Under conditions of low oxygen tensions, the alternative pathway may regenerate NADH and NADPH. Table 1.5 shows *Saccharomyces* and *Schizosaccharomyces* species cytochrome P-450 systems.
The chemical hydroxylation at the aromatic ring is an expensive step in the synthesis of a specific aromatic chemical because of the non-specificity of the hydroxylation reaction forming unwanted by-products and thus creating an inefficient use of the starting material. Chemical hydroxylations require extreme conditions and so consume large quantities of energy. Also there is the possibility of the formation of undesirable contaminants in non-specific hydroxylation reactions. Cytochrome P-450 has a wide range of substrates, and selective hydroxylation or side chain cleavage reactions of organic compounds on a preparative scale would be useful in the pharmaceutical industry (Mohr et al., 1984).

Cytochrome P-450 may be used in vitro to determine the potential carcinogenic or mutagenic activities of prospective drugs. The Ames test already employs cytochrome P-450 in this way (Ames, 1974). An extracorporeal metabolic detoxification of blood system using cytochrome P-450 enzymes which catalyse the conversion of drugs and other foreign substances into water soluble compounds, which could then be excreted by the kidney, is a possibility.

Cytochrome P-450 may also be used as a test system for antifungal agents many of which are cytochrome P-450 inhibitors (van den Bossche, 1985). Cytochrome P-450 could further be used in the design of new antifungal agents, allowing the mechanism of action to be precisely investigated using isolated cytochrome P-450.

All the applications of cytochrome P-450 necessitate knowledge on the accumulation and degradation of this enzyme to facilitate production in
large amounts. For this purpose mass culture of microorganisms for cytochrome P-450 would be best and to consider in which organism mammalian cytochrome P-450 is best suited all the characteristics of the systems must be known.

1.11.1 Applications of Yeast Cytochrome P-450

The concentration of cytochrome P-450 in the yeast microsomal fraction is about 1-10% of that from mammalian liver microsomal fraction. Thus for practical applications (see section 1.11) of yeast cytochrome P-450 a higher concentration of the enzyme is required. There are possible approaches to enhance cytochrome P-450 expression:

(i) To clone the cytochrome P-450 genes and put them under the control of a more avid promoter (such as the alcohol dehydrogenase I promoter (Ammerer, 1983)).
(ii) To select the most suitable yeast strain (see chapter 2).
(iii) To optimise cultivation conditions not only in the media (Salihon, 1983, 1985) but also of the inoculum (see chapter 6).
(iv) To prevent degradation of cytochrome P-450 already present, whilst awaiting a greater accumulation (see chapters 4 and 8).

Yeasts are suitable as hosts for mammalian cytochrome P-450. In 1985 Oeda et al. constructed an expression plasmid for the cloned cytochrome P-450 complementary DNA under the control of yeast alcohol dehydrogenase I promoter. The yeast microsomal cytochrome P-450 was subsequently found to exhibit benzo(a)pyrene hydroxylase activity (Oeda et al., 1985). This experiment indicates that electron transport to foreign cytochrome P-450 can be effected with yeast NADPH-cytochrome P-450 reductase. It may therefore be possible that electron transport and monooxygenase activity will be achieved with
### Table 1.7: Consensus sequences in introns involved in splicing in Saccharomyces cerevisiae, Schizosaccharomyces pombe and higher eukaryotes

(from Langford et al., 1984; and Piskelny et al., 1983)

<table>
<thead>
<tr>
<th>Organism</th>
<th>5' Splice Site</th>
<th>Internal Sequence</th>
<th>3' Splice Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>G GTA$_C^{T}$GT</td>
<td>TACTAAC</td>
<td>TAG</td>
</tr>
<tr>
<td>Sch. pombe</td>
<td>G GTAAGT</td>
<td>CT$_G^{A}$A$_C^{T}$T$_A$</td>
<td>TAG</td>
</tr>
<tr>
<td>Higher eukaryote</td>
<td>G GTA$_G^{A}$AGT</td>
<td>CT$_G^{A}$A$_C^{T}$</td>
<td>TAG</td>
</tr>
</tbody>
</table>
other mammalian cytochrome P-450 isoenzymes expressed in yeasts. It may be possible that particular catalytic activities of cytochrome P-450 isoenzymes be obtained from yeasts.

_S. cerevisiae_ has been used in the traditional biotechnology of baking and brewing which has led to it being characterised genetically and biochemically more extensively than any other yeast. Furthermore, it has become, next to _E. coli_, the host microorganism for cloned genes and in some laboratories the model eukaryote for research on eukaryotic gene structure, function and regulation.

_Schizosaccharomyces pombe_ has been employed much less, principally in cell division cycle research and industrially in removing malic acid from lesser quality wine musts (Beelman and Gallander, 1979). More recently, however, Kaufer et al. (1985) have shown that _Schizosaccharomyces pombe_ may be more useful, in certain genetic manipulations, than _S. cerevisiae_. _Schizosaccharomyces pombe_ can accurately excise an intervening sequence from the transcript of a higher eukaryotic gene. Beggs et al. (1980) showed that in some instances cloning in _S. cerevisiae_ gave rise to an unspliced and incorrectly terminated transcript. This is due to differences in the specificity of splicing between _S. cerevisiae_ and higher eukaryotes (see Table 1.7) and probably reflects the stringency of the requirement for the TACTAAC sequence located near the 3' end of the intron (Langford et al., 1984; Pikielny et al., 1983).

If mammalian cytochrome P-450 genes could be cloned and expressed in yeast then media and inoculum optimisation procedures would still be necessary as would inhibition of the enzymes degradation.
The reaction mechanism of cytochrome P-450 has still to be resolved in precise detail. Because yeast cells under certain conditions express only one form of cytochrome P-450, this is easier to research than the multiple forms present in hepatic systems. The one cytochrome P-450 isoenzyme can be studied with greater ease with regard to the biochemical interactions of the individual components of the electron transport chain.

The expression of mammalian cytochrome P-450 genes in yeasts may help in the understanding of cytochrome P-450 specificity.

1.11.2 Application of Yeasts in Research on Carcinogens

The most widely used short-term method for screening environmental carcinogens is the Ames test (Ames, 1974). The method involves treating the possible carcinogens with liver homogenate extracted from rats which were pretreated with Aroclor to induce a mixture of cytochrome P-450 isoenzymes. The liver homogenate (S9 fraction) is capable of metabolizing most carcinogens to mutagens, the degree of mutagenicity is then indicated with *Salmonella typhimurium*. The test has proved very helpful because many carcinogens are also mutagenic (McCann *et al.*, 1975). What is not helpful is that since the carcinogen is applied to the indicator cell in vitro thus before interaction with DNA can occur the carcinogen may lose its activity, if it is highly labile or reactive. Another disadvantage is the binding or reaction of the carcinogens to proteins in the S9 fraction.

With yeasts as indicator cells the carcinogens can be metabolized inside the cell (Callen and Philpot, 1977) and it was found that a higher concentration of cytochrome P-450 gave better activation of the
carcinogen (Kelly and Parry, 1983). The problem facing mutagenic testing with yeast is whether yeast cytochrome P-450 systems give the same metabolites as the hepatic S9 fraction (since the yeast cytochrome P-450 isoenzymes have a narrower specificity). Current research on cloned mammalian cytochrome P-450 enzymes expressed in yeast might overcome this problem (Oeda et al., 1985).

Another method of testing carcinogens is to use the yeast mitochondrial system. Yeast cells can grow anaerobically by employing the glycolytic pathway, provided that a fermentable carbon source, such as glucose, is present. Under aerobic conditions yeast mitochondria can use the pyruvate from glycolysis to produce more ATP with the Krebs cycle and respiratory chain. However, if the mitochondria are damaged or inhibited by anti-mitochondrial agents, then the yeast can only grow in the presence of a fermentable carbon source. A yeast mitochondrial mutant is known as a petite because when plated on 2% glucose agar medium they are smaller than normal yeast colonies (Ephrussi, 1949). It is well known that acriflavine and ethidium bromide induce petite mutations very efficiently in yeasts, but most chemical carcinogens induce the petite mutations and depress organelle protein synthesis (Egilsson et al., 1979). In addition, chemical carcinogens could be detected by their effect on certain yeast cell surface characteristics or by monitoring oxygen uptake in yeasts (Wilkie, 1982).
CHAPTER 2

ASPECTS ON THE CONDITIONS REQUIRED FOR
MAXIMUM CYTOCHROME P-450 ACCUMULATION
2.1 INTRODUCTION

In batch culture of unicellular organisms there are three types of changes in physiological activity expressed in terms of cell population (see Figure 2.1). Type 1 is growth associated and type 2 is non-growth associated (Yanagita, 1966). But growth associated changes in physiological activity can also be type 3.

In Type 1 the duration of the phase is longer than in type 2. In the growth associated change, the highest activity per cell is exhibited at the transition stage from the lag to exponential phases, explained by the tendency of the cells in the lag phase to grow rather synchronously (Prescott, 1937). At the end of the exponential phase the population is composed of actively dividing cells which are expected to be most active metabolically on a cell number basis.

When the culture enters the steady state stationary phase, the population is composed of cells of various phases of the cell cycle under a fixed cell-age distribution pattern (Maruyama, 1955). In the exponential phase the metabolic activity exhibited by the population is at the average level of these cells of different cell ages. The protein in type 1 represents the average metabolic activity.

In type 3 such a steady state cannot be maintained because of the short duration of the exponential phase. In that stationary phase, eventually most of the cells become senescent or dormant (Brown and Zainudeen, 1978) and the average metabolic activities drop to the lowest values.

In the case of non-growth associated metabolic changes in unicellular organism cultures the activities are expected to increase as the culture enters the stationary phase (Yanagita, 1966).
Figure 2.1. The three types of changes in physiological activity expressed in terms of unicellular population.

(From Yanagita, 1966)
From these three types it can be seen that the accumulation of cytochrome P-450 in *S. cerevisiae* matches type 3 best. The growth of a culture is therefore important in determining the time of maximum accumulation of an enzyme. During the exponential phase of growth the population of yeast increases at a constant rate, the rate of increase of biomass (dx/dt) being proportional to the amount of biomass (x) present;

\[
dx/dt = \mu x\tag{Equation 1}
\]

where the proportionality constant \( \mu \) is the specific growth rate of the culture; \( \mu \) is a constant for a given medium, yeast strain and growth conditions but may depend also on the age of the inoculum slope (see chapter 6).

The growth rate of a culture is often expressed in terms of the doubling time \( t_D \) which is derived from equation 1 thus;

\[
dx/x = \mu dt
\]

integrating,

\[
\ln_e x - \ln_e x_0 = \mu t
\]

\[
\ln_e (x/x_0) = \mu t
\]

but if,

\[
x/x_0 = 2
\]

then,

\[
t_D = \log_{10} 2/\mu = 0.301/\mu
\]

Lindenmayer and Smith (1964) were the first to report the presence of cytochrome P-450 in yeast. These workers found that the enzyme was present in higher concentrations under semi-anaerobic conditions in 4% (w/v) glucose growth media and in lower concentration under aerobic conditions in 4% glucose growth media. Ishidate et al. (1969a) found that in 1% (w/v) glucose growth media under aerobic conditions no
<table>
<thead>
<tr>
<th>Yeast</th>
<th>Cytochrome P-450 nmol. (g. wet wt.)^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brettanomyces anomalous</td>
<td>7.82</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td></td>
</tr>
<tr>
<td>glucose grown</td>
<td>0.10</td>
</tr>
<tr>
<td>alkane grown</td>
<td>5.11</td>
</tr>
<tr>
<td>Candida utilis</td>
<td></td>
</tr>
<tr>
<td>glucose grown</td>
<td>0.00</td>
</tr>
<tr>
<td>alkane grown</td>
<td>0.00</td>
</tr>
<tr>
<td>Debaromyces hansenii</td>
<td>0.13</td>
</tr>
<tr>
<td>Hansenula anomala</td>
<td>4.07</td>
</tr>
<tr>
<td>Kluyveromyces fragilis</td>
<td>1.87</td>
</tr>
<tr>
<td>Pichia fermentans</td>
<td>0.95</td>
</tr>
<tr>
<td>Saccharomyces bayanus</td>
<td>3.68</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae NCYC 240</td>
<td>4.66</td>
</tr>
<tr>
<td>Saccharomyces chevalieri</td>
<td>1.62</td>
</tr>
<tr>
<td>Schizosaccharomyces japonicus</td>
<td>1.11</td>
</tr>
<tr>
<td>Torulopsis dattila</td>
<td>3.95</td>
</tr>
<tr>
<td>Torulopsis glabrata</td>
<td>6.22</td>
</tr>
</tbody>
</table>

Table 2.1: Occurrence of cytochrome P-450 values in some yeasts. Values represent the highest concentration observed. Grown in 5% glucose growth media.

(from Karenlampi et al., 1980, 1986; and Sanglard et al., 1984)
cytochrome P-450 could be found. When cytochrome P-450 was grown semi-anaerobically the cytochrome P-450 which accumulated was rapidly lost in aerobic conditions because of the formation of active mitochondria (Ishidate et al., 1969b). If glucose was added at high concentrations then mitochondrial respiration could be prevented and cytochrome P-450 would accumulate at a higher concentration.

Several workers have shown that cytochrome P-450 accumulates rapidly during the exponential phase of growth but begins to decline soon after the onset of the stationary phase (Woods, 1979; Karelnampi et al., 1981; Blatik et al., 1987). Karelnampi et al. (1981) have shown the levels of cytochrome P-450 accumulation in various yeast strains (Table 2.1). The first part of the chapter surveys the different ways in estimating the growth of yeast which is important in determining the time of maximum accumulation of cytochrome P-450 (as discussed above). This chapter considers certain aspects concerning the conditions necessary for maximum cytochrome P-450 accumulation. The monitoring of growth in S. cerevisiae is first investigated then the effect of glucose on cytochrome P-450 accumulation in whole yeast and protoplasts (the effect of oxygen in various glucose concentrations is dealt with in chapter 5). The accumulation of cytochrome P-450 from different strains is considered, and the interconversion of cytochrome P-450 and cytochrome P-420. Lastly discovery of assay error arising from experiments in the chapter is dealt with.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Various strains of yeast Saccharomyces cerevisiae, as mentioned in the text, were obtained from the National Collection of Yeast Culture,
Nutfield, Surrey. Powdered yeast extract was obtained from Oxoid Co. Ltd. Mycological peptone was obtained from London Analytical and Bacteriological Media Ltd. Snail gut enzyme was obtained from Micro-Bio Ltd., London. Glucose (Analar Grade) sodium dithionite was obtained from BDH Chemicals Ltd. Sodium chloride was obtained from May and Baker Ltd. Chloramphenicol, cycloheximide, 2,4-dinitrophenol and all other chemicals mentioned in the text were obtained from Sigma Chemical Corporation Ltd. (London).

2.2.2 Growth of Yeast

The strains of *S. cerevisiae* were maintained on slopes of Sabouraud-dextrose agar, these were subcultured at regular intervals. The growth medium consisted of yeast extract (1% w/v), mycological peptone (2% w/v), sodium chloride (0.5% w/v) and glucose at various stated concentrations. The cultures were inoculated from slopes with a wire loop and grown at 30°C in a Mickie shaking water bath at 90 rpm, stroke 5 cm, or Gallenkamp orbital shaker. Yeast was grown in 250 ml flasks each containing 100 ml of medium unless stated. The yeast growth medium was previously autoclaved at 15 p.s.i. for 15 minutes, the glucose was autoclaved separately to prevent the formation of breakdown products which would interfere with the cytochrome P-450 assay (Lim, 1976). Yeast was harvested by centrifugation in a M.S.E. bench centrifuge (Measuring and Scientific Equipment Ltd.), washed three times in buffer and then assayed.

2.2.3 Cytochrome P-450 Assay

Cytochrome P-450 levels were measured by a modification of the difference spectrum method of Omura and Sato (1964). Yeast was suspended to a concentration of 0.1 g wet weight/ml in 0.1 M phosphate
Figure 2.2. The carbon monoxide difference spectrum of a yeast suspension.

Typical peaks at 420 nm and 450 nm are shown.

Spectrum from Pye-Unicam SP 1800.
buffer pH 7.0 and the sample divided into two cuvettes. Sodium dithionite was added to reduce each cuvette and a baseline was drawn in the range 400-500 nm using a Pye-Unicam SP 1800 or a Varian Cary 219 or 2200 spectrophotometer. The sample cuvette was removed and carbon monoxide bubbled through it for 30 seconds. The difference spectrum was then recorded again between 400-50 nm. A typical yeast cytochrome P-450 assay is shown in Figure 2.2. The concentration of cytochrome P-450 was calculated from the difference in absorbance between 450 and 490 nm with reference to the baseline, using the same extinction coefficient as for mammalian cytochrome P-450 of 91 mM$^{-1}$cm$^{-1}$.

2.2.4 Cytochrome P-420 Assay

The procedure is as for the cytochrome P-450 assay. The concentration of cytochrome P-420 being calculated from the difference in absorbance between 400 nm and 450 nm with reference to the baseline using an extinction coefficient of 110 mM$^{-1}$cm$^{-1}$. A typical yeast cytochrome P-420 assay is shown in Figure 2.2.

2.2.5 Mitochondrial Cytochromes Assay

Mitochondrial cytochromes were assayed by a modification of the method of Williams (1964). Yeast was suspended at a concentration of 0.1 g wet weight/ml in 0.1 M phosphate buffer pH 7.0, and divided into two cuvettes. The sample cuvette was reduced with sodium dithionite and the reference oxidised by the addition of 50 µl of hydrogen peroxide (20 vol.). The spectrum was then recorded from 500-640 nm using a Pye-Unicam SP 1800 or a Cary Varian 219 or 2200 spectrophotometer. A typical spectrum obtained is shown in Figure 2.3. The absorbance between four wavelength pairs was measured and used to calculated the
Figure 2.3. A typical yeast reduced-oxidized difference spectrum, showing mitochondrial cytochromes. A yeast suspension of 0.1g/ml (wet weight) was used.
concentrations of the individual cytochromes, cytochrome c, c<sub>1</sub>, b and 
a + a<sub>3</sub>, using four simultaneous equations which allow for the spectral 
overlap of the cytochromes.

The four wavelength pairs were;

\[ a_{15} = 550-535 \text{ nm} \]
\[ a_{25} = 554-540 \text{ nm} \]
\[ a_{35} = 563-577 \text{ nm} \]
\[ a_{45} = 605-630 \text{ nm} \]

The cytochromes c (X<sub>1</sub>), c<sub>1</sub> (X<sub>2</sub>), b (X<sub>3</sub>) and a + a<sub>3</sub> (X<sub>4</sub>) were then 
calculated from the simultaneous equations below.

\[
21.0 X_1 + 10.3 X_2 - 3.12 X_3 + 0.63 X_4 = a_{15} \\
6.51 X_1 + 18.8 X_2 + 2.55 X_3 + 0.95 X_4 = a_{25} \\
-1.16 X_1 + 0.91 X_2 + 14.3 X_3 - 0.326 X_4 = a_{35} \\
-0.22 X_1 - 0.59 X_2 + 0 X_3 + 12.0 X_4 = a_{45}
\]

A computer programme was used in obtaining answers to the above 
calculations (see Appendix 1).

2.2.6 Preparation of Protoplasts

The following procedure was taken from Eddy and Williamson (1957). The 
yeast cells were grown in 0.5% glucose growth media and were then 
washed three times with 0.1 M phosphate buffer pH 7.0. The cells were 
then resuspended to 100 mg/ml and incubated for 30 minutes at 30°C 
with the following media:

- 0.14 M 2-mercaptoethanol
- 0.04 M EDTA
- 0.2 M citrate phosphate buffer, pH 5.8

The cells were then resuspended in 5 ml sodium phosphate buffer, pH 5.8 
and 0.4 ml of snail gut enzyme per gramme wet weight of yeast cell was
added. The incubation was then continued for 30 minutes at 30\textdegree C. The suspension was then centrifuged for 5 minutes at 1,000 g to sediment the protoplasts from the cell wall debris. The sediment containing the protoplasts were resuspended in 0.66 M phosphate buffer, pH 6.8 which prevented lysis of the protoplast, at a concentration of 1 ml of medium/50 mg of original yeast.

2.2.7 The Scanning Electron Microscope

\textit{S. cerevisiae} NCYC 754 and 753 cells were grown as described above in 20\% glucose growth medium, the yeast cells were then washed three times in de-ionised water and transferred onto aluminium caps and allowed to dry to 24 hours. The yeast cells were then transferred to a Nanotech Gold Splutter where a thin layer of gold was deposited on their surface. The yield cells were then viewed with a Cambridge Stereoscan 100.

2.2.8 Optimised Media Experiment

The following optimised media components were made up (Salihon \textit{et al.}, 1983).

- 11.6\% (w/v) glucose
- 1.69 \% (w/v) yeast extract
- 1.04\% (w/v) mycological peptone
- 0.9\% (w/v) yeast extract

2.2.9 Preparation of Yeast Microsomal Fraction

Yeast was harvested by centrifugation (10 minutes at full speed on a bench centrifuge) and transferred to a stainless steel Vibro-mill vessel for disruption. The yeast was mixed with glass beads (1.00-1.05 mm diameter) in the Vibro-mill vessel and milled for a total of 6 minutes in a
Figure 2.4:  The carbon-monoxide difference spectrum of the microsomal fraction.
water-cooled Vibro-mill disruptor. The disruptor was stopped after 1 minute for topping up with glass beads and then milling was carried out in one minute bursts, allowing a one minute interval between bursts for cooling. After milling, the glass beads were washed with 0.1 M tris/HCl buffer pH 7.0 and the washings centrifuged at 10,000 g for 10 minutes on an M.S.E. high speed centrifuge to remove unbroken cells, debris and large cellular organelles. The supernatant was then spun at 160,900 g, for one hour in a Beckman L5-65 ultracentrifuge to obtain a microsomal pellet. The microsomal pellet was then resuspended in 0.1 M phosphate buffer pH 7.0 containing 20 % (v/v) glycerol, 1 mM EDTA and 0.1 % (w/v) reduced glutathione, by use of a hand-held Potter-Elvesjhem homogeniser. Triton X-100 may also be added to improve microsomal cytochrome P-450 stability. Figure 2.4 shows a typical difference spectrum of the microsomal fraction.

2.2.10 Yeast Cell Protein Determination

A modification of the method described by Lowry et al. (1951) was used for the determination of yeast cell protein.

Reagents:

A. 2% Na₂CO₃ in 0.1 N NaOH
B. 1% CuSO₄ mixed with an equal volume of NaK tartrate before use.
C. 50 ml of solution A + 1 ml of solution B mixed immediately before use.
D. 1 ml Folin Ciocalten reagent added to 1 ml H₂O.
E. 1 mg/ml Bovine Serum Albumin (BSA) stock solution to act as standards.

Procedure:

The blank consisted of 1 ml 0.1 N NaOH. The standards consisted of suitably diluted BSA stock solution to give concentrations ranging from 10 to 100 µg/ml.
Samples consisted of 1 ml of yeast suspension which had been diluted to give a suspension of approximately 100 μg/ml dry weight of yeast.

4 ml of reagent C was added to each of the samples and standards. This was allowed to stand for 10 minutes.

0.5 ml of reagent D was then added to the mixture. Each tube was mixed immediately and the absorbance at 750 nm read after 90 minutes on a Cecil CE 292 spectrophotometer.

The standard curve of absorbance against protein concentration was used to estimate protein in the sample suspensions. On the protein standard curve the linear region was between 20 and 100 μg/ml, the yeast cell suspensions were diluted (100 μl yeast cell suspension in 10 ml) to obtain readings in the linear range.

2.2.11 Protein Determination for Microsomal Fraction

For the yeast microsomal determination the procedure was modified as shown below.

The 1 ml volume of microsomal yeast suspension was added to 1 ml of ice cold trichloroacetic acid 15% (w/v) and stood on ice for 5 minutes. The precipitated protein was recovered by centrifugation (10 minutes at 3,000 RPM in a Beckman J6 centrifuge) and re-dissolved in 1 ml of 0.1 M NaOH. This solution was then used for the Lowry determination.

The microsomal fraction solution was diluted (500 μl of the solution in 2 ml) to obtain readings in the linear range of the protein standard curve.
Cell count. \(10^6/\text{ml}\).

Figure 2.5. Cell count of *S. cerevisiae* NCYC 240 grown in 20% glucose growth medium at 30°C. in shaking water bath. Results are the mean of two determinations.
Figure 2.6. The turbidity of yeast at 600 nm.

*S. cerevisiae NCYC 240* grown in 20% glucose growth media in shaking water bath at 30°C.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.8. S. cerevisiae NCYC 240 growth as represented by dry weight.

Cells were grown in 20% glucose growth media in a shaking water bath at 30°C.

Values are the mean of six determinations, bars indicate standard deviations.
Figure 2.7. Wet weight of S.cerevisiae NCYC 240 grown in 20% glucose growth media in shaking water bath at 30°C. Values are the mean of two determinations.
2.3 RESULTS AND DISCUSSION

2.3.1 Growth of *S. cerevisiae* in Batch Cultures

Figures 2.5, 2.6, 2.7 and 2.8 show different ways of measuring the growth of *S. cerevisiae* in batch culture using 100 ml of 20% glucose growth media in 250 ml flasks. Figure 2.5 shows the cell count, Figure 2.6 shows the yeast turbidity, Figure 2.7, the yeast wet weight and Figure 2.8 the yeast dry weight.

It was discussed in section 2.1 that the growth of yeast was important in determining the time of maximum accumulation of cytochrome P-450. The results obtained here are somewhat lower than those obtained by Hough *et al.* (1982) although these workers do not mention the growth temperature which may account for the difference in number. Hough *et al.* obtained a maximum cell count just over $1 \times 10^8$, here a figure of $3.2 \times 10^7$ was obtained (see Figure 2.5). Hough *et al.* reached the maximum count in approximately 30 hours, whereas here it took almost twice as long. Hough *et al.* obtained a maximum specific growth rate, $\mu_{\text{max}}$ of 2.62 per hour and a doubling time, $t_D$ of 2.62 hours as compared to a $\mu_{\text{max}}$ of 0.08 per hour and a $t_D$ of 12 hours obtained here. Obviously the yeast used by Hough *et al.* grows more quickly, the differences between the results representing different conditions of growth and may also be strain related. Results presented on Figure 2.5 agree closely with those of Karenlampi *et al.* (1981).

Figure 2.7 displays some decline in growth after 70 hours. The decline in yeast growth, termed autolysis may not be shown in other figures. The turbidity and dry weight may have included the yeast cell debris from dead cells which may have been left in suspension when centrifuging for wet weight. Autolysis is important because it can be used for the
Figure 2.9. The accumulation of cytochrome P-450 and wet weight in *S. cerevisiae* NCYC 240 grown in 20% and 0.5% glucose growth media. No cytochrome P-450 could be found in 0.5% glucose growth media. Results are the mean of two determinations.
extraction and purification of certain enzymes. During autolysis amino acids, protein and DNA are released from yeast cells. Industrially, autolysis of yeast cells can lead to unwanted off-flavours in beverages and can increase susceptibility to bacterial contamination (Hough and Maddox, 1970).

The protein content of yeast cells was determined at 40 hours and found to be 38% of the dry weight. When referring to enzyme concentration it may be more accurate to define the protein content of the yeast sample. The drawback may be the lengthy protein determination procedure which may detract from the already time-consuming cytochrome P-450 assays involved in time course plotting. The yeast samples could be frozen and protein determinations performed at a later date.

2.3.2 The Effect of High and Low Glucose Concentrations on Cytochrome P-450 Accumulation

Figure 2.9 shows the accumulation of cytochrome P-450 and wet weight in *S. cerevisiae* NCYC 240 in 20% glucose growth media. Figure 2.9 also shows the wet weight in *S. cerevisiae* NCYC 240 in 0.5% glucose growth media in which no cytochrome P-450 was detected.

Results from growth of yeast in glucose concentrations substantiate the theory of cytochrome P-450 accumulation occurring only under conditions of mitochondrial repression. In the above experiments mitochondrial repression was successfully maintained by high glucose concentrations. The regulation of cytochrome P-450 accumulation is considered, by Wiseman et al. (1978) to be via the level of intracellular cyclic AMP which is inversely related to the glucose concentration in growing yeast. At high glucose concentrations (allowing the
<table>
<thead>
<tr>
<th>Glucose concentration in yeast growth medium.</th>
<th>Level of cytochrome accumulation (nmol./g.wet weight of yeast)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a   a_3  b  c_1  c  P-450</td>
</tr>
<tr>
<td>20%</td>
<td>8.2</td>
</tr>
<tr>
<td>0.5%</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Table 2.2. Mitochondrial cytochrome accumulation in aerobically grown *S. cerevisiae* NCYC 240 taken after 42 hours.

Results are the mean of two determinations.
Cytochrome P-450 n.mol/g.wet wt.

Figure Z.to-

The effect of added glucose on the cytochrome P-450 level in _S.cerevisiae_ NCYC 754 incubated aerobically with shaking in phosphate buffer.

Values are the mean of six determinations, bars indicate standard deviations.
accumulation of cytochrome P-450) the cyclic AMP concentration is low, hence Wiseman et al. (1978) suggested that cyclic AMP may have a repressive effect on cytochrome P-450 accumulation. Wiseman et al. (1978) demonstrated a direct effect of cyclic AMP in repressing cytochrome P-450 synthesis in yeast protoplasts, although 5'-AMP and 2'-((3')-AMP did not have an effect. An interpretation of these results might be that under conditions of mitochondrial repression by a high glucose concentration in the growth medium, the cyclic AMP remains in low concentration, hence the repressive effect of cyclic AMP is removed and de novo synthesis of cytochrome P-450 can occur. Qureshi et al. (1980) demonstrated a direct relationship between cytochrome $a + a_3$ and cyclic AMP and an inverse relationship to the cytochrome P-450 level at various glucose concentrations.

2.3.3 Mitochondrial Cytochrome Levels in S. cerevisiae grown in Low and High Glucose Growth Media

To confirm that mitochondrial repression was occurring at high glucose concentration the levels of mitochondrial cytochrome were measured after 42 hours of aerobic growth in 0.5% and 20% glucose growth media. Table 2.2 shows that in 20% glucose growth media the cytochrome $a + a_3$ concentration is considerably lower at 8.2 nmol/g wet weight of yeast than in 0.5% glucose growth medium with 19.6 nmol/g wet weight. These results support the concept of mitochondrial repression occurring at 20% glucose concentration.

2.3.4 The Effect of Added Glucose on Cytochrome P-450 Levels in S. cerevisiae

Figure 2.10 shows the effect on cytochrome P-450 accumulation and degradation when incubating the yeast after transfer to non-growth
Figure 2.11 The effect of incubating yeast protoplasts in 1% glucose growth medium on cytochrome P-450 accumulation and wet weight.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.12. The effect of incubating yeast protoplasts in 5% glucose growth medium on cytochrome P-450 accumulation and wet weight.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.13. The effect of incubating yeast protoplasts in 20% glucose growth medium on cytochrome P-450 accumulation and wet weight.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.4. The effect of incubating yeast protoplasts in 20% glucose growth medium on cytochrome P-450 accumulation and wet weight. The yeast had previously been grown in 20% glucose growth medium.

Values are the mean of three determinations, bars indicate standard deviations.
media in phosphate buffer, pH 7.0 with different concentrations of glucose added. With 8% (w/v) glucose a clearly marked increase in an already high existing level of cytochrome P-450 to 150% of the original level after 5 hours occurs. At 1% (w/v) glucose after 1 hour there is less degradation than with the phosphate buffer control but after two hours this effect declines, and the levels of degradation become similar. At a higher glucose concentration of 12% (w/v), less protection against degradation was seen after 5 hours but after 1 hour the cytochrome P-450 value was 20% higher than the original level. Under these conditions fermentation of the glucose occurs, it is possible that at 8% to 12% (w/v) glucose fermentation may result in an optimum level of ethanol to further induce de novo cytochrome P-450 production (Blatiak et al., 1987) (see chapters 4 and 8).

2.3.5 The Effect on Cytochrome P-450 Accumulation of Incubating S. cerevisiae Protoplasts in Various Glucose Concentrations with Growth Media

Figures 2.11 to 2.14 show the effect on cytochrome P-450 accumulation and growth when yeast protoplasts were incubated in a shaking water bath at $30^\circ$C in 1%, 5% and 20% glucose growth media having previously been grown in 0.5% glucose growth media. From Figure 2.11 it can be seen that in 1% glucose the cytochrome P-450 reaches a maximum of only just under 0.5 nmol/g wet weight. Growth of yeast protoplasts as shown on Figure 2.11 is slight, starting at 45 mg/ml wet weight and after 8 hours reaching 63 mg/ml wet weight.

Figure 2.12 shows the effect of incubating yeast protoplasts in 5% glucose growth medium on cytochrome P-450 accumulation and wet weight. Here the cytochrome P-450 accumulates to a value of
2.5 nmol/g wet weight of yeast but declines after 4 hours to a level of just under 2.0 nmol/g wet weight of yeast in 8 hours. The growth, as measured by wet weight of yeast, is more substantial than in 1% glucose. Figure 2.12 shows the increase between 4 and 6 hours from just under 100 mg/ml to 150 mg/ml which may indicate cell wall regeneration.

Woods (1979) found a similar result with yeast protoplast incubation in 5% glucose growth medium. Woods obtained a slightly higher cytochrome P-450 value of 3.0 nmol/g wet weight of yeast after 6 hours. Woods also had a lag phase in cytochrome P-450 accumulation between 0 and 2 hours which was not apparent from Figure 2.12 and would explain an earlier cytochrome P-450 maximum shown in Figure 2.12. Woods obtained very similar growth in the yeast protoplasts, the only difference being the lag phase of growth which Woods obtained, but again, was not apparent in Figure 2.12. The small differences in the two sets of results may be due to the ages of the slopes used (see section 6.3).

Figure 2.13 shows the effect of incubating yeast protoplasts in 20% glucose growth medium on cytochrome P-450 accumulation and yeast wet weight. The accumulation of cytochrome P-450 continues throughout the 8 hour incubation reaching a maximum of 2.3 nmol/g wet weight of yeast. Yeast growth as well is significant, the weight at 6 and 8 hours being over 150 mg/ml suggesting, again, that cell wall regeneration may have occurred.

There is little difference between the maximum levels of cytochrome P-450 accumulation as shown in Figures 2.12 and 2.13. In yeast protoplast incubation in 20% glucose growth media it is possible that the maximum accumulation of the enzyme may have occurred after 10 or
<table>
<thead>
<tr>
<th>Strain</th>
<th>Maximum c.P-450 value in nmol/g wet wt. of yeast</th>
<th>Time of maximum c.P-450 value (h)</th>
<th>Maximum wet wt. (g.)</th>
<th>Time of maximum wet wt. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP 100</td>
<td>0</td>
<td>-</td>
<td>0.87</td>
<td>48</td>
</tr>
<tr>
<td>NCYC 396</td>
<td>0</td>
<td>-</td>
<td>1.13</td>
<td>48</td>
</tr>
<tr>
<td>Formerly S. carlsbergensis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>NCYC 108</td>
<td>1.8</td>
<td>24</td>
<td>3.23</td>
<td>42</td>
</tr>
<tr>
<td>NCYC 147</td>
<td>0.88</td>
<td>42</td>
<td>4.41</td>
<td>48</td>
</tr>
<tr>
<td>NCYC 406</td>
<td>1.5</td>
<td>24</td>
<td>12.01</td>
<td>48</td>
</tr>
<tr>
<td>NCYC 700</td>
<td>3.1</td>
<td>24</td>
<td>3.2</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 2.3. Maximum cytochrome P-450 accumulation and maximum wet weight in various strains of yeast.
12 hours. It is of interest that at a 5% glucose concentration with growth medium (Figure 2.13) the rate of cytochrome P-450 accumulation was as good as in 20% glucose growth media. It is possible that the 5% glucose growth media may provide a less hostile osmotic environment although in 20% glucose growth media there is more energy available for cytochrome P-450 biosynthesis and the higher concentration of remaining glucose protects against cytochrome P-450 degradation (Blatiak et al., 1980, see chapter 4).

Figure 2.14 shows the effect of incubating yeast protoplasts in 20% glucose growth medium on cytochrome P-450 accumulation and yeast wet weight, when the yeast had previously been grown in 20% glucose growth medium. In the previous incubations shown above (Figures 2.11, 2.12 and 2.13) the yeast had previously been grown in 0.5% glucose growth medium. The differences between 2.13 and 2.14 are considerable, there being less growth and much less cytochrome P-450 accumulation shown in Figure 2.14. However, a point of similarity is the continuing accumulation of cytochrome P-450 throughout the 8 hour incubation.

It is not known why previous growth in 0.5% or 20% glucose growth medium should make a difference and this question is raised again in chapter 6.

2.3.6 Cytochrome P-450 Accumulation in Various Strains of Saccharomyces cerevisiae

Table 2.3 shows the maximum cytochrome P-450 accumulation and maximum wet weights obtained with various strains. Karenlampi et al. (1980) reported a cytochrome P-450 accumulation of 9 nmol/g wet
weight of yeast in *Saccharomyces italicus* which has been renamed *Saccharomyces cerevisiae* strains NCYC 108, 406 and 700. As can be seen from Table 2.3, the highest cytochrome P-450 accumulation obtained was only 3.1 nmol/g wet weight of yeast which is similar to that obtained from NCYC 240.

Karenlampi et al. (1980) obtained a cytochrome P-450 value of 4.66 nmol/g wet weight of yeast with NCYC 240 with 20% glucose growth media which is higher than the value of 2.5 nmol/g wet weight of yeast shown in Figure 2.9, but the growth conditions were different, Karenlampi et al. employing 0.5% peptone, 0.3% yeast extract and 0.3% malt extract with 20% glucose.

It was important to evaluate all high cytochrome P-450 accumulations reported since microsomal preparations (and degradation effects) are more difficult with low initial cytochrome P-450 accumulations.

Figure 2.15 shows the time course of cytochrome P-450 accumulation in *S. cerevisiae* NCYC 239 in 20% glucose growth medium. The highest value was 5.8 nmol/g wet weight of yeast after 40 hours growth which is more than in *S. cerevisiae* NCYC 240. However, Qureshi et al. (1980) reported a maximum cytochrome P-450 value of 11 nmol/g wet weight of yeast obtained after 110 hours.

The accumulation of cytochrome P-450 on other fermentable carbon sources has also been reported (Karenlampi et al., 1981). The effect of growing *S. cerevisiae* NCYC 239 on maltose has been compared to growth on glucose as normally used. Figure 2.16 shows the time course of cytochrome P-450 accumulation in *S. cerevisiae* NCYC 239 in 20%
Cytochrome P-450
nmol./g.wet. wt.

0 20 40 60 80
Time (h)

Figure 2.15. Time course of cytochrome P-450 accumulation in
*S. cerevisiae*NCYC 239 grown on 20% glucose growth
media.

Values are the mean of eight determinations,
bars indicate standard deviations.
Figure 2.16. Time course of cytochrome p-450 accumulation in *S. cerevisiae* NCYC 239 grown on 20% maltose growth media.

Values are the mean of eight determinations, bars indicate standard deviations.
maltose growth medium. It can be seen that the maximum cytochrome P-450 value is 2.8 nmol/g wet weight of yeast, less than half of the value obtained with 20% glucose growth media. This is in broad agreement with Karenlampi et al. (1981) who employed S. cerevisiae NCYC 240 in their comparison of fermentable carbon sources.

2.3.7 The Accumulation of Cytochrome P-450 in S. cerevisiae NCYC 240, 753 and 754

The yeast S. cerevisiae NCYC 240 has been shown to be a mixture of two different strains named NCYC 753 and 754. The strain NCYC 753 has a minor 20% component in NCYC 240 and NCYC 754 has a major 80% component. The strains differ in their ability to grow on maltotriose (Dr. B. Kirsop, personal communication).

Figure 2.17 shows the time course of cytochrome P-450 accumulation in the S. cerevisiae strains NCYC 240, 753 and 754. The strain NCYC 754 has a much higher value of cytochrome P-450 (8 nmol/g wet weight of yeast) than either NCYC 240 (2.5 nmol/g wet weight of yeast) or NCYC 753 (0.8 nmol/g wet weight of yeast). The time taken to reach the highest enzyme value is similar, NCYC 240 took 40 hours, NCYC 753 took 44 hours and NCYC 754 took 42 hours. NCYC accumulated an intermediate amount of cytochrome P-450 but since this strain consists of approximately 80% of NCYC a higher enzyme accumulation could have been expected (King, Blatiak and Wiseman, 1983b). The reason for NCYC 754 strain having more than three time the accumulation of cytochrome P-450 than NCYC 240, especially since NCYC 754 is the major component, is not known.
Fig. 2. Time course of cytochrome P-450 production in Saccharomyces cerevisiae.NCYC 240, 753 and 754 grown in 20% glucose media. Values are the means of 8 determinations. Bars indicate standard deviations.
Cytochrome P-450 (nmol/g. wet wt.)
Dry wt. (g./100 ml. of media)

Figure 2.18. Cytochrome P-450 accumulation and growth of rho+ JD1 strain in 20% glucose growth media.

Values are the mean of three determinations, bars indicate standard deviations.
Cytochrome P-450 (nmol/g wet wt.)
Dry wt. (g./100 ml of media)

![Graph showing cytochrome P-450 accumulation and growth of rho JD1 strain in 20% glucose growth media.]

- Cytochrome P-450 nmol/g wet wt. of yeast
- Dry weight of yeast in grams per 100 ml of media.

Figure 2.19 Cytochrome P-450 accumulation and growth of rho JD1 strain in 20% glucose growth media.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.2. Cytochrome P-450 accumulation and growth of rho-JD1 strain in 20% glucose growth media.

Values are the mean of three determinations, bars indicate standard deviations.
Figure 2.2: Time course of petite mutant dry weights (JD1) grown in 0.5% glucose growth media.

(No cytochrome P-450 was found.)

Values are the mean of two determinations.
The ability of cytochrome P-450 from both 753 and 754 to metabolise benzo(a)pyrene has been tested (King et al., 1983b). No difference was found in the kinetic parameters for benzo(a)pyrene metabolism with these strains which was identical to cytochrome P-450 from NCYC 240.

Plates 2.1 and 2.2 show scanning electron micrographs of *S. cerevisiae* NCYC 753 (Plate 1) and NCYC 754 (Plate 2). No difference between the two strains was externally visible, which was not unexpected.

2.3.8 The Accumulation of Cytochrome P-450 in *S. cerevisiae* JD1 petite mutant strains

Figure 2.18 to 2.20 show cytochrome P-450 accumulation and growth of *S. cerevisiae* JD1 petite mutant (see chapter 3) strains in 20% glucose growth media. The results indicate generally poor cytochrome P-450 accumulation and the enzyme concentration did not appear to be growth related. Cytochrome P-450 is only present at high levels in the very early stages of growth and declines as growth proceeds. One of the reasons may be the rather poor rate of growth of these strains, consequently the cytochrome P-450 may not be required in higher concentrations to cope with ergosterol biosynthesis.

Figure 2.21 shows the time course of *S. cerevisiae* JD1 petite mutant growth in 0.5% glucose growth media as monitored by dry weight readings. No cytochrome P-450 could be detected. From Figure 2.21 it can be seen that the strain rho+ has the best growth almost reaching 0.3 g/100 ml media. The petite mutant strain rho− did give very interesting electron micrographs and for that reason constitutes the next chapter in this thesis.
A Comparison of Cytochrome P-450 Accumulation in S. cerevisiae NCYC 754 with Optimised and Non-Optimised Media

Media has been optimised by Salihon et al. (1983) for maximum cytochrome P-450 accumulation. Figure 2.22 shows a comparison of the cytochrome P-450 accumulation in S. cerevisiae NCYC 754 with optimised and non-optimised media. It can be seen that the optimised media allows for a faster rate of accumulation of the enzyme culminating with a maximum of 9.5 nmol/g wet weight of yeast as compared to a maximum of 7.0 nmol/g wet weight of yeast in the non-optimised media. The optimised media allows for the maximum cytochrome P-450 accumulation to be reached after 38 hours, in non-optimised media the peak was reached slightly later at 40 hours. The optimised media was slightly less viscous since it contained a 14% glucose concentration rather than a 20% glucose concentration, consequently there may be a better diffusion of oxygen into the yeast cells aiding a higher cytochrome P-450 accumulation (A. Blatiak et al., 1985, 1987, see also chapter 5). After 48 hours the optimised media may have a lower glucose concentration remaining which may help explain the higher rate of enzyme degradation (A. Blatiak, 1980, see also chapter 5). With optimised media Salihon et al. (1983) using NCYC 240 found significant increases in cytochrome P-450 yield per unit culture volume, using 100 ml medium in 250 ml flasks. When these increases in enzyme yield were analysed in terms of costs then the optimised media gave 326 nmol of cytochrome P-450 per £ and non-optimised media gave only 144 nmol of the enzyme per £. Thus the optimised media provides a 125% increase in yield per unit cost. Figure 2.22 shows that with NCYC 754 a further increase in cytochrome P-450 accumulation is obtainable with optimised media.
Figure 2.22. Comparison of cytochrome P-450 accumulation in *S. cerevisiae* NCYC 754 with optimised and non-optimised media.

Values are the mean of two determinations.
2.3.10 Accumulation of Cytochrome P-420 in *S. cerevisiae*

Figure 2.23 shows a time course for cytochrome P-450 and cytochrome P-420 in *S. cerevisiae* whole yeast grown in 20% glucose growth media. It can be seen that the level of cytochrome P-420 rises when that of cytochrome P-450 falls. After 40 hours the cytochrome P-450 has accumulated at 5.9 nmol/g wet weight of yeast, at the same time as the dry weight maximum of 397 mg/100 ml medium. After 46 hours the cytochrome P-420 accumulation increases suddenly to 5.3 nmol/g wet weight of yeast just after the cytochrome P-450 accumulation falls significantly to less than 4.0 nmol/g wet weight of yeast.

Callen and Philpot (1977) discussed the ability of cytochrome P-420 to convert procarcinogens into carcinogens hence the accumulation of cytochrome P-420 may well prove to be important.

Von Borstel et al. (1985) proposed that in yeast cytochromes P-450 and P-420 are interconvertible forms of the same enzyme. Yoshida and Kumaoka (1975) indicated that in yeast cytochrome P-450 is the membrane bound form and cytochrome P-420 is free in the cytosol. Von Borstel et al. used a diploid strain of *S. cerevisiae* which can accumulate relatively high cytochrome P-450 levels. During the logarithmic phase of growth the workers reported more cytochrome P-450 than P-420, a situation which was reversed after stationary growth phase had been reached. The results presented here are in general agreement with Von Borstel et al. It is possible that the solubilisation of cytochrome P-450 to P-420 is a prerequisite for the degradation of the enzyme and that knowledge of cytochrome P-420 accumulations may be valuable in understanding degradation of cytochrome P-450. However, at 420 nm it is possible that other haem containing proteins contribute to the
Cytochrome P-450 and P-420 nmol./g. wet wt.

Figure 2.23. The accumulation of cytochromes P-450 and P-420 during growth of S. cerevisiae NCYC 754 in 20% glucose growth media.

Values are the mean of six determinations, bars indicate standard deviations.
Von Borstel et al. claims that cytochrome P-450 and P-420 are interconvertible; whilst it can be demonstrated that cytochrome P-450 converts to cytochrome P-420 there is no evidence to suggest the reverse. In all the experiments performed here, it has never been observed that cytochrome P-420 accumulation converts to give cytochrome P-450.

Another suggestion made by Von Borstel et al. is that "much (sic) of the cytochrome P-450 and P-420 is either maintained in, or is regulated by, the mitochondria". The only evidence for this supposition being that petite mutant strains from S. cerevisiae D5 contained less cytochrome P-450 and P-420. Furthermore, Von Borstel refer to a paper by Blatik et al. (1980) which suggests an "involvement" of the mitochondria. The reference by Blatik et al. states that anaerobiosis, chloramphenicol and 20% glucose all promote the biosynthesis of cytochrome P-450 in yeast and also prevent the biosynthesis of cytochrome a + a₁ in the yeast mitochondrion (see chapter 5). Hence the supporting evidence for the supposition made by Von Borstel et al. in the regulation of cytochrome P-450 by mitochondria appears rather speculative at this time.

Microsomal cytochrome P-450 when left at 20°C will slowly degrade (see Table 2.3) and cytochrome P-420 values slowly rise, presumably as a result of the degradation of cytochrome P-450.

Table 2.3 shows that when Triton X-100 is added to the microsomal cytochrome P-450 fraction the rate of degradation to cytochrome P-420
Table 2.3: Conversion of cytochrome P-450 and P-420 in yeast microsomal fraction with and without Triton X-100 at 20°C.

Values are the means of two determinations for yeast microsomal and one determination for the purified fraction.
slows down (see section 2.3.11 for assay error in this method). However, when the degradation of purified cytochrome P-450 (a gift from Dr. D. King) was monitored at 20°C there was no commensurate rise in cytochrome P-420. However, this preparation was obtained in 20% glycerol, a known stabilizing agent for cytochrome P-450. Thus the question of cytochrome P-450 conversion to cytochrome P-420 remains controversial.

The protein content of the yeast microsomal fraction used in this experiment was found to be 8.3 mg/ml which compares quite well with the preparation used by King (1982) of 6.5 mg/ml. The protein content of the yeast cytochrome P-450 purified fraction was 50 µg/ml (D. King, personal communication).

2.3.11 Error in Cytochrome P-450 Assay due to Time Dependency of Carbon Monoxide Difference Spectrum in Yeast Microsomal Fraction

Figure 2.24 shows the difference in cytochrome P-450 in yeast microsomal fraction with time. It can be seen that the peak reaches its highest value after 6 minutes. The increase in cytochrome P-450 accumulation is significantly greater than in whole yeast. These results agree closely with Azari and Wiseman (1981) who worked on these effects at the same time. Yoshida and Kumaoka (1975) demonstrated that yeast S. cerevisiae cytochrome P-450 in the presence of Triton X-100 has an absolute spectrum identical to that of the low spin state (which corresponds to the Soret peak at 416 nm). Mitani and Horie (1969a; 1969b) demonstrated that yeast cytochrome P-450 can be in both low and high spins states (the high spin state corresponds to the Soret peak of the absolute spectrum at 395 nm) and that the equilibrium between the two states depends on the environmental conditions.
Figure 2.24. Carbon monoxide difference spectrum of reduced cytochrome P-450. The microsomes were treated with Triton X-100 (0.1% v/v). Lines A to D were recorded 2, 4, and 6 minutes after initial scanning. The experiment was performed at 20°C.
and Horie (1971) demonstrated that the reduction of cytochrome P-450 from adrenocortical mitochondria by sodium dithionite or by NADPH-dependent enzymatic system is not instantaneous. When the spin state of the haem iron is modulated from high to low, the rate of reduction is decreased; conversely, when the spin state is modulated from low to high the rate of reduction increases. Substrates such as Triton X-100, producing type II spectral changes decreasing the rate of reduction and substrates producing type I spectral changes to hepatic cytochrome P-450 could increase the rate of electron flow from NADPH-reductase to cytochrome P-450 by modulating the spin state from low to high (Gigon et al., 1969; Sligar et al., 1979).

Azari and Wiseman found that the addition of Triton X-100 affected the carbon monoxide difference spectrum of yeast cytochrome P-450. It was considered that the time dependency of the carbon monoxide difference spectrum with Triton X-100 treated yeast cytochrome P-450 could not be due to slow solubilisation of carbon monoxide (and thus slow binding of the carbon monoxide to the cytochrome P-450), but is due to an effect on the rate of reduction as discussed above.
CHAPTER 3

INVESTIGATION OF MICROSTRUCTURE IN YEAST CELLS
WHICH ACCUMULATE DIFFERENT LEVELS OF CYTOCHROME P-450
3.1 INTRODUCTION

3.1.1 Cell Morphology

The cells of \textit{Saccharomyces cerevisiae} are round, ovoid or ellipsoidal in shape and vary from \(2.5-10\) \(\mu\text{m}\) in width and \(4.5-21\) \(\mu\text{m}\) in length. Unstained cells exhibit little detail under the light microscope.

Although more information can be obtained by using specific stains, it is only since the advent of the electron microscope that a clear picture of the yeast cell has emerged. The characteristic features of a typical yeast cell are shown in Figure 3.1. It can be seen that the cell is bounded by a thick cell wall. Inside this it is possible to recognize many of the features of a typical cell; a plasmalemma, a nucleus, mitochondria, endoplasmic reticulum, vacuoles, vesicles and granules (see Figure 3.1).

The distinguishing feature of a growing population of yeast cells is the presence of the buds which are produced when the cell divides. The daughter cell is initiated as a small bud which increases in size throughout most of the cell cycle, until it is the same size as the mother cell. Most growth in yeast occurs during bud formation, so the bud is more or less the same size as the mature cell before it separates.

Cell separation may occur soon after cell division; however, often new rounds of cell division take place before cell separation has occurred, so groups of cells are produced. The site of cell separation is marked on the mother cell by a structure referred to as the bud scar and on the daughter cell by the birth scar. No two buds arise at the same site on the cell wall in \textit{Saccharomyces cerevisiae}, so each time a bud is produced a new bud scar is produced in the cell wall of the mother cell. By counting the number of bud scars, it is possible to establish the
Figure 3.1  Section through typical yeast cell showing main features and distribution of cell components.
number of buds which have been produced by a particular cell. This can be used as a measure of the age of the cell. In any yeast population, 50% of the cells were produced by the last generation of cell divisions so possess a birth scar but no bud scar. Of the remaining 50%, 25% have one bud scar, 12.5% two bud scars and 12.5% more than two bud scars.

In some strains, cells growing in liquid culture adhere together to form clumps which settle to the bottom of the growth vessel. This phenomenon, which is referred to as flocculence, is of considerable importance in the brewing industry, and in cytochrome P-450 assays, since with the settlement the peak disappears.

3.1.2 Cell Wall
The cell wall is a rigid structure which is 25 nm thick and constitutes approximately 25% of the dry weight of the cell (see Figure 3.2). Chemical analysis of the cell wall indicates that the major components are glucan and mannann; however chitin and protein are also present. Glucan is a complex branched polymer of glucose units and is located in the inner layer of the yeast cell wall adjacent to the plasmalemma. It appears to be the major structural component of the wall, since removal of the glucan results in total disruption of the cell wall. Mannan, which is a complex polymer of mannose occurs mainly in the outer layers of the cell wall. Since it is possible to remove the mannann without altering the general shape of the cell, it appears that it is not essential to the integrity of the cell wall. Protein constitutes 10% of the dry weight of the cell wall. At least some of this protein is in the form of wall bound enzymes. Several enzymes have been described as being associated with the cell wall in yeast, including glucanase and mannannase, which are probably involved in the softening of the cell wall to permit bud
Figure 3.2 STRUCTURE OF YEAST CELL WALL

Figure 3.3 STRUCTURE OF YEAST PLASMALEMMMA
formation; invertase, alkaline phosphatase and lipase. Several of these enzymes, e.g. invertase are manno-proteins and contain up to 50% of mannan, as an integral part of the enzyme molecule. Much of the remaining protein in the cell wall is also associated with mannan, so it is possible that this plays a structural rather than enzymic role in the cell wall. The detailed organization of the cell wall is not fully understood.

3.1.3 The Cell Membrane

The cell membrane of plasmalemma of the yeast cell can be observed using electron microscopy as a three-layered structure which is closely associated with the inner surface of the cell wall (see Figure 3.3). It usually has a smooth appearance but at certain stages of the growth of the cell, invaginations can be seen. An understanding of the chemical composition of the plasmalemma requires isolation techniques which produce plasmalemma membrane free of other cellular components, including other membranes. One technique involves the formation of protoplasts.

The plasmalemma is composed of lipids in more or less equal amounts, together with a small amount of carbohydrates. The main lipids present are mono-, di- and triglycerides, glycerophosphatides and sterols such as ergosterol and zymosterol. The nature of the protein in the plasmalemma is less well understood but probably includes the enzymes which are involved in the uptake of sugars and amino acids.

3.1.4 The Nucleus

The nucleus is usually situated between the vacuole and the bud. Chromatic bodies can be recognized in the nucleus using specific stains such as acid fuchsin. The nuclear membrane remains intact throughout
the cell cycle. It is visible in electron micrographs as a double membrane which is perforated at intervals with pores. Associated with the nuclear membrane is a structure referred to as a plaque which appears to function in a similar manner to the centriole of animal cells. The characteristic structure of a plaque is a multilayered disc from which microtubules extend into both the nucleus and the cytoplasm. These plaques are considered to represent the spindle apparatus of the yeast nucleus and their behaviour has been monitored to follow the different stages of nuclear division. The nucleus is composed of an optically dense crescent-shaped portion (nucleolus) and a more translucent portion that contains various species of RNA and a type of polyphosphate with a chain length of 20 to 40 orthophosphate residues.

3.1.5 Other Cytoplasmic Structures

The cytoplasm of the yeast cell contains a system of double membranes known as the endoplasmic reticulum. Some of the membranes are associated with ribosomes as in other organisms; however, the endoplasmic reticulum appears to be involved in many other cellular activities. The relationship between endoplasmic reticulum and other organelles is not clear; however, there is continuity between the endoplasmic reticulum, the outer membrane of the mitochondrion and the plasmalemma. The endoplasmic reticulum is also involved in the formation of vesicles which are present in the cell. In this it behaves in a manner akin to the Golgi apparatus of some other organisms. It is not clear, however, whether a true Golgi apparatus is present in yeast; membranous discs have been observed in yeast cells but they are few in number and not clearly recognizable as a Golgi apparatus.
Lipid granules are also present in the cytoplasm and again these appear to be derived from the endoplasmic reticulum.

Mature yeast cells contain a large vacuole; however at the point in the cell cycle when the bud formation is initiated, the vacuole appears to fragment into smaller vacuoles which become distributed between the mother cell and the bud. Later in the cell cycle, these small vacuoles fuse again to produce a single vacuole in the mother and daughter cell.

The function of the vacuole is not well established. Evidence has been presented that it contains hydrolytic enzymes, polyphosphates, lipids and low molecular weight cellular intermediates, and metal ions. It may act as a storage reservoir for nutrients and for hydrolytic enzymes. It has also been suggested to act as the "lysosome" of the yeast cell responsible for degradation of many proteins (Enter and Wolf, 1984).

3.1.6 Mitochondria

Mitochondria are readily recognizable in electron micrographs of aerobically grown yeast as spherical or rod-shaped structures surrounded by a double membrane (Palade, 1952).

They contain cristae which are formed by the folding of the inner membrane (Sjöstrand, 1953). In cross sections their diameters range from about 0.3-1 μm, and their lengths from 0.5-3 μm, much of course, depends on the angle of the section through a mitochondrion.

Rapidly growing yeast cells contain only a few mitochondria and these are transformed into a population of many smaller organelles in stationary phase cells (Stevens, 1977).
The size of the mitochondrial DNA varies in different species of yeast from 6 μm-26 μm (Gillham, 1978) and with *S. cerevisiae* from 21-25 μm (Peters *et al.*, 1973 and Sanders *et al.*, 1976).

### 3.1.7 Petite Mutations

The majority of spontaneous and induced petite mutations exhibit non-mendelian inheritance and are termed vegetative petites (Ephrussi *et al.*, 1949). A minority are inherited in a mendelian fashion and are called segregational petites (Chen, S.-Y., Ephrussi, and Hottinguer, 1950).

Vegetative petites are non-reverting pleiotropic mutations that either have grossly altered mt DNA or have no mt DNA at all. Thus vegetative petites cannot carry out mitochondrial protein synthesis because components of the mitochondrial protein synthesis system, particularly mitochondrial r RNA and t RNA are coded by mt DNA (see Figure 3.1).

This absence of a functional mitochondrial protein-synthesizing system results in pleiotropic defects in the respiratory electron transport chain because certain components which include cytochromes aa₃, b and the attendant ATP-generating system require mitochondrial protein synthesis for their assembly and function. Thus mitochondrial morphology in vegetative petites is modified.

Slonimski *et al.* (1968) found that ethidium bromide causes quantitative conversion of wild type cells to vegetative petites. Ethidium bromide induces vegetative petites because of specific covalent binding of the dye to *S. cerevisiae* mt DNA, which triggers a series of steps during which the mt DNA is progressively degraded into smaller pieces until it is eliminated. The cleavage of mt DNA in the presence of ethidium bromide is traceable to the probable covalent bonding of the dye to
mt DNA (Mahler & Bastos, 1974). Since the dye molecules are known to intercalate between adjacent base pairs, conformational distortion of the helix quite probably occurs at the same time. Following dye binding double-strand scission takes place and the quarter molecules are formed.

Acriflavine induces mutations only in dividing cells, these dyes causing petite induction only in buds and not mother cells. All petite mutations lack mitochondrial function and manage to survive because of the yeast's ability to grow by fermentation.

The term rho (p) was first used by Marquardt (1952), then adopted by Sherman and Ephrussi (1962) to designate the cytoplasmic factor required for respiration. Respiratory competent grande cells were designated p+ and vegetative petites, p−. Nagley and Linnane (1972) designated p0 to vegetative petites which lacked mt DNA allowing all other petites containing mt DNA to be designated p−.

There are 3 types of _S. cerevisiae_ petite mutations:

(a) Nuclear petites where mitochondrial activity is abolished.

(b) Neutral (rho0) petites where all mitochondrial DNA is absent.

(c) Suppressive (rho−) petites with grossly abnormal mitochondrial DNA.

Rho− mitochondria contain circles of DNA that are made smaller than the usual genome ranging from 0.2% to 36% in different cases. Thus more of the mitochondrial genome is deleted than is retained. The sequence in rho− petite is often amplified to generate a number of copies. Amplification is either by increase of the ploidy or by the production of multimeric DNA molecules, each containing many copies of the sequence.
<table>
<thead>
<tr>
<th>Species</th>
<th>Complexity of DNA in kb</th>
<th>Genomes/organelle</th>
<th>Organelles per cell</th>
<th>mt DNA/total DNA as a %</th>
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</thead>
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<td>2</td>
<td>500</td>
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<td>22</td>
<td>18</td>
</tr>
<tr>
<td><em>P. sativum</em></td>
<td>110</td>
<td>not known</td>
<td>not known</td>
<td>not known</td>
</tr>
</tbody>
</table>

Table 3.1 Variation of the mitochondrial genome

(Modified from Lewin 1985)
Saccharomyces cerevisiae mitochondria has 84 kb whereas the mammalian mitochondria only 16, a fivefold discrepancy. Bernardi (1979) helps to explain the additional genetic material present in yeast mitochondria by noting that there are non-coding regions present with ATP rich segments making up 25% of the genome. The mitochondrial genes coding for cytochrome b and subunit 1 of cytochrome oxidase are almost as large as the entire mammalian genome.

Mitochondrial genomes vary in size (see Table 3.1). In yeast there are on average 22 mitochondria per cell, in growing cells the proportion of mitochondrial DNA to total cell DNA can be as high as 18%. In mitochondria no passage is permitted for nucleic acids in either direction through the organelle membrane.

As illustrated in Figure 3.4, each mitochondria engages in protein synthesis. A small number of proteins are produced, each of which is a component of an oligomeric aggregate that includes protein subunits imported from the cytoplasm (Hurt et al., 1984). More specifically proteins which make up the mitochondrial components, including components for replication and expression of mt DNA and a distinct set of ribosomal proteins are, with few exceptions, synthesized outside the organelle and then correctly located within it (Hay et al., 1984) (see Figure 3.4).

In the yeast S. cerevisiae, less than a dozen of the 250 known proteins of the isolated organelle are encoded by mt DNA (Dujon, 1981).

The yeast mitochondrial system now provides the best available genetically characterised model for the analysis of nuclear gene products.
Figure 3.4 Mitochondrial protein aggregates are assembled from the products of expression of nuclear genes and from mitochondrial genes.

(Modified from Lewin 1985)
involved in the transcription and translation of a eukaryotic gene (Dujon, 1981). Since the accumulation of cytochrome P-450, in S. cerevisiae is a little known process, the study of the nuclear genes associated with the induction of and degradation of cytochrome P-450 can only help in our understanding of the system. Just such a study was carried out on different haploid strains of S. cerevisiae by King, Wiseman and Wilkie (1983a) who suggested that the action of the regulatory gene controlling cytochrome P-450 accumulation is modified by a second nuclear gene which enhances the amount of the enzyme produced.

From previous work (see chapter 2) there are different accumulations of cytochrome P-450 at high and low glucose concentrations of yeast grande and petite strains. It was considered of interest to compare the internal structures of the different strains grown in different glucose concentrations to evaluate the degree of various organelle development, or lack of it, with accumulation of cytochrome P-450. There was no literature available on this specific subject.

3.2 Materials and Methods

3.2.1 Transmission Electron Microscope

The transmission electron microscope has three essential systems: (1) an electron gun, which produces the electron beam, and the condenser system, which focusses the beam onto the object; (2) the image-producing system, comprising the objective lens, movable specimen stage, and intermediate and projector lenses, which focus the electrons passing through the specimen to form a real, highly magnified image; and (3) the image-recording system, which converts the electron image into some form perceptible to the human eye. It usually consists of a fluorescent screen for viewing and focussing the image and camera for permanent records (see Figure 3.5).
Figure 3.5. Schematic section through a six lens transmission electron microscope.
Additional requirements are a vacuum system, comprising at least two pumps and their associated valves, and electric-power supplies with essential stabilisers and control equipment.

The source of electrons, the cathode, is a heated, V-shaped tungsten filament with its apex, from which the bulk of the emission occurs, towards the condenser lenses. The final electrode of the electron gun is the anode, which takes the form of a disc with an axial hole. Electrons leave the cathode and shield, accelerate towards the anode, and, if the stabilization of the high voltage is adequate, pass through the central aperture at a uniform speed. The control and design of the electron gun are critical in ensuring satisfactory operation.

The specimen, mounted on a copper grid, is carried in a small holder in a movable specimen stage. The operator can control it by a system of micrometer screws and bell cranks working through vacuum seals, so that a large area of the specimen can be examined systematically.

For practical reasons of image stability and brightness, the microscope is often operated to give a final magnification of 1,000-50,000 magnitudes on the screen. If a higher magnification is required, it may be obtained by photographic enlargement. The quality of the final image in the electron microscopes depends largely upon the accuracy of the various mechanical and electrical adjustments with which the various lenses are aligned to one another and to the illuminating system. The lenses require stabilization provided electronically for a satisfactory standard resolution.
The gun, specimen chamber, and column and camera are evacuated so that the electron beam can pass freely. At the place where the specimen and photographic plates are inserted, air locks provide that only a minimal amount of air is introduced into the column; this reduces pumping time before the microscope is again operational.

The electron image must be made visible to the eye by allowing the electrons to fall on a fluorescent screen fitted at the base of the microscope column. Such a screen is satisfactory for quick observations and for focussing and aligning the instrument. A low-power optical microscope fitted outside the column allows the image on the screen to be inspected at a magnification of about ten magnitudes, but the image suffers from poor contrast, lack of brightness, and the inherent grain of the screen. By allowing the electrons to fall upon the emulsion of the photographic plate, the latent image can then be developed and fixed exactly as in ordinary photography, and prints and any degree of further enlargement may be obtained from the negative. Such further enlargement allows all the detail present in the image to be resolved by the eye and also allows some control over the contrast.

3.2.2 Preparation for Transmission Electron Microscope

Saccharomyces cerevisiae NCYC 754 and JD1 rho^- (p^-) were grown in 0.5%, 5% and 20% glucose growth media (as described in section 2.2.1). The cells were harvested after 40 hours, late exponential phase. For microscopy the yeast cells were placed directly onto slides, after washing the cells twice in phosphate buffer pH 7.0. For transmission electron microscopy the cells were fixed in 1% (w/v) unbuffered potassium permanganate at 0°C for one and a half hours (modified after Gay and Martin, 1971). Potassium permanganate was preferred to
osmium tetroxide since it is safer and fixes membranous structures well (D.G. Smith et al., 1969). The yeast cells were then dehydrated ready for embedding by washing in the following solutions sequentially:

1. 50% alcohol, 10 minutes
2. 70% alcohol, 10 minutes
3. 70% alcohol, 10 minutes
4. 90% alcohol, 10 minutes
5. 95% alcohol, 10 minutes
6. 100% alcohol, 10 minutes (three times)
7. Propylene oxide : Alcohol (1 : 1), 10 minutes
8. Propylene oxide, 10 minutes
9. Propylene oxide : Epon 812 (1 : 1), 10 minutes

The cells were then spun down, the supernatant poured away and the cells scraped out with a microspatula and embedded in 100% Epon 812 (Luft, 1961) in truncated capsules (Taab Laboratories, Reading, Berkshire). The yeast was pelleted by centrifugation and incubated at 60°C for 48 hours for resin polymerisation. The capsules were removed from the oven, the resin blocks taken out of the plastic capsules. The resin blocks were initially trimmed to expose the cells. The cells were then sectioned with a Reihardt OmU3 (Reihardt-Jung, Austria) ultramicrotome using a diamond knife (Du Pont UK Ltd., Herts.) to a thickness of 700 Å (silver-grey in colour). The sections were floated in distilled water and mounted onto copper grids, blotted dry and placed in a numbered grid box. They were then counterstained with lead citrate (Reynolds, 1963) for 10 minutes and saturated uranyl acetate in alcohol (Gibbons and Grimstone, 1960) for 5 minutes. A JEOL JEM 100B (Joel, Japan) transmission electron microscope was then used to examine the material.
The lead citrate was prepared by dissolving lead nitrate 1.33 g and trisodium citrate 1.76 g in 30 ml of distilled water. The solution was left in the Gallenkamp orbital shaker for 30 minutes at 40 rpm at 30°C. 8 ml of 1 M sodium hydroxide was added to the solution to dissolve any precipitate that may have formed. The solution was then made up to 50 ml with distilled water and centrifuged at 3,000 rpm for 20 minutes in an MSE bench centrifuge to bring down any particulate matter.

The uranyl acetate solution was prepared by dissolving 0.2 g of uranyl acetate in 10 ml distilled water with the aid of an ultrasonicator. Before use the solution was centrifuged at 3,000 rpm for 20 minutes using an MSE bench centrifuge to bring down any undissolved particles.

3.3 RESULTS AND DISCUSSION
3.3.1 Effect of Different Glucose Concentration on Ultrastructure in S. cerevisiae NCYC 754

Plates 3.1 and 3.2 are micrographs taken with a transmission electron microscope and show the difference between S. cerevisiae (NCYC 754) grown at 0.5% glucose and 20% glucose respectively. As can be seen under glucose repression the mitochondrial development has been severely inhibited with no apparent intact mitochondria, as also reported by Wallace et al. (1968). Transcription of nuclear genes encoding most mitochondrial proteins is repressed by a fermentable carbon source and derepressed by a non-fermentable carbon source (Szekely and Montgomery, 1984).

As described earlier, cytochrome P-450 is only produced in cells grown at high glucose concentration. These cells contain apparently no active mitochondria (as confirmed here by electron microscopy) however the
link between glucose repression and cytochrome P-450 production in *S. cerevisiae* is unknown. As cytochrome P-450 is located in the endoplasmic reticulum of *S. cerevisiae* (Ishidate et al., 1969a) it is possible that during glucose repression with subsequent high cytochrome P-450 levels proliferation of endoplasmic reticulum may occur as is known to occur in mammalian systems on induction of cytochrome P-450. The extensive cytoplasmic membranes seen in plate 2 may show some preliminary evidence for this.

3.3.2 Effect of Different Glucose Concentration on Structure in JD1

The petite mutant strain JD1 rho minus when grown in 5% glucose and harvested for microscopy after 40 hours exhibited an unusual deformation in about 20 out of every 100 cells viewed, the most extreme deformations in the cell wall are shown here (plates 3.3 to 3.7). The other petite mutant strains and the rho minus strain grown in 5% or 20% glucose growth media showed no such deformations.

As a result of very low available nutrients the petite mutants grown in 0.5% glucose may have constructed cell walls lacking in some of the carbohydrate components perhaps as a result of low ATP levels enabling only a 'skeleton' cell wall.

The unusual morphology exhibited by the petite mutants may possibly be as a result of the lack of mitochondrial DNA which affects the properties of the cell wall polymers in low nutritional-status cells. Alternatively an unknown intermediate compound, not produced from the mitochondria (as a result of the missing mt DNA) may in some way affect the plasticity of the cell wall just as indole acetic acid delays the setting of the cellulose plant cell wall allowing elongation just behind apical meristems to occur.
The deformations could be due, in part, to the method used in the preparation of the sample (Prof. A.H. Rose, personal communication) yet other yeast samples prepared in the same way have not exhibited such cell wall changes. Plate 3.8 shows a yeast cell with a bud scar. It can be seen that the ridges are totally different to the cell walls shown in plates 3.3 to 3.7.

Zaborowska et al. (1982) reported abnormalities in cell wall separation during budding in *S. cerevisiae* diepoxibutane induced rad 1-1 and rad 3 mutants but the changes which caused anucleate mother cells, were on a much smaller scale. Zaborowska et al. offered no explanation for the lack of cell wall separation.

Plate 3.9 is most interesting, it offers yet another possible explanation for the unusual morphology. If during the growth of the petite mutant the cell wall polymers were in a plastic state then during harvesting by centrifugation the yeast cells being compacted may have caused the changes to the cell wall. But this may be peculiar to the petite mutant strain at low nutritional level because the same centrifugation speeds and times were applied to other yeast without such changes to their cell walls. To examine this hypothesis further the petite mutants were observed under a light microscope to determine whether any changes in morphology could be seen before or after centrifugation. Plates 3.10 and 3.11 show the petite mutants before and after centrifugation and there are no visible changes in the external morphology. It is possible the mitochondria may have a role in the biogenesis of cell membranes; it is known that calcium is sequestered in the mitochondria and that calcium activates certain proteins. This may be another explanation for the deformation of the cell wall at low nutritional levels, if the lack of calcium were to affect the proteins in the cell wall.
At low glucose concentrations the yeast cell may flocculate more readily as a result of the excretion of proteins which cross-link with other cells and so help binding. In petite mutants the flocculation properties can change perhaps as a result of different proteins in the plasma membrane (Wilkie and Nudd, 1981).

Another possibility is that the cell walls are in a degradative phase and consequently deformation is a symptom of the break-up of the cell during autolysis.
CHAPTER 4

THE EFFECTS OF ETHANOL ON CYTOCHROME P-450

ACCUMULATION AND DEGRADATION
4.1 **INTRODUCTION**

_Saccharomyces cerevisiae_ has been employed by men for thousands of years to produce ethanol and carbon dioxide in the brewing and baking industries. Today, not only is the ethanol toxicity to yeast in high gravity brewing important but ethanol itself is increasingly important as an energy source. As the fossil fuels are in finite quantity man's interest in seeking alternatives may well focus on yeast. Research on the factors influencing ethanol tolerance in yeast may facilitate ethanol production on an industrial scale.

The study of the role ethanol has in the metabolism of yeast may help in the optimization of cytochrome P-450 accumulation so that this enzyme may be produced on a large scale for industrial purposes such as the stereospecific insertion of oxygen into particular macromolecules.

4.1.1 **Ethanol Production and Tolerance**

During yeast culture, growth may stop because of:

1. Depletion of nutrients
2. Accumulation of products of the organism's own metabolism which are toxic to the cell at high concentration, such as ethanol, or
3. Other environmental resistance parameters.

In the commercial production of ethanol by fermentation there is a certain ethanol concentration - the value of which depends on the strain - which cannot be exceeded regardless of the environmental conditions. Different yeasts vary in the amount of ethanol they produce, the differences being in the yeasts' ability to tolerate ethanol. This is illustrate by the data shown in Table 4.1 for several industrial yeast strains. Gray (1941) defined ethanol tolerance as the concentration of ethanol in the growth medium that reduced the rate of sugar utilisation
<table>
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<td></td>
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<td><em>S. carlsbergensis</em></td>
<td>NCYC 1324</td>
<td>11</td>
</tr>
<tr>
<td>Sake brewing yeasts</td>
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<td></td>
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<tr>
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<td>NCYC 478</td>
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</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>CBS 1198</td>
<td>9</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Kyokai No. 7</td>
<td>20</td>
</tr>
<tr>
<td>Sugar tolerant yeasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. rouxii</em></td>
<td>AB 197</td>
<td>7</td>
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<tr>
<td><em>S. rouxii</em></td>
<td>AB 283</td>
<td>7</td>
</tr>
<tr>
<td><em>S. diastaticus</em></td>
<td>AB 88</td>
<td>11</td>
</tr>
<tr>
<td><em>S. bailii</em></td>
<td>NCYC 464</td>
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</tr>
</tbody>
</table>

Table 4.1. The Ethanol Tolerance of Yeasts

(From Day et. al. 1975 and Hayashida 1974)
by a stipulated amount. Ethanol tolerance is a reproducible property of a yeast strain (Table 4.1).

The amount of ethanol produced during the course of brewery fermentations is quite low (2.5 - 5% v/v). Beers containing 8 - 10% (v/v) ethanol can be produced although the yeast crops resulting from such fermentations tend to leave low viabilities, this is not that important since such beers constitute only a minor proportion of the total production of most breweries and so it has not been found necessary to re-use the yeast from such fermentations.

4.1.2 Effects of Ethanol on Membranes

Ethanol is an amphipathic molecule i.e. one end of the molecule is hydrophobic and the other end is hydrophilic and the primary toxic effects on the yeast cell may be exerted at the plasma membrane.

It has been suggested (Day et al., 1975) that the importance of unsaturated fatty acids in alcohol tolerance does suggest a role in membrane function which may include the control of leakage of intracellular metabolites.

Ethanol is likely to interact with all of the components of biological membranes, however, most data is available on its interactions with the fatty acid side-chains of phospholipids. It has been shown that the incorporation of unsaturated fatty acids into the membranes of an anaerobically-grown strain of S. cerevisiae used in sake production resulted in an increased ability of the yeast to produce and tolerate ethanol levels as high as 20% (v/v) (Hayashida et al., 1974). The enrichment of the plasma membrane lipids of a strain of S. cerevisiae
with certain sterols and unsaturated fatty acid residues has been shown to result in increased viability of the yeast in ethanol (Thomas, 1977). These findings are due to the tight packing of sterols and phospholipids in the yeast plasma-membrane (promoted especially by the presence of sterols with an unsaturated side chain, which makes the membrane a more effective barrier to ethanol molecules). Also important in this increased viability is the increased solubility of ethanol in the membrane resulting from the presence of unsaturated fatty acid residues in the membrane interior (Thomas, 1977).

For the yeast cell to synthesise unsaturated fatty acids molecular oxygen is required (Andreasen et al., 1954). Therefore under anaerobic conditions unsaturated fatty acids become essential growth requirements for yeast, the addition of unsaturated fatty acids was shown to result in enhanced yeast viability not only in culture but also when suspended in aqueous ethanol (Day et al., 1975).

The structure of biological membranes consists of a bilayer of phospholipid molecules, embedded in which are functional proteins such as enzymes (Singer and Nicolson, 1972). Each phospholipid molecule consists of a hydrophilic phosphate-containing head group attached via a glycerol unit to a hydrophobic tail consisting of two fatty acid residues. Instead of being aligned in a rigid crystalline-lattice structure, the fatty acids tails are flexible and quasi-fluid in character. The fluidity of the membrane is determined largely by the structure and relative proportion of unsaturated fatty acid to saturated. In phospholipids consisting only of saturated fatty acids the tails are aligned in a rigidly stacked array at physiological temperatures. When both saturated and unsaturated fatty acids are present in the double bonds of the unsaturated fatty acids
Phospholipids within biological membranes cause structural deformation. Packing is then less orderly and the fatty acids more fluid. This dynamic state of a membrane is also influenced in yeast by its content of sterols. These have been found in both the free and esterified form in yeast membranes, the most abundant being ergosterol and are believed to fulfill a loose-fit or 'filler' role between phospholipid molecules (Proudlock et al., 1968). They therefore influence the degree of packing of the phospholipid molecules and in general stabilise their arrangement in the membranes.

The binding or absorption of solvents to membranes appears to depend on the following factors:

a. Partition coefficient in a non-aqueous/aqueous system.
b. The charge distribution of the molecule.
c. The molecular weight or volume.
d. pH: Increasing the pH may increase the membrane/buffer partition coefficient.
e. Temperature - the membrane binding of alcohols increases with temperature (Seeman, 1969).
f. Content of membrane sterols.
g. Reversibility of binding. Generally the binding of solvents to membranes is reversible ($10^{-8}$ to $10^{-3}$M), but high ($10^{-3}$M plus) concentration of solvent may irreversibly expose new binding sites in the membrane (Seeman, 1969).

Seeman et al. (1972) working on anaesthetic effects found that the activity of alkanols correlated much better with the non-aqueous/aqueous partition coefficient than with any other parameter (e.g. polarisability, molecular weight, or molar attraction constant).
Solvents have been found to expand biological membranes (Buchi, 1967). J. Traube (1908) found that amyl alcohol inhibited the haemolysis of erythrocytes in hypotonic solutions. Solvent-induced expansion increases the surface area: volume ratio of a cell and erythrocytes with higher area: volume ratios are associated with lower osmotic fragility.

The amount of membrane area expansion is of the order of 10 times the bulk volume of the solvent molecules absorbed in the membrane phase (Seeman et al., 1972). Seeman et al. (1972) found that $10^{-4}$ concentrations of either chloroform or ether expand human erythrocyte membranes by 0.4% in area, which is equivalent to 0.6% in membrane volume.

The following mechanisms may be considered for membrane expansion.

a. Solvent being absorbed in the membrane phase.

b. Disordering and expansion of lipid regions of membrane.

c. Membrane expansion by solvent induced hydration in the membrane (Seeman, 1972).

d. Solvent induced conformation changes in membrane globular proteins (Balasubramian and Wetlaufer, 1966).

e. Solvent-induced alteration in membrane calcium. When $Ca^{2+}$ absorbs to the cytoplasmic aspect of the membrane, the membrane becomes rigid and difficult to deform (Weed et al., 1969). So a displacement of the $Ca^{2+}$ may expand the membrane. ($Ca^{2+}$ and $Mg^{2+}$ affect membrane fluidity by forming ionic bonds with neighbouring phosphoryl headgroups tending to tie phospholipid molecules together and decrease their mobility). However, it has been found that members of the alkanols series increase membrane $Ca^{2+}$ which might promote contracture of the membrane (Chau-Wong and Seeman, 1971).
f. Solvents might absorb to the membrane, altering the amount of water in contact with either side of the membrane and consequently altering the interfacial tension at the membrane-water interface (Schneider, 1968). The net result of these effects would be membrane expansion.

g. Solvents inhibition of contractile enzymes or proteins in the membrane. One of the mechanisms for solvent-induced membrane expansion may be that the solvent inhibits a contractile membrane-associated ATPase (Palek et al., 1971).

As well as dissolved oxygen tensions and medium composition two other environmental factors are known to affect the lipid composition and thus ethanol tolerance, the growth temperature, growth rate and pH. In common with most living organisms, yeasts will tend to raise their lipid content and degree of unsaturation as the growth temperature drops (Rattray, 1975).

F.H. White (1978) found that periodic aeration/oxygenation, or the addition of unsaturated fatty acids or ergosterol, increased ethanol tolerance of yeast.

Ingram (1976) working on E. coli suggested that in the presence of alcohols the fatty acid composition of the plasma membrane changes. The addition of 1-4% ethanol stops growth. As the fatty acid chain length is increased then growth re-starts.

Thomas et al. (1978, 1979) working on S. cerevisiae NCYC 366 found similar results to Ingram in that the addition of fatty acids to the medium offered some protection to the cells against ethanol.
More recently Watson (1982) found that unsaturated fatty acids such as oleic acid rather than sterols such as ergosterol are essential for high ethanol production (13-15% w/v) in *S. cerevisiae* CBS 1171. However there was still distinct protection by ergosterol at lower ethanol concentration. Yet again, Casey (1983) showed that the supplementation with ergosterol and tween 80 enabled yeast to grow in much higher levels of ethanol.

The membrane fluidity has been considered an important factor in regulating certain membrane functions. An alteration of the lipid environment in the plasma membrane by ethanol and other alkanols has a deleterious effect on uptake of solutes (Leao and Van Uden, 1982), amino acids (Leao and Van Uden, 1984a), passive proton influx across the plasma membrane (Leao and Van Uden, 1984b). However, effects on the various proteins involved in these processes are also likely. Membrane fluidity may be particularly important in the endoplasmic reticulum which synthesizes its own lipid components (Garda, 1984). Yeast cytochrome P-450 is known to be a key enzyme required for ergosterol biosynthesis (Aoyama et al., 1984). So if ethanol was adversely affecting ergosterol biosynthesis at the endoplasmic reticulum then ergosterol supplementation would be expected to help overcome this. This is especially important if the increased exposure of the cells to ethanol requires the yeast cells to change their composition within the cellular membrane so as to prevent disintegration of the cytoplasmic matrix (Kappeli, 1986). An increase in the incorporation of ergosterol in the membrane may be one answer. If this were so then it is possible to envisage a role for cytochrome P-450 in the presence of ethanol.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half-life (hr.)^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>0.2</td>
</tr>
<tr>
<td>-Aminolevulinate synthetase (mitochondrial)</td>
<td>1.1</td>
</tr>
<tr>
<td>RNA polymerase I</td>
<td>1.3</td>
</tr>
<tr>
<td>Tyrosine aminotransferase</td>
<td>2.0</td>
</tr>
<tr>
<td>Tryptophan oxygenase</td>
<td>2.5</td>
</tr>
<tr>
<td>Deoxythymidine kinase</td>
<td>2.6</td>
</tr>
<tr>
<td>Serine dehydratase</td>
<td>4.0</td>
</tr>
<tr>
<td>Amylase</td>
<td>4.3</td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>12</td>
</tr>
<tr>
<td>RNA polymerase II</td>
<td>12</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>15</td>
</tr>
<tr>
<td>3-Phosphoglycerate dehydrogenate</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4.2. Proteins degraded most rapidly in rat liver

(modified from Goldberg et al. 1976)
4.1.3 Degradation of Proteins

The average protein in rat liver has a half-life of approximately one day, in bacteria regulatory proteins may be completely hydrolysed within minutes of their synthesis. This continuous turnover of cell proteins is of major importance in the regulation of enzyme levels, in protecting the organism against the accumulation of abnormal proteins, in the control of tissue mass and in the organism's ability to adapt to poor nutritional conditions.

The degradation of specific proteins in cells has been found to obey first order kinetics (Liu et al., 1984), the rate of degradation is defined by the half life of the protein, the time at which 50% of the protein is degraded. The rate of degradation may vary under different physiological conditions, for example with starvation or in response to hormones.

The concentration of any protein is determined by the balance between its rate of synthesis and degradation. So a rapid rate of degradation would ensure that the concentration of an enzyme would fall very quickly when its synthesis is reduced.

Key rate-limiting enzymes that are important in metabolic control may have evolved especially short half-lives (see Table 4.2). Included in the group of rapidly degraded proteins are the rate-limiting enzymes for haem synthesis, RNA synthesis, amino acid breakdown and cholesterol production. Metabolic instability of such proteins allows for rapid adjustment of their intracellular concentration through regulated changes in rates of their synthesis and/or degradation (Pontremoli and Melloni, 1986). Most of the selective turnover of intracellular proteins
under normal metabolic conditions is ATP-dependent and in eukaryotes non-lysosomal (Finley and Varshavsky, 1985). Recent evidence indicates that, in eukaryotes, covalent conjugation of ubiquitin to short lived intracellular proteins is essential for their selective degradation (Finley and Varshavsky, 1985). The half-life of a protein has recently been found to be controlled by its N-terminal amino acid (Bachmair, Finley and Varshavsky, 1986).

Liu et al. (1984) found that the degradation of normal proteins was suppressed by glucose in S. cerevisiae. These workers also found that in the presence of ethanol (1-2% v/v) abnormal proteins which may have been found were degraded at a faster rate. Cytochrome P-450 accumulates in large quantities in S. cerevisiae only under specific growth conditions. This enzyme accumulation is under complex regulatory control and is influenced by several environmental factors including oxygen glucose and ethanol. The work described in this chapter was undertaken in an attempt to define the role of ethanol in the regulation of cytochrome P-450 accumulation in S. cerevisiae.

4.2 MATERIALS AND METHODS
4.2.1 Time Course Experiments
S. cerevisiae NCYC 240 or 754 as described in the text was grown on growth media under specific conditions stated, then harvested by centrifugation for 10 minutes in the M.S.E. bench centrifuge. The yeast cells were washed, centrifuged again and resuspended in 0.1 M phosphate buffer pH 7.0. The cytochrome P-450 assay and dry weight determinations were performed as described in chapter 2.
4.2.2 Measurement of Ethanol

Ethanol was measured enzymically by using alcohol dehydrogenase and measuring the increase in absorbance at 340 nm of NADH, using a kit from BCL Boehringer Corporation, London.

\[ \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \xrightarrow{\text{alcohol dehydrogenase}} \text{CH}_3\text{CHO} + \text{NADH} + H^+ \]

A typical standard curve for this assay is shown in Figure 4.1.

4.2.3 Measurement of Glucose

Glucose was measured by the glucose oxidase method using a Boehringer kit and measuring the absorbance of the coloured complex at 436 nm.

\[ \text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2 \]

\[ H_2O_2 \xrightarrow{\text{POD}} \text{coloured complex} + H_2O \]

ABTS = di-ammonium 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonate)

A typical standard curve for this assay is shown in Figure 4.2.

4.2.4 Determination of Reduced Cytochrome P-450 Values

The cytochrome P-450 assay was performed as before (described in chapter 2) except that the aliquot additions of buffer or n-alkanol were made to the cuvette with the sodium dithionite already present. The sodium dithionite reduces the cytochrome P-450 (Fe$^{III}$ $\rightarrow$ Fe$^{II}$).

The cuvettes were sealed to prevent evaporation of n-alkanols.

4.2.5 Determination of Glutathione Effect

In the glutathione experiments (Figure 4.8, section 4.3.4), 1% glutathione (w/v) was added to the glucose growth media at the same time as the dithionite.
Figure 4.1. Ethanol standard curve
Figure 4.2. Glucose standard curve.
4.2.6 Filtration of Growth Media with Activated Carbon

Yeast was grown as described in chapter 2, and harvested at 42 hours. The media separated from the yeast culture (100 ml) was then poured into a sterile 250 ml conical flask containing 50 g of activated carbon granules (Sigma). This flask was then returned to the orbital shaker and shaken at 120 RPM at 30°C for one hour. The carbon was separated from the media by centrifuging for 10 minutes at 3,000 RPM on a bench centrifuge. The yeast that had been separated from this medium was added to a flask containing yeast in 20% glucose growth medium for the duration of the hour. After one hour the yeast was centrifuged, weighed, washed and divided into two portions; one portion to be resuspended in the remaining glucose growth medium and the other portion was resuspended in the carbon-treated glucose growth medium. The flasks were then incubated in the orbital shaker at 120 RPM at 30°C.

4.2.7 Filtration of Growth Media with Zeolite

The procedure was as for activated carbon (section 4.2.6). Forty grammes of zeolite SP-115 1.6 mm (Union Carbide) per flask, was used.

It was not necessary to separate the zeolite from the medium by centrifugation, simple paper filtration with a Buchner funnel sufficed.

4.2.8 Production of Alginate Beads

A 3% (w/v) Manugel DJB (Kelco/AIL International Ltd.) alginate solution was prepared in sterile distilled water. The solution was stored in a cool place for three hours to homogenise, was then stirred and then poured into a 25 ml pipette (previously autoclaved) from which droplets were allowed to agglomerate in a 0.05 M CaCl₂ solution, the distilled water from which had been previously autoclaved. The beads were then washed
in sterile distilled water. The whole operation was carried out in close proximity to a Bunsen burner to maintain sterile conditions. The beads, so formed were used for the experiments.

4.2.9 Production of Alginate Beads with Glass and Zeolite

The procedure was similar to that described in section 3.2.8 except that into 25 ml of 3% (w/v) alginate gel 10 g of zeolite or 10 g or 2mm glass ballatini were added prior to pouring but after homogenisation of the alginate.

4.2.10 Preparation of Media with n-Alkanols

Members of the n-alkanol series were added in separate experiments and incubated with sealed flasks (to avoid evaporation of alkanol) at 30°C for several hours. Samples were withdrawn at timed intervals for determination of residual cytochrome P-450 and therefore half life of this enzyme in the 6% (v/v) n-alkanol suspension.

4.3 RESULTS AND DISCUSSION

4.3.1 The Accumulation of Cytochrome P-450 during Growth of S. cerevisiae correlated with the Rise in Ethanol Concentration and Loss of Glucose in the Medium

Figure 4.3.1 shows a time course of the growth of S. cerevisiae NCYC 754 in 20% glucose medium as represented by its dry weight.

Figure 4.3.2 shows a time course of the levels of glucose ethanol and cytochrome P-450 accumulation during growth of S. cerevisiae NCYC 754 on 20% glucose medium. It can be seen from Figure 3.1.2 that the cytochrome P-450 value declines after approximately 40 hours, which coicides exactly with a levelling off and then a fall in dry weight. This is
Figure 4.3. Time course of *S. cerevisiae* NCYC 754 dry weight per 100 ml medium.

Values are the mean of six determinations, bars indicate standard deviations.
Figure 4.3.2. The accumulation of cytochrome P-450 during growth of *S. cerevisiae* NCYC 754 in 20% (w/v) glucose growth medium correlated with the rise in ethanol concentration and loss of glucose in the medium.

Values are the mean of six determinations, bars indicate standard deviations.
in agreement with Woods (1979) and Karenlampi et al., (1981) who also observed a fall in the enzyme level at the end of the exponential phase of growth. This rapid fall in cytochrome P-450 level has been partially attributed to the decline in the glucose concentration in the growth medium (Wiseman et al., 1975a). However, Figure 4.3.2 shows that 10% glucose still remains at this time. A glucose level of 10% is usually sufficient to ensure a high level of cytochrome P-450 (Lim et al., 1975). Thus the glucose concentration alone cannot be responsible for the loss of cytochrome P-450 or the reduction in growth rate at this stage.

It is possible that there may be reduced growth due to the depletion of other nutrients from the media such as a nitrogen source or vitamins.

From Figure 4.3.2 it can be seen that after 40 hours the ethanol concentration approaches 5%, after 120 hours it is at a maximum of 7%, after that the level falls probably as a result of diauxic growth where some ethanol is used as a carbon source.

Ethanol has pleiotropic effects on yeast including inhibiting cell multiplication rate (G. Moulin et al., 1984), fermentation rate and yeast viability (Troyer, 1953; Thomas and Rose, 1979; Brown et al., 1981; Jones and Greenfield, 1985). The increase in ethanol may affect the accumulation of cytochrome P-450 activity directly or indirectly by inhibiting growth rate of the yeast or both. Del Carroto re et al. (1984) have suggested that the decline in cytochrome P-450 in *S. cerevisiae* at the end of the exponential growth phase is due to the increase in ethanol concentration. A possible direct effect of ethanol on cytochrome P-450 in *S. cerevisiae* has been investigated in further experiments described in this chapter.
Cytochrome P-450 n.mol/g. wet wt.

Percentage content (v/v) ethanol, tris, buffer.

Ml. aliquots of ethanol, tris, buffer added.

- △ Tris pH 9
- □ Ethanol pH 9
- ○ Buffer pH 7

Figure 4.4. The effect of ethanol, tris pH 9 and buffer pH 7 on reduced cytochrome P-450 values from \textit{S. cerevisiae} NCYC 240 taken after 40 hours growth.

Values are the mean of two determinations.
Figure 4.5. Degradation of reduced cytochrome P-450 (taken from *S. cerevisiae* NCYC 240 after 40 hours) with different concentrations of ethanol. Whole yeast used at 20°C. Values are a mean of two determinations.
Leao and van Uden (1982) reported that ethanol inhibits the glucose transport system in *S. cerevisiae*. From Figure 4.3.2 at the highest ethanol concentration, 7% after 120 hours there seems to be little glucose utilization the glucose concentration being almost constant but at the time point there is only 2% glucose left; it is possible that the ethanol build up by this mechanism is preventing glucose utilization.

4.3.2 Effect of Ethanol on Reduced Cytochrome P-450

Figure 4.4 shows the immediate effect that ethanol has on reduced cytochrome P-450 in whole yeast. Thirteen per cent ethanol caused immediate total loss of cytochrome P-450 as measured spectrophotometrically. Lower concentrations also resulted in significant losses of the enzyme. The phosphate buffer pH 7.0 acting as control caused little loss of reduced cytochrome P-450. The addition of ethanol in this system inevitably changes the pH to approximately 9. However, this was not the major cause of the losses seen, as a control at pH 9 (tris buffer 0.1 M) resulted in only small losses of the reduced cytochrome P-450 enzyme.

Figure 4.5 shows a time course of the loss of reduced cytochrome P-450 in whole yeast in the presence of 3% and 7% ethanol added at zero time. Figure 4.5 indicates that the reduced cytochrome P-450 declines more rapidly at the higher ethanol concentration and that in the absence least degradation occurs.

It may be that after a certain ethanol concentration is reached in the growth medium enzymes such as hexokinase which are sensitive to ethanol may be inactivated and thus diminish the rate of glycolysis.
<table>
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<tr>
<th>COMPOUND</th>
<th>FERMENTED MATERIAL FOUND IN</th>
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<tr>
<td>ISOButyl Alcohol</td>
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</tr>
<tr>
<td>Methanol</td>
<td>BEER, WINE</td>
</tr>
<tr>
<td>Propanol</td>
<td>BEER, WINE</td>
</tr>
<tr>
<td>Butanol</td>
<td>BEER, WINE</td>
</tr>
<tr>
<td>Amyl Alcohol</td>
<td>BEER, WINE</td>
</tr>
<tr>
<td>IsoAmyl Alcohol</td>
<td>BEER, WINE</td>
</tr>
<tr>
<td>Pentanol</td>
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</tr>
<tr>
<td>Hexanol</td>
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<td>Heptanol</td>
<td>POTATO, WINE BRANDY</td>
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</tr>
<tr>
<td>Decanol</td>
<td>CANE MOLASSES, POTATO</td>
</tr>
</tbody>
</table>

TABLE 4.3. SOME OF THE COMPOUNDS PRESENT IN FUSEL OILS FROM VARIOUS SOURCES

(Modified from Webb and Ingraham 1963)
Israel et al. (1969) have suggested that ethanol may have an inhibitory effect on amino acid transport, but the observed effects from Figures 4.4 and 4.5 are immediate. It is possible that amino acid availability may affect yeast growth rate and enzyme biosynthesis but it does not help explain a sudden decline in reduced cytochrome P-450 values.

The addition of ethanol inhibits membrane enzymes such as adenosine triphosphatase (Coldwell and Sever, 1974). ATP may be required for the biosynthesis of the proteases responsible for enzyme degradation and also for transport of enzymes across membranes into appropriate vacuoles for degradation (Murakami et al., 1979). As ethanol inhibits ATP synthesis ethanol may therefore inhibit degradation either by slowing protease synthesis or inhibiting membrane transport. However, at the lowest concentrations used (3%) ethanol was found to increase the rate of cytochrome P-450 degradation and showed no protective effect. It is not known whether the loss of cytochrome P-450 observed is due to ethanol induced conformational change and loss of the specific environment around the haem group of the enzyme which gives rise to the characteristic P-450 spectrum or, to haem loss or to increased proteolytic degradation.

4.3.3 Degradation of Reduced Cytochrome P-450 by n-Alkanes

Table 4.3 shows the fusel oils which can be found in yeast fermentations. Castor and Guymon (1952) report that there may be as much as 35 mg of fusel oil per 100 ml of media in the fermentation of grape juice by some strains of yeast. As can be seen from the Table 4.1 alkanols are well represented, two major components are methanol and propanol, butanol is a minor component as is pentanol. Leao and van Uden (1982) have reported that alkanols inhibit the glucose transport system in yeast. The
Percentage alkanol added to destroy cytochrome P-450 in 1 minute at 20°C.

![Graph showing degradation of reduced cytochrome P-450 in whole yeast by alkanols at 20°C. Values are the mean of eight determinations, bars indicate standard deviations.](image)

Figure 46. Degradation of reduced cytochrome P-450 in whole yeast by alkanols at 20°C. Values are the mean of eight determinations, bars indicate standard deviations.
Figure 4.7. The effect of glycerol and glucose as protective agents against reduced cytochrome P-450 degradation with ethanol. S. cerevisiae harvested after 40 hours strain NCYC 240 used at 20°C.

Values are the mean of 8 determinations, bars indicate standard deviations.
Figure 4.8. The effect of glutathione on the degradation of cytochrome P-450 in whole yeast, strain NCYC 240 at 20°C. Values are the mean of 2 determinations.
fusel oils may be an important factor in yeast fermentations but nothing has been published about their effects on cytochrome P-450 accumulation or degradation in *S. cerevisiae*.

Figure 4.6 indicates the concentrations of different *n*-alkanols required to completely degrade cytochrome P-450 immediately. The longer chain alkanols seem more effective in degrading cytochrome P-450. Only about 2% butanol, pentanol or hexanol being required to immediately and completely degrade the cytochrome P-450 present. The addition of alkanols causes an increase in the fluidity of a membrane (Grisham and Barnett, 1972). The level of fluidity is dependent on the length of the carbon chain of the alkanol (Ingram, 1976).

4.3.4 **The Protective Effect of Glucose, Glycerol and Glutathione against Reduced Cytochrome P-450 Degradation by Ethanol**

Figure 4.7 shows the protective effect of 10% glucose and 10% glycerol against degradation of cytochrome P-450 by ethanol. Twice as much ethanol is required to completely degrade cytochrome P-450 with glucose present compared to the phosphate control. The presence of high concentrations of these protective agents may simply prevent the entry of ethanol into the yeast cell by blocking its uptake mechanism. Alternatively, the presence of glucose may enable the yeast cells to carry out some glycolysis and thus stabilize cytochrome P-450 levels. The glycerol may protect cytochrome P-450 preventing loss of the cytochrome P-450 haem group and/or degradation of the protein chain, as is known to be the case in solutions of solubilized cytochrome P-450 (Azari, 1984). However, a mechanism for this in intact cellular membranes is difficult to envisage. Figure 4.8 shows the protective effect of 1% glutathione on the degradation of cytochrome P-450. Very
Figure 4.9. Degradation of microsomal cytochrome P-450 with 5% and 10% ethanol and phosphate buffer. Values are the mean of 2 determinations.
Figure 4.10. Distribution of pore diameters in zeolite and carbon.

(Modified from Breck 1974)
little degradation of cytochrome P-450 occurs with the 1% glutathione up to the addition of 0.5 ml of ethanol (equivalent to 13% ethanol v/v). Without the glutathione, Figure 4.8 indicates that at 13% ethanol concentration, all of the cytochrome P-450 has been degraded. The glutathione may be protecting against haem loss and/or degradation of the protein chain of the cytochrome P-450.

4.3.5 Degradation of Reduced Microsomal Cytochrome P-450 by Ethanol

Figure 4.9 shows the degradation of microsomal cytochrome P-450 with various ethanol concentrations. Degradation of the microsomal cytochrome P-450 took longer than with the whole yeast cytochrome P-450 because of the introduction of various chemicals such as glutathione and glycerol with a protective influence, during the microsomal preparation. However, Figure 4.9 still indicates that at 10% ethanol concentration there is a more rapid cytochrome P-450 degradation than at 5% ethanol; after 4 hours with phosphate buffer the value of microsomal cytochrome P-450 was 0.47 nmol.ml\(^{-1}\), with 5% ethanol the value was 0.30 nmol.ml\(^{-1}\) and with 10% ethanol the value was as low as 0.13 nmol.ml\(^{-1}\).

4.3.6 The Effect of Filtering the Growth Media with Activated Carbon on Cytochrome P-450 Degradation

Activated charcoal is a carbon which exhibits ultraporosity and is generally used for the separation of gas and vapour mixtures. Activated carbons do not have an ordered crystal structure and consequently the pores are non-uniform. The distribution of the pore diameters may range widely from 20\(\AA\) to several thousand \(\AA\) (see Figure 4.10). Thus most molecules may enter and be retained in the pores (Breck, 1974). Thus activated carbon can be used as a non-specific adsorbent.
Cytochrome P-450
n.mol/g wet wt.

Figure 4. The effect of filtration with activated carbon on cytochrome P-450 in \textit{S. cerevisiae} NCYC 754.

Grown in orbital shaker at 120 RPM at 30°C.

Values are the mean of 4 determinations, bars indicate standard deviations.
This activated carbon can be used to remove ethanol from yeast growth medium in an attempt to see whether any effect on cytochrome P-450 levels is achieved. If ethanol is involved in cytochrome P-450 loss at the end of exponential growth then removal of high concentrations of ethanol might be expected to increase the half life of the cytochrome P-450, i.e. slow down degradation.

Figure 4.11 shows the effect on cytochrome P-450 accumulation when the growth medium is filtered with activated carbon to, principally, eliminate the ethanol. As can be seen, the difference between the filtered and non-filtered media is not significant but the filtered media does exhibit a steeper gradient in Figure 4.11. After filtration through activated carbon the glucose growth medium had become almost colourless and it would seem that more than just the ethanol had been adsorbed. As would be expected from the non-specific nature of this adsorbent, indeed the wide range of pore sizes that the carbon has would confirm that this was probable.

Since after incubation of the media through with activated carbon there was a slight 0.27 ml decrease in volume from the original 99.4 ml volume it is possible that this may have had an effect on the degradation of cytochrome P-450 in that the reconstituted yeast and medium had a slightly smaller volume in which to grow and hence may have encountered greater environmental resistance.

4.3.7 The Effect of Filtering the Growth Media with Zeolite on Cytochrome P-450 Degradation

Zeolites have wide application in chemical engineering process technology including separation of air components, recovering
Cytochrome P-450
n.mol/g.wet wt.

0 2 4 6
0 1 2 3 4 5

Incubation time (h)

- Control yeast
- Yeast in growth medium filtered with zeolite.

Figure 4.12. The effect of filtration with zeolite on cytochrome P-450 in *S. cerevisiae* NCYC 754. Grown in orbital shaker at 120 RPM at 30°C. Values are the mean of 4 determinations, bars indicate standard deviations.
radioactive ions from radioactive waste solutions and the removal of atmospheric pollutants such as sulphur dioxide. Zeolites have an empirical formula, $\text{Na}_2\text{O}.\text{Al}_2\text{O}_3\times\text{SiO}_2.\text{yH}_2\text{O}$.

Unlike activated carbons, zeolite has a more ordered crystal structure with consequently uniform pores ranging from $3\AA$ to $10\AA$, the pores will exclude molecules which are larger than their diameter. Figure 4.8 shows the distribution of the pore diameter for activated charcoal and for zeolite.

Dehydrated crystalline zeolites have a high internal surface area available for adsorption due to the channels or pores which uniformly penetrate the entire volume of the solid. The external surface of the adsorbant particules or only a small part of the total available surface area. The rate of adsorption of alkanols by zeolites decreases with the number of carbon atoms in the series (Breck, 1974) but since the author's prime interest was ethanol this seemed acceptable.

Figure 4.12 shows the effect on cytochrome P-450 degradation when the glucose growth medium is filtered with zeolite to, primarily, eliminate the ethanol. 40 g of zeolite was used since 1 g of zeolite was estimated to adsorb 0.15 g of ethanol (M. Winkler, personal communication) hence if a maximum of 7 ml of ethanol is produced (see Figure 4.3.2) then approximately 37 g of zeolite would be required. A slightly higher figure of 40 g was used to allow for the standard deviation from Figure 4.3.2.

The difference between the filtered media and non-filtered media in cytochrome P-450 accumulation values is not significant. This was surprising, if previous findings have shown the ethanol to be deleterious
to cytochrome P-450 accumulation then on the removal of ethanol one would have expected reduced cytochrome P-450 degradation. The zeolite adsorbed fewer nutrients from the media which inhibited yeast growth and cytochrome P-450 accumulation because of uniform pore size (see Figure 4.10). It can be said, however, that the gradient of the slope showing cytochrome P-450 degradation after media filtration with zeolite (Figure 4.12) is less steep than the gradient with activated carbon filtration (Figure 4.11). The smaller pore sizes to be found on the zeolite (Figure 4.10) which may have preferentially adsorbed the ethanol may be responsible for this. Also the zeolite retained less media (0.15 ml) than the activated carbon (0.27 ml) in the filtration process which may have caused less environmental resistance in the zeolite filtered media as compared to the carbon filtered media. But the significance of the gradients in Figures 4.11 and 4.12 is obscured by the standard deviations.

It is possible that incubation was not continued long enough to see any effects since in this experiment (and in the previous experiment with activated carbon) relatively little loss of cytochrome P-450 is seen even in the controls. Thus incubation to the extent where all of the cytochrome P-450 in the control has been lost may have shown some difference to the medium with ethanol removed.

4.3.8 The Effect of the Addition of Alginate Beads with Zeolite on Cytochrome P-450 Accumulation and Degradation

Alginate is most often used for thickening, stabilisation and film forming in the food industry being effective at low concentrations and having minimal influence on the taste of the food product. Manugel DJB was used which has an approximate particle size of 106 µm, the gel being formed with the presence of calcium forming a homogenous mass of
The effect of alginate beads on the accumulation of cytochrome P-450 in *S. cerevisiae* NCYC 754.

Grown in orbital shaker at 120 RPM at 30°C.

Values are the mean of 4 determinations, bars indicate standard deviations.
Cytochrome P-450
n.mol/g.wet wt.

Figure 4.3.2. The effect of alginate beads with glass and zeolite on the accumulation of cytochrome P-450 in S. cerevisiae NCYC 754. Grown in orbital shaker at 120 RPM at 30°C. Values are the mean of 4 determinations, bars indicate standard deviations.
insoluble, hydrated alginate which remains stable at 30°C, unlike gelatine gels. Using alginate beads with zeolite offered an opportunity to examine the commercial possibility of extracting ethanol in the production of alcohol-free beverages (M. Winkler, personal communication).

The results drawn on Figures 4.13.1 and 4.13.2 were from the same experiment but were not drawn on the same graph because the details would be obscured. Figure 4.13.1 shows the effect of the addition of 5 g of alginate beads per flask on the accumulation of cytochrome P-450 in \textit{S. cerevisiae} NCYC 754. In the control flask the peak cytochrome P-450 accumulation of 5.1 nmol/g wet weight seems to have been reached after 24 hours, but in this experiment no results were taken after 40 hours so it is possible that a peak may have occurred then. In the flasks with the alginate a distinctly higher cytochrome P-450 accumulation has occurred at 7.5 nmol/g wet weight after 24 hours.

The alginate with glass was a control for the alginate with zeolite. Glass being chosen because it is inert and has a similar relative mass to zeolite.

From Figure 4.13.2 it can be seen that the initial cytochrome P-450 values are different, the flasks with alginate and zeolite beads accumulating 7.2 nmol/g wet weight after 24 hours, with alginate and glass the value is 5.0 nmol/g wet weight which is very similar to the control value from Figure 4.13.1. After 44 hours the enzyme value in the flasks with alginate and zeolite beads decline to 6.8 nmol/g wet weight whereas the flasks with alginate and glass accumulate 7.1 nmol cytochrome P-450 per gramme wet weight of yeast. The error bars
overlap at the 44 hour time point as they do at 72 hours when the flasks with alginate and zeolite have enzyme values just over 2.0 nmol/g wet weight of yeast and the alginate and glass flasks have slightly less. Although both sets of flasks contained 10 g of alginate beads, the alginate and glass beads had a slightly higher relative mass, consequently in the course of the experiment - the beads rotated closer to the periphery of the flasks than the alginate and zeolite beads. This phenomenon may explain the slower accumulation of cytochrome P-450 in the alginate and glass flasks. The presence of zeolite seemed to do little to enhance the accumulation of cytochrome P-450, although the zeolite was present in very small amounts and the surface area for adsorption was masked by the alginate. In the 5 g of alginate present only 2 g of zeolite was present which, if not masked by the alginate, could adsorb only 0.4 ml of ethanol. At low concentrations of ethanol, such as prior to 24 hours where the ethanol does not exceed 1% (w/v) then the zeolite may have exerted an influence in possibly being able to adsorb some of the ethanol but this cannot be the entire explanation for the increase in cytochrome P-450 value in the alginate and zeolite flasks at 24 hours because the alginate alone achieved a similar enzyme accumulation. The alginate beads may be selectively adsorbing small molecular weight toxins and by their removal help in the growth of the yeast, consequently increasing the accumulation of cytochrome P-450. Alternatively, or as well, the presence of the beads may assist in the agitation of the medium in the flask and possibly optimise the oxygen tension which, again, would increase the accumulation of cytochrome P-450.

The rate of degradation of cytochrome P-450 in the four conditions examined appeared to be remarkably similar. It was anticipated that the
flasks containing alginate beads with zeolite, having adsorbed ethanol, may have delayed cytochrome P-450 degradation, however, in view of the small amount of zeolite available this may not have been possible.

As a result of the experiments above and others it was possible for the author to advise M. Winkler about a patent for ethanol-free beverage production.

4.3.9 The Effect of Ethanol on Oxidised Cytochrome P-450 Degradation

Figure 4.14 shows the effect of ethanol on oxidized cytochrome P-450 degradation during the incubation of *S. cerevisiae* NCYC 754. It can be seen that the oxidized cytochrome P-450 is less labile than the reduced form. 6% ethanol was used as this approximates to the concentration seen at the end of a shake-flask experiment in 20% glucose growth medium. It can be seen that initially the 6% ethanol degrades cytochrome P-450 at a slightly faster rate until 4 hours. After 5 hours the yeast cytochrome P-450 with the 6% ethanol has stopped degradation but continues at 7 hours. In control phosphate buffer incubations the yeast cytochrome P-450 level declined to approximately 45% of its original level in seven hours. With the phosphate buffer supplemented with 6% ethanol the level of cytochrome P-450 degradation was less, after 7 hours 65% of the original level was still present. The reason that degradation appears to slow in the presence of ethanol after approximately 4 hours may be due to the induction of some new enzyme by the ethanol after this time (Blatik *et al.*, 1987, see also 4.3.11). The 4 hour period may thus represent the time taken for new enzyme synthesis to occur. It is interesting in this regard that 4 hours is approximately the same length of time taken for the induction of new cytochrome P-450 more specific for benzo(a)pyrene hydroxylation by benzo(a)pyrene (King, 1982).
○ Yeast incubated aerobically with shaking in 100 mM phosphate buffer pH 7.0

● Yeast incubated aerobically with shaking in 100 mM phosphate buffer pH 7.0 containing 6% (v/v) ethanol.

Figure 4. The effect of ethanol on cytochrome P-450 degradation during the incubation of *S. cerevisiae* NCYC 754 resuspended in phosphate buffer.

Values are a mean of six determinations, bars indicate standard deviations.
<table>
<thead>
<tr>
<th>Solution</th>
<th>Half-life of cytochrome P-450 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control phosphate buffer alone</td>
<td>420</td>
</tr>
<tr>
<td>Methanol</td>
<td>130</td>
</tr>
<tr>
<td>Ethanol</td>
<td>510</td>
</tr>
<tr>
<td>Propanol</td>
<td>100</td>
</tr>
<tr>
<td>Butanol</td>
<td>20</td>
</tr>
<tr>
<td>Pentanol</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 4.4. Half-life of the cytochrome P-450 peak when whole yeast is incubated at 30°C in the presence of the first five members of the n-alkanol series.
4.3.10 \textbf{The Effect of n-Alkanols on Cytochrome P-450 Degradation}

Table 4.4 shows the half-life of the cytochrome P-450 peak when whole yeast is incubated at 30°C in the presence of the first five members of the alkanol series at 6\% (w/v) concentration. Following on from the previous experiment 4.3.9 it can be seen from Table 4.6 that there was less cytochrome P-450 degradation in ethanol than in the buffer control or any other \textit{n}-alkanol. Again suggesting either a protective effect or the induction of some new enzyme by ethanol. An increasing chain length of the alkanol accelerated degradation. This result reflects the lipid solubility of the alkanols; the more lipid-soluble the alkanol, the faster the degradation caused by its addition. The lipid solubility of alkanols also correlates well with many other effects on yeast such as glucose transport and fermentation (Leao and van Uden, 1985), ammonium transport (Leao and van Uden, 1983) and thermal death (Leao and van Uden, 1982). This suggests that the alkanols interfere with hydrophobic membrane regions, including the endoplasmic reticulum in which cytochrome P-450 is located. Ethanol stands out as remarkably non-destructive towards cytochrome P-450 at this concentration.

Recently it has been noted that in 5\% batch fermentations no cytochrome P-450 appears until 6-9 hours after inoculation (A. Dorr, personal communication), and it is possible that this late appearance may coincide with the presence of ethanol in sufficient concentration to induce the enzyme.

4.3.11 \textbf{Induction of Cytochrome P-450 in S. cerevisiae with 1\% Ethanol}

Although at higher concentrations ethanol may degrade cytochrome P-450 at low concentrations it may induce cytochrome P-450 accumulation. This would be analogous to the situation found with
Figure 4.15. The accumulation of cytochrome P-450 in *S. cerevisiae* NCYC 754 during growth in 0.5\% (w/v) glucose medium containing 1\% (v/v) ethanol.

In the absence of ethanol no cytochrome P-450 could be detected. Values are the mean of 6 determinations, bars indicate standard deviations.
oxygen (see chapter 5). To investigate this possibility experiments were performed with the addition of low concentrations of ethanol to yeast growing in low glucose growth media. Low glucose concentrations were employed because at high glucose concentrations ethanol is rapidly produced by fermentation making it difficult to maintain consistent concentrations of ethanol throughout growth.

Figure 4.15 shows the accumulation of cytochrome P-450 in \textit{S. cerevisiae} NCYC 754 during growth in 0.5\% (w/v) glucose medium containing 1\% (v/v) ethanol. In the absence of ethanol no cytochrome P-450 could be detected. The cytochrome P-450 peak occurs after 24 hours and is 0.22 nmol/g, wet weight of yeast, this is a very low value compared with the 9.8 nmol/g, wet weight which could be obtained in 20\% glucose growth medium but it represents a significant induction of cytochrome P-450 since in the absence of ethanol in 0.5\% glucose growth medium no cytochrome P-450 accumulation could be detected.

Although actually published about a year earlier, the work by Morita and Mifuchi (1984) and Del Carratore et al. (1984) has many similarities with the work presented here on ethanol enhancement and induction in the yeast \textit{S. cerevisiae} (also Blatiak et al., 1985b, 1987), Morita and Mifuchi cultured \textit{S. cerevisiae} D7 in 1\% Difco yeast extract, 2\% Difco protease peptone No. 3 with 2\% glucose liquid medium and supplemented with 1.5\% ethanol, (the work reported here was 0.5\% glucose growth media supplemented with 1\% ethanol for best ethanol induction results).

Morita and Mifuchi preincubated yeast cells and inoculated them in 4 l of liquid media at $5 \times 10^4$ cells/ml at 30\degree C (here 100 ml of media, inoculated with 2 loops from dry slopes was used). Morita and Mifuchi
found 1.5 nmol cytochrome P-450 per $10^9$ yeast cells after 16 hours incubation compared with the maximum of 0.23 nmol/g. wet weight of yeast, after 24 hours growth reported here.

The results are comparable because the number of yeast cells present in the author gram wet weight, approximate to $10^8$ (see chapter 2).

However Morita and Mifuchi did find 1.0 nmol/10$^9$ cells cytochrome P-450 without the ethanol supplement; the author did not find any cytochrome P-450 in 0.5% glucose growth media without the ethanol in agreement with King (1982). Hence Morita and Mifuchi report an entrancement effect whereas the author's is an induction effect. Unless of course, there is a small amount of cytochrome P-450 present during growth in 0.5% glucose medium not detectable spectrophotometrically, as would be needed to carry out its role in ergosterol biosynthesis. The reservation being that the work was limited by the sensitivity of the spectrophotometer and that if a more sensitive instrument had been employed cytochrome P-450 accumulation in 0.5% glucose growth media may be found. Morita and Mifuchi do not mention how long the yeast cells were preincubated or in what phase of growth the cells were at the moment of transfer to the incubation medium.

Morita and Mifuchi do not disclose the value of cytochrome P-450 at the inoculation point and this value is not apparent from the graph although it could be assumed to be zero, or close to zero.

Morita and Mifuchi found that if no glucose was added to the ethanol growth medium then less than 0.05 nmol/10$^9$ cells of cytochrome P-450 was found after 18 hours incubation. In work reported here it was found
that without glucose there was no cytochrome P-450 at any concentration of ethanol with supplemented growth media.

In agreement with Morita and Mifuchi no detectable cytochrome P-450 accumulation could be observed from yeast grown on ethanol as a sole carbon and energy source. Although in this work and in that of Morita and Mifuchi (1984) it was found that some yeast growth took place under these conditions.

Yeast growth was also found in other alkanols (see Table 4.5) methanol and pentanol with decreasing quantities as the alkanol series increased but in no instance could cytochrome P-450 accumulation be detected.

It has been found that ethanol increases the cytochrome P-450 content in higher plant tissues (D. Reichhart et al., 1979), in cultured hepatocytes (J.F. Sinclair et al., 1981), in rats (E. Rubin et al., 1971; J.-P. Villeneuve, 1976) and in hamsters (G.D. McCoy et al., 1979). The mechanism of ethanol enhancing or inducing cytochrome P-450 accumulation in yeast is difficult to interpret from the results. It is possible that at such low glucose concentration the ethanol provides a better viscosity and thus a more suitable oxygen concentration for cytochrome P-450 since ethanol decreases the oxygen concentration in the medium. In the flask there may be a high initial oxygen concentration which the ethanol decreases and hence favours cytochrome P-450 accumulation. In all the cytochrome P-450 assays before 12 hours only very low values were obtained, perhaps some oxygen utilization by the growing yeast may be required to remove the higher oxygen concentration before cytochrome P-450 can accumulate.
<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Dry weight in mg per 100 ml of media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>162</td>
</tr>
<tr>
<td>Ethanol</td>
<td>240</td>
</tr>
<tr>
<td>Propanol</td>
<td>93</td>
</tr>
<tr>
<td>Pentanol</td>
<td>0</td>
</tr>
<tr>
<td>Hexanol</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>470</td>
</tr>
</tbody>
</table>

(growth in 20% glucose growth media)

Table 4.3: The effect of 3% alkanol (in place of glucose) with growth media on *S. cerevisiae* NCYC 240 growth.

Yeast harvested after 42 hours

Values are a mean of 2 determinations.
Del Carratore et al. also cultured *S. cerevisiae* by cells as did Morita and Mifuchi but the former employed a different medium using 1% yeast extract, 2% bacto peptone and varying concentrations of glucose. The use of bactopeptone is interesting, the author has found higher cytochrome P-450 accumulation with mycological peptone rather than bacto-peptone and the absence of salt is noteworthy from previous finding a small addition of salt was found to increase cytochrome P-450 accumulation. Del Carratore et al. do not mention whether the flask sizes used or the level of agitation, if any; this is very important as the level of agitation will greatly affect cytochrome P-450 accumulation (Blatiak et al., 1985a, 1987). Del Carratore et al. inoculated with 150-300 cells on 100 ml of liquid medium, this is lower than the inoculation levels used by the author. Very low inoculation levels give poor growth and lower levels of cytochrome P-450 accumulation (see chapter 6).

Del Carratore et al. found that when yeast was grown on medium containing only ethanol as the carbon source no cytochrome P-40 was found in the cells. This agreed with Morita and Mifuchi (1984) and Karenlampi et al. (1981) and the author.

Using 0.5% glucose growth media and supplementing with 2.0% (v/v) ethanol Del Carratore found 7 pmol of cytochrome P-450 from $10^8$ cells (although the time after inoculation at which the cytochrome P-450 assays were performed was not mentioned). Without the 2.0% ethanol supplement there was no cytochrome P-450 detected. The result agrees with the author's findings on ethanol induction in yeast cells but the levels of cytochrome P-450 were much lower and Del Carratore et al. used 2% ethanol supplement whereas the author used 1% ethanol.
The highest cytochrome P-450 value Del Carratore et al. found was 0.02 nmol/10^8 cells using 20% glucose growth media, compared with over 9 nmol/g. wet weight as found by the author (number of yeast present in 1 gram wet weight was 2 x 10^8). Thus Del Carratore et al. were investigating a different magnitude of yeast cytochrome P-450 levels.

It is interesting to compare the cytochrome P-450 values of Del Carratore with those of Morita and Mifuchi, both were culturing S. cerevisiae strain D7 but Morita and Mifuchi obtained much higher values with ethanol, 1.5 nmol/10^9 cells as compared with 7 pmol/10^8 cells obtained by Del Carratore et al., admittedly under different conditions but using similar media.

It is not known why there should be a large accumulation of cytochrome P-450 in yeast during rapid fermentation growth on high glucose media especially when sufficient cytochrome P-450 for lanosterol-14α-demethylation can be made even aerobically in low glucose media (Aoyama et al., 1981). The increased amount of the enzyme in high glucose may be related to the level of ethanol produced. In mammalian systems ethanol is known to induce a specific form of cytochrome P-450 which has a very high activity towards the oxidation of ethanol to acetaldehyde (Coon et al., 1984). It is possible that a similar system is present in S. cerevisiae. Several forms of cytochrome P-450 are thought to exist in S. cerevisiae (King et al., 1984) and their regulation is complex involving not only oxygen (Blatiak et al., 1985a) and glucose but also ethanol (Blatiak et al., 1985b). The increased exposure of yeast cells to ethanol may require an adapted composition of cellular membranes. This may involve an increased incorporation of ergosterol which may help to explain the high cytochrome P-450 content of cells.
subjected to strongly fermentative growth conditions (Kappeli, 1986). Investigations by the author (Blatiak et al., 1985 and Blatiak et al., 1987), Morita and Mifuchi (1984) and Del Carratore (1984) support this hypothesis. However, degradation of the enzyme cytochrome P-450 occurs in the presence of high concentrations of ethanol during the yeast growth stationary phase by which time, perhaps, the yeast is no longer capable of adapting its composition of cellular membranes. There are also many yeasts which seem not to have any cytochrome P-450 in them; it would be of interest to determine how they adapt their cellular membrane composition.
CHAPTER 5

EFFECTS OF OXYGEN ON CYTOCHROME P-450

ACCUMULATION AND DEGRADATION
INTRODUCTION

The pollution of the early atmosphere of the earth caused by the appearance of oxygen due to the photosynthetic activity of the blue-green algae created a serious survival problem for the life forces then in existence. Many fermentative anaerobes may have disappeared, victims of this dangerous corrosive gas.

The adaptive changes of the successful life forces were chiefly the elaboration of the respiratory chain enzymes, peroxidases and catalase, though superoxide dismutase may also have been important in controlling oxygen toxicity. Cytochrome P-450 also interacts with molecular oxygen, acting as an oxygenase enzyme.

Cytochrome P-450 may also act as a microsomal peroxidase (Hrycay et al., 1972). Molecular oxygen is itself damaging to living matter e.g. enzymes labile to aerobic conditions, autoxidation of lipids and oxidation of free sulphydryl groups of proteins (Wickramsinghe, 1973).

There are many electrons present in the cytoplasm of all cells. The electron reservoir in the form of cytochromes ensures reduction of the peroxo complex to water (see Figure 5.1). However, since the reduction of dioxygen probably proceeds in every instance by a series of one electron transfer reactions (see Figure 5.1) and unless the intermediate reduction products are retained within the active site of an enzyme or coordinated to a metal complex there is every likelihood that most oxidation reactions will generate superoxide as the initial reduction product.

The reduction products of dioxygen are all food oxidants. In aqueous solution they form hydrogen peroxide and water quite rapidly but with
Figure 5.1. The relationship between dioxygen and its reduction products.

(from C. Greenwood et al. 1982)
reduced metal ions give hydroxyl radicals which oxidise superoxide to dioxygen and hydrogen peroxide to superoxide. The properties of all the reduction products are influenced by solvent, pH and metal ions.

If toxicity is equated with reactivity then the hydroxyl radical, or the metal complexes of its conjugate base i.e. \( H^+ + O^- \rightarrow HO^+ \) pose the greatest threat. So superoxide, hydrogen peroxide or even dioxygen may be precursors to the toxins. Cytochrome P-450 may couple with superoxide dismutase and catalase to convert excess \( O_2 \) to \( H_2O \).

Oxygen is important in the accumulation of cytochrome P-450 in *S. cerevisiae*. Lindenmayer and Smith (1964) found cytochrome P-450 when *S. cerevisiae* was grown under anaerobic conditions in 4% (w/v) glucose growth media. Less of the enzyme was found when the yeast was grown aerobically. *S. cerevisiae* produces cytochrome P-450 only under fermentative conditions such as when grown at high glucose concentration or under semi-anaerobic conditions (Wiseman, 1980). Under these conditions repression of mitochondrial cytochromes is achieved and the intracellular concentration of cyclic AMP is low. The production of yeast cytochrome P-450 is thought to be regulated by the level of intracellular cyclic AMP by repression, thus when the cyclic AMP is low, accumulation of cytochrome P-450 can occur (Wiseman et al., 1978).

Several groups of workers have previously used semi-anaerobic conditions to obtain cytochrome P-450 in *S. cerevisiae*. Ishidate et al. (1969) reported that high levels of cytochrome P-450 were produced in yeast grown semi-anaerobically in 4% (w/v) D-glucose medium. Yoshida et al. (1974) have used semi-anaerobic conditions with 6% (w/v)
D-glucose media and 3% (w/v) D-glucose media (1984). Rogers and Stewart (1973) found that when S. cerevisiae was grown on a medium containing 4% (w/v) D-galactose as sole carbon and energy source cytochrome P-450 production was optimum at a dissolved oxygen concentration of 0.25-0.5 µmol.l⁻¹. Trinett et al. (1982) working with S. cerevisiae in continuous culture in a medium containing 3% (w/v) D-glucose, found that a low aeration rate was required for the production of high cytochrome P-450 values. Thus relatively low dissolved oxygen concentrations are required for optimum cytochrome P-450 production but under strictly anaerobic conditions. Rogers and Stewart (1973) found no detectable cytochrome P-450. The optimum dissolved oxygen level (0.25-0.5 µmol.l⁻¹, Rogers and Stewart, 1973) is well below the sensitivity level of most commercially available dissolved oxygen sensors.

Aoyama et al. (1981) have shown that S. cerevisiae grown aerobically in 1% D-glucose media contains a very low level of cytochrome P-450, not detectable spectrophotometrically, but capable of catalysing lanosterol 14α-demethylation. This was subject to inhibition by antibodies to purified yeast cytochrome P-450 from yeast grown under fermentative conditions. When Rogers and Stewart (1973) grew S. cerevisiae strictly anaerobically, they could detect no cytochrome P-450 so this suggests a requirement for O₂-induction (Blatiak et al., 1985). In this chapter the role of oxygen in relation to agitation in the accumulation of cytochrome P-450 in S. cerevisiae is reported.
5.2 MATERIALS AND METHODS

5.2.1 Semi-anaerobic Growth Experiments

Saccharomyces cerevisiae strains NCYC 754, 240 were used throughout the experiments discussed in this chapter. Yeast was grown from wire loop inoculations at 30°C in a Galenkamp orbital incubator in 100 ml media (the composition of which has been described in section 2.2). For semi-anaerobic experiments flasks were bubbled through with oxygen-free nitrogen for 10 minutes and sealed under nitrogen with alternating layers of parafilm and aluminium foil each kept in place with polyvinyl tape. Cytochrome P-450 was determined as described in chapter 2. For oxygen determinations a battery powered Griffin DOS-240-M oxygen meter was used.

5.2.2 Protective Agents against Degradation

In the experiments on degradation of cytochrome P-450 in whole yeast in 0.1 M phosphate buffer and protection by various agents as described in the text, the yeast *S. cerevisiae* NCYC 240 was grown as described above. After 42 hours the de-adaptation studies were performed at 30°C by aerobic or anaerobic (as described) shaking incubation in 0.1 M phosphate buffer, pH 7.0 alone, or in the presence of the additives. Samples were taken at 2, 4, 6 and 8 hours for spectrophotometric assay of cytochrome P-450.

5.3 RESULTS AND DISCUSSION

5.3.1 The Effect of Semi-anaerobic Growth Conditions on Cytochrome P-450

Accumulation and Degradation

The levels of cytochrome P-450 produced under semi-anaerobic and aerobic conditions were studied at glucose concentrations of 0.1%, 0.5%, 1%, 5%, 20% (w/v). Very low oxygen levels are difficult to achieve,
Figure 52. The decrease in percentage saturation of oxygen with time of bubbling nitrogen into 20% glucose growth medium and phosphate buffer pH 7.0. at 21°C.
Cytochrome P-450
(n mol/g wet weight of yeast)

Figure 5.3.
Cytochrome P-450 accumulation in *S. cerevisiae* grown semi-anaerobically in 1% glucose growth media.
Values are the mean of eight determinations.
NCYC 240 grown at 30°C in shaking water bath.
Cytochrome P-450 (nmol/g wet weight of yeast)

**Figure 5.4.**
Cytochrome P-450 accumulation in *S. cerevisiae* grown aerobically and semi-anaerobically in 5% glucose growth media. Values are the mean of eight determinations. NCYC 240 grown at 30°C in shaking water bath.
Figure 5.2 shows the length of time nitrogen was required to bubble through buffer pH 7.0 and 20% glucose growth media to achieve very low oxygen percentage saturation. Lower oxygen levels were not possible presumably because oxygen is bound, in the media, to various molecules. Thus semi-anaerobic conditions were used because the conditions with other workers (Ishidate et al., 1969; Yoshida et al., 1974). Rogers and Stewart (1973) have reported that under strictly anaerobic conditions there is no cytochrome P-450 accumulation. This may be because oxygen is required for several biosynthetic reactions during yeast growth. At glucose concentrations of 0.1% and 0.5% no cytochrome P-450 was produced, irrespective of aerobic or semi-anaerobic growth conditions.

Figure 5.3 shows the accumulation of cytochrome P-450 in 1% glucose growth media grown semi-anaerobically, a maximum of 2.5 nmol/g wet weight was found after 62 hours. Under aerobic conditions cytochrome P-450 was not found at the time points employed, which is not in agreement with Wiseman et al. (1975, 1976) who have reported a rapid accumulation of the enzyme under these conditions reaching a maximum after 20 hours, which was equally rapidly lost. However, the first time point in Figure 5.3 is at 24 hours thus it is possible that some cytochrome P-450 may have accumulated and been lost before that first 24 hour time point.

Figure 5.4 shows the accumulation of cytochrome P-450 in S. cerevisiae grown in 5% glucose growth media. A maximum enzyme accumulation of only 1.2 nmol/g. wet weight after 24 hours under aerobic conditions can be seen from Figure 5.3. Wiseman et al. (1976) reported that yeast grown in 5% glucose growth media accumulated at a concentration of
Cytochrome P-450 accumulation in *S. cerevisiae* grown semi-anaerobically and aerobically in 20% glucose growth media. Values are the mean of eight determinations. NCYC 240 grown at 30°C in shaking water bath.
3.5 nmol/g. wet weight of yeast after 32 hours. The difference in results may be because of the different age of slopes used in the separate experiments (see chapter 6), or that a higher growth rate resulted in Figure 5.4 in which case the cytochrome P-450 would accumulate to a maximum value earlier, since the maximum cytochrome P-450 accumulation has always been found to occur at the end of the exponential phase of growth (see chapter 5 and Blatias et al., 1985b).

Under semi-anaerobic conditions in 5% glucose growth media yeast is slower, the lower oxygen concentration may be inhibiting the synthesis of relevant metabolites or macromolecules; the maximum cytochrome P-450 accumulation is higher at 1.4 nmol/g. wet weight of yeast and is reached after 48 hours. From Figure 5.4 it can be seen that the degradation of cytochrome P-450 takes place at a faster rate aerobically, 0.057 nmol/g. wet weight of yeast/hr. being lost, than semi-anaerobically which loses 0.016 nmol/g. wet weight of yeast/hr. So aerobically the enzyme is lost at a rate more than three times faster than semi-anaerobically. This may reflect the oxygen requirement for cytochrome P-450 degradation in yeast (Blatias et al., 1980; Blatias et al., 1985a; Blatias et al., 1987).

It is of interest that there was greater cytochrome P-450 accumulation with 1% glucose growth media under semi-anaerobic conditions than with 5% glucose growth media. It is possible that the age of the slope may be critical in enzyme accumulation values (see chapter 6).

Figure 5.5 shows the time course of cytochrome P-450 accumulation in yeast grown aerobically and semi-anaerobically in 20% glucose growth media. There is little difference in the maximum cytochrome P-450
Cytochrome P-450

(n mol/g wet weight of yeast)

![Graph showing Cytochrome P-450 production over time](image)

- ■ aerobically growing yeast
- • semi-anaerobically growing yeast after 24 hours
- ▲ semi-anaerobically growing yeast after 48 hours

**Figure 5.6.**
Cytochrome P-450 production in aerobically growing yeast made anaerobic after 24 or 48 hours and growth continued. Values are the mean of six determinations.

NCYC 240 grown at 30°C in shaking water bath.
values about 3 nmol/g. wet weight of yeast, achieved at about the same 40 hour time point.

The above data suggests that maximum cytochrome P-450 accumulation occurs under conditions of repression of the mitochondrial cytochromes either by high glucose concentration or by semi-anaerobic conditions. Figure 5.5 indicates that the two aforementioned mitochondrial repressive conditions are not additive suggesting that cytochrome P-450 accumulation occurs via a similar mechanism (see chapter 2).

5.3.2 The Effect on Cytochrome P-450 Accumulation in Aerobically Growing Yeast made Semi-Anaerobic after 24 or 48 hours

Figure 5.6 shows the change in cytochrome P-450 accumulation in aerobically growing cultures of \textit{S. cerevisiae}, when made semi-anaerobic during the exponential growth phase (24 hours growth) and during the stationary phase (48 hours growth). When a yeast culture growing exponentially is made anaerobic the biosynthesis of cytochrome P-450 is inhibited, with an increase in cytochrome P-450 level between 24 and 48 hours, of less than 10% of that in the aerobic control. This effect is not due to an altered growth rate as very similar growth rates were observed under both sets of conditions with sufficient glucose still present (at 24 hours) to ensure actively fermenting yeast cells under glucose repression, as required for optimal cytochrome P-450 biosynthesis (Wiseman \textit{et al.}, 1978).

One effect of oxygen may be in acting as a substrate inducer of yeast cytochrome P-450. This may also explain why cytochrome P-450 cannot be detected in yeast grown under strictly anaerobic conditions (Rogers and Stewart, 1973).
Trinn et al. (1982) working with yeast in continuous culture have shown that without glucose repression it is not possible to achieve cytochrome P-450 production by oxygen limitation. Lim (1976) has demonstrated that in steady state continuous culture of *S. cerevisiae* cytochrome P-450 production is optimal at dissolved oxygen concentration between 16-30% of saturation in a medium containing 20% glucose (dilution rate 0.1).

Oxygen solubility decreases exponentially with increasing concentration of dissolved substances due to the restriction of free water volume by the specific partial volume of the dissolved substances (Zander, 1976).

If \( \alpha \) = oxygen solubility coefficient

\[ \alpha_0 = \text{oxygen solubility coefficient in distilled water} \]

then \( \alpha = \alpha_0 e^{-kc} \)

where \( c \) = concentration of dissolved substances (g/100 ml)

\[ \alpha_0 = 0.0241 \text{ at } 37^\circ C \]

\[ k = \text{constant which for glucose } = 0.01562 \]

\( \alpha \) is the "Bunsen coefficient" being the volume of gas at 273°K and 1 atmosphere absorbed by unit volume of solvent under a partial pressure of 1 atmosphere.

\[
\beta = \frac{\text{Volume of gas}}{\text{Volume liquid}} \times \frac{273.15}{T^0K}
\]

<table>
<thead>
<tr>
<th>Glucose concentration % wt. vol.</th>
<th>( \alpha )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0241</td>
</tr>
<tr>
<td>2</td>
<td>0.0230</td>
</tr>
<tr>
<td>10</td>
<td>0.0205</td>
</tr>
<tr>
<td>20</td>
<td>0.0178</td>
</tr>
</tbody>
</table>

The above is by volume but for pure oxygen the decrease in solubility would be,
However, when considered from the viewpoint of falling glucose concentrations the oxygen solubility changes are even more marked.

<table>
<thead>
<tr>
<th>Glucose concentration</th>
<th>Δα</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5%</td>
</tr>
<tr>
<td>2</td>
<td>15%</td>
</tr>
<tr>
<td>10</td>
<td>15%</td>
</tr>
<tr>
<td>20</td>
<td>26%</td>
</tr>
</tbody>
</table>

The oxygen solubility may therefore be crucial in determining the level of cytochrome P-450 accumulation especially if oxygen is a substrate inducer.

The endogenous role of cytochrome P-450 in *S. cerevisiae* is thought to be in the 14α-demethylation of lanosterol, which is the first step in the biosynthetic pathway of ergosterol, the major sterol in yeast membranes (Yoshida and Aoyama, 1980). When yeast is grown under strictly anaerobic conditions ergosterol needs to be added to the growth medium as a supplement because oxygen is required for ergosterol biosynthesis. Therefore during growth under these conditions cytochrome P-450 is not required for sterol synthesis and does not appear to be produced (Rogers and Stewart, 1973). When oxygen is introduced in small quantities cytochrome P-450 is produced, presumably for use in sterol biosynthesis. So, oxygen may be acting as a substrate inducer of cytochrome P-450 in this system (Blatiak et al., 1983).
5.3.3 The Effect of Culture Shake Speed on Cytochrome P-450 Accumulation in *S. cerevisiae*

Figures 5.7 to 5.14 show the time courses at different revolutions per minute in the Gallenkamp orbital shaker and indicate the accumulation of cytochrome P-450, the wet weight and the dry weight with standard deviations at each time point taken. Dry weight units were converted to grammes per litre.

At 0 RPM (Figure 5.7) the wet weight peaked after only 25 hours and remained at the same level until 160 hours. The cytochrome P-450 maximum was 1.2 nmol/g wet weight, quite a high value especially as it peaks from 48 to 95 hours. It is the highest value determined at this time point, even at 120 RPM (Figure 5.10) the cytochrome P-450 value is approaching zero value.

At 80 RPM (Figure 5.8) the cytochrome P-450 peaks at 40 hours but the dry and wet weight at 48 hours. At 100 RPM (Figure 5.9) the maximum accumulation of cytochrome P-450, wet and dry weight all peak at the same time of 40 hours.

At 120 RPM (Figure 5.10) there is an interesting divergence of gradients at 48 hours when the dry weight continues to rise and yet the wet weight falls being also the time point at which cytochrome P-450 peaks.

At 140 RPM (Figure 5.11) the wet and dry weight peak at 48 hours whilst the cytochrome P-450 peaks after 40 hours. At 180 RPM (Figure 5.12) the values all peak at 48 hours. At 220 RPM (Figure 5.13) there is a greater time difference between peaks, cytochrome P-450 being highest in value after 24 hours, wet and dry weights peaking after 48 hours. At
Cytochrome P-450 n.mol/g wet wt.
Wet weight (g.) per 50ml.

Dry wt. (mg.)

Figure 5.7. Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 0 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Figure 5.8 Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with *S. cerevisiae* NCYC 754 at 30°C in 20% glucose growth medium at 80 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Figure 59. Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 100 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Figure 5.1a Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 120 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Cytochrome P-450 n.mol/g. wet weight of yeast.

Wet wt. (g.)/50 ml.

Dry wt. (mg.) per 50 ml.

Time (hours)

- - Cytochrome P-450 n.mol/g. wet weight of yeast.
- - Dry weight of yeast mg. per 50 ml. of medium.
- - Wet weight of yeast g. per 50 ml. of medium.

Figure 5.1. Time course of the wet weight, dry weight, and accumulation of cytochrome P-450 with S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 140 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Cytochrome P-450 n.mol/g. wet wt.
Wet wt.(g.) per 50 ml.
Dry wt.(mg.) per 50 ml.

Time (hours)

Figure 5.12 Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S.cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 180 RPM. Values are the mean of six determinations, bars indicate standard deviation.
Cytochrome P-450 n.mol/g. wet wt.
Wet wt.(g.) per 50 ml.

Dry wt.(mg.) per 50 ml.

Figure 5.13 Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S.cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 220 RPM.
Values are the mean of six determinations, bars indicate standard deviation.
Figure 5.4. Time course of the wet weight, dry weight and accumulation of cytochrome P-450 with S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 260 RPM. Values are the mean of six determinations, bars indicate standard deviation.
260 RPM (Figure 5.14) there is less cytochrome P-450 accumulation than at 220 RPM.

From Figures 5.7 to 5.14 it can be seen that *S. cerevisiae* grown in 20% glucose growth medium produce cytochrome P-450 rapidly during the exponential growth phase, up to a maximum level at about the end of the exponential phase and the beginning of the stationary phase. The accumulation of cytochrome P-450 in *S. cerevisiae* is strongly associated with growth here, the enzyme level declining when rapid growth ends.

Figure 5.15 shows the effect of growing *S. cerevisiae* in batch culture, at a series of shake speeds in an orbital shaker in 20% glucose growth media, on the accumulation of cytochrome P-450 and the growth of yeast. Dry weight units were converted to grammes per litre. A clear optimum in cytochrome P-450 is observed at 120 RPM, with the enzyme level being lower at both faster and slower speeds. The maximum yield of cytochrome P-450 occurs at the same shake speed as the maximum growth rate and therefore the maximum yield of yeast biomass.

Agitation is known to increase the homogeneity in a system and enhances the transfer of solutes between biomass and the bulk medium and between the liquid medium and the gas phase with which it is in contact. If this improves the availability of nutrients and the disposal of metabolites in the microbial microenvironment. Generally transfer rates increase as the intensity of agitation increases, with the effect being most pronounced in the case of sparingly soluble gaseous solutes. Their concentration in the bulk medium is the result of dynamic balance between the rate of their dissolution or excretion into the medium and the rate of their uptake or desorption from it.
Figure 5.15. The determining effect of culture shake speed on the peaking of *S. cerevisiae* NCYC 754 growth rate, cytochrome P-450 and dry weight.

Cytochrome P-450 levels are the peak levels measured at the end of the exponential growth phase in each case. Values are the means of six determinations, bars indicate standard deviations.
<table>
<thead>
<tr>
<th>RPM</th>
<th>Max. wet wt./50ml.</th>
<th>Max. dry wt./50ml.</th>
<th>Max.c.P-450</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g.</td>
<td>Time (h)</td>
<td>g.</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>165</td>
<td>0.13</td>
</tr>
<tr>
<td>80</td>
<td>2.5</td>
<td>48</td>
<td>0.37</td>
</tr>
<tr>
<td>100</td>
<td>2.6</td>
<td>31</td>
<td>0.54</td>
</tr>
<tr>
<td>120</td>
<td>3.2</td>
<td>40</td>
<td>0.66</td>
</tr>
<tr>
<td>140</td>
<td>3.8</td>
<td>48</td>
<td>0.62</td>
</tr>
<tr>
<td>180</td>
<td>2.8</td>
<td>48</td>
<td>0.49</td>
</tr>
<tr>
<td>220</td>
<td>2.7</td>
<td>48</td>
<td>0.42</td>
</tr>
<tr>
<td>260</td>
<td>2.4</td>
<td>40</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**TABLE 5.1. SUMMARY OF THE TIME POINTS AT WHICH MAXIMUM DRY WEIGHT, WET WEIGHT AND ACCUMULATION OF CYTOCHROME P-450 OCCURRED.**
Agitation increases the dissolution rate of a sparingly soluble gas such as oxygen by replacing saturated liquid elements at the gas-liquid interface with elements from the bulk liquid. This maintains a high activity driving force for gas dissolution between the gas and liquid phases. The rate of interface element renewal increases as the intensity of agitation increases. A similar mechanism would describe the desorption of gaseous metabolites.

The appearance of an optimum agitation intensity would suggest that there is a corresponding optimum solute concentration and the most critical solute may well be oxygen, although other factors, such as homogeneity and viscosity may be relevant, the peak in cytochrome P-450 level may be dependent on the degree of aeration of the culture, with an optimum aeration level being required to produce the maximum cytochrome P-450 value.

Table 5.1 summarizes the time points at which maximum dry weight, wet weight and accumulation of cytochrome P-450 occurred. From Table 5.1 it can be noted that the time taken for the maximum cytochrome P-450 accumulation and maximum dry weight was not always the same at any single shake speed. From the data presented the maximum dry weight occurs after the maximum cytochrome P-450 value except at RPM 120 and 180 when they coincide. It is not known why this should be so, possibly at the agitation speed of 120 RPM and 180 the aeration level allows for the concurrent growth of yeast and enzyme production. The times at which maximum dry weight occurred decrease from 95 to 40 hours with increase in RPM from 0-260. Although most of the maximum values (from 80 to 220) are after 48 hours. Perhaps the availability of oxygen influences not only the quantity of yeast produced but also the time taken to reach its maximum value.
Figure 5.16. Time course of the changes in the percentage dry weight of the wet weight in *S. cerevisiae* NCYC 754 at 0 RPM and 80 RPM. Grown at 30°C in orbital shaker. Values are the mean of six determinations, bars indicate standard deviations.
Figure 5.17. Time course of the changes in the percentage dry weight of the wet weight in *S. cerevisiae* NCYC 754 at 100 RPM and 120 RPM. Grown at 30°C in orbital shaker. Values are the mean of six determinations, bars indicate standard deviations.
Figure 5.18. Time course of the changes in the percentage dry weight of the wet weight in S. cerevisiae NCYC 754 at 140 RPM and 180 RPM. Grown at 30°C in orbital shaker. Values are the mean of six determinations, bars indicate standard deviations.
Figure 5.17. Time course of the changes in the percentage dry weight of the wet weight in S. cerevisiae NCYC 754 at 220 RPM and 260 RPM. Grown at 30°C in orbital shaker. Values are the mean of six determinations, bars indicate standard deviations.
Figure 5.2. The determining effect of culture shake speed on the maximum percentage dry weight/wet weight in *S. cerevisiae* NCYC 754. Grown at 30°C. Values are the mean of six determinations. Bars indicate standard deviations.
The peak times for wet weight match the peak dry weights quite well although there are exceptions at 0, 100 and 120 RPM. Between shake speeds 0-180 the maximum cytochrome P-450 occurred between 40-48 hours but at the two higher shake speeds the maximum enzyme value occurred after only 24 hours. It is possible that the increased availability of oxygen at the higher shake speeds inhibited the accumulation of cytochrome P-450 (Rogers and Stewart, 1973).

Figures 5.16 to 5.19 show the variation in percentage dry weight to wet weight at different shake speeds.

Figure 5.16a (top) shows at 0 RPM how small (less than 3%) the dry weight is as a percentage of the wet weight after 25 hours, reaching a maximum of only 13% after 96 hours.

Figure 5.16b (lower) shows how at 80 RPM there is a substantial increase in the percentage dry weight of wet weight after 24 hours, the amount being 8%. It is not known why after 65 hours there is an increase in the percentage figure which does not appear again at 100 RPM (Figure 5.17a).

Figure 5.18 shows how at 120 RPM the percentage figures remain high after 48 hours until 96 hours which has the last time point in the experiment. Figures 5.18 and 5.19, showing time courses for 140 to 260 RPM are similar except, as Figure 5.19b shows, the maximum percentage value reached at 260 RPM was the highest since 120 RPM but since the error bars overlap this is not significant. Figure 5.20 summarises the effects.
5.3.4 The Effect of Culture Shake Speed on Degradation of Cytochrome P-450

Figure 5.2 summarises the effect at shake speed on degradation of cytochrome P-450. It can be seen that at 120 RPM the greatest rate of degradation occurs and that at 0 RPM the least degradation happens.

Table 5.2 emphasises the difference in the rate of degradation at 120 RPM - it takes 48 hours for 9.87 nmol/g.wet weight cytochrome P-450 to be degraded to zero at a degradation rate of 0.21 nmol/g.wet weight/hr (Data taken from graphical extrapolation).

At 0 RPM the time of degradation of only 1.2 nmol/g.wet weight is 142 hours, this is a slower degradation rate of 0.008 nmol/g.wet weight/hr which is 26 times slower.

Possibly the greater oxygen presence at 120 RPM assists degradation, it has been reported that anaerobic conditions protect against degradation of cytochrome P-450 (Blatiak et al., 1980). The level of ethanol is not known at 0 RPM or the amount of glucose, which may also have a protective effect against cytochrome P-450 degradation (Blatiak et al., 1980). Similar peak values of cytochrome P-450 occur at 80 RPM (4.29 nmol/g.wet weight) and 180 RPM (4.82 nmol/g.wet weight) and it can be seen from the graph that the times of degradation to zero value are markedly different. At 80 RPM the time is 60 hours and at 180 RPM only 32 hours, almost half the time. So it is feasible that the higher rate of agitation which provides a greater dissolved oxygen tension may be influencing the rate of degradation. It has been noted that before that oxygen may aid the degradation of cytochrome P-450 (Blatiak et al., 1980).
Figure 5.21. The determining effect of culture shake speed on the degradation of cytochrome P-450 in S. cerevisiae NCYC 754. Grown at 30°C. Values are the mean of six determinations.
### Table 5.1: The Effect of Shake Speed on the Rate of Degradation of Cytochrome P-450 in *S. cerevisiae*

<table>
<thead>
<tr>
<th>RPM</th>
<th>Max. Cyt. P-450 n.mol/g. wet wt.</th>
<th>Cyt.P-450 peak at (h)</th>
<th>Degradation time from peak (h)</th>
<th>Rate of degradation n.mol/g.wet wt./h.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.20</td>
<td>48</td>
<td>142</td>
<td>0.008</td>
</tr>
<tr>
<td>80</td>
<td>4.29</td>
<td>40</td>
<td>60</td>
<td>0.071</td>
</tr>
<tr>
<td>100</td>
<td>8.19</td>
<td>40</td>
<td>46</td>
<td>0.178</td>
</tr>
<tr>
<td>120</td>
<td>9.87</td>
<td>48</td>
<td>48</td>
<td>0.210</td>
</tr>
<tr>
<td>140</td>
<td>7.43</td>
<td>40</td>
<td>46</td>
<td>0.160</td>
</tr>
<tr>
<td>180</td>
<td>4.82</td>
<td>48</td>
<td>32</td>
<td>0.151</td>
</tr>
<tr>
<td>220</td>
<td>2.62</td>
<td>24</td>
<td>42</td>
<td>0.062</td>
</tr>
<tr>
<td>260</td>
<td>2.00</td>
<td>24</td>
<td>41</td>
<td>0.048</td>
</tr>
</tbody>
</table>
Dry wt. mg per 50 ml. media

Dry weight in mg. per 50 ml. of media at 120 RPM.

Dry weight in mg. per 50 ml. of media at 260 RPM

Figure 5.22 Time course of the dry weight of *S. cerevisiae* NCYC 754 at 30°C in 20% glucose growth medium at 120 RPM and 260 RPM. Values are the mean of six determinations.
Figure 2: S. cerevisiae NYC 254 at 150 and 250 RPM. Growth at 30°C. Values are a mean of four determinations ± standard deviations.
Figure 5.2.2c Time course of the accumulation of cytochrome P-450 n.mol/g.wet weight in S. cerevisiae NCYC 754 at 30°C in 20% glucose growth medium at 120 RPM and 260 RPM. Values are the mean of six determinations, bars indicate standard deviations.
5.3.5 The Effect of Culture Shake Speed on Glucose and Ethanol Content in the Media

Figure 5.22 shows the decline in glucose values at 120 RPM and 260 RPM of \textit{S. cerevisiae} NCYC 754 at 30°C in 20% glucose growth medium. From Figure 5.22 it can be seen that at 120 RPM the percentage glucose declines at a faster rate than at 260 RPM.

The differences in the values of percentage glucose become significant after 40 hours, prior to this there is some overlap in the standard deviations.

After 60 hours the rate of decline of the percentage glucose slows down at both 120 and 260 RPM. At 60 hours at 260 RPM there is about 7% glucose remaining, at 120 RPM about 4% glucose remains.

Also, Figure 5.22b shows the production of ethanol at 120 and 260 RPM in \textit{S. cerevisiae} NCYC 754. It can be seen from Figure 5.22b that there is no significant difference, at any time, between the various shake speeds in the production of ethanol by the yeast used. The maximum ethanol value obtained at 96 hours were about 9% at both 120 and 260 RPM.

Figure 5.22c shows time courses of the accumulation of cytochrome P-450 in \textit{S. cerevisiae} NCYC 754 at 30°C in 20% glucose growth media at 120 RPM and 260 RPM. If Figures 5.22b and 5.22c are overlaid it can be seen that at the height of cytochrome P-450 accumulation at 120 RPM (which is after 48 hours) the ethanol value is 6% and the glucose value is 8%. This is similar to previous findings (Figure 4.3.2). The effect of additional agitation as shown by the results at 260 RPM from Figures 5.22b and 5.22c, at the height of cytochrome P-450
accumulation (being after 40 hours) the ethanol value is 5% and the glucose value approaches 10%.

From Figures 5.22b and 5.22c it can be seen that the difference in oxygen levels affects not only the accumulation of cytochrome P-450 in yeast but also the rate at which glucose is used in the media, this is as expected from the variation seen in yeast growth rate (Figure 5.15).

Figure 5.22a shows the time course of the dry weight of *S. cerevisiae* NCYC 754 at 30°C in 20% glucose growth medium at 120 RPM and 260 RPM. The standard deviations have been omitted for the sake of clarity. Again, the accumulation of cytochrome P-450 can be seen to be growth related. When growth ceases then so does the accumulation of cytochrome P-450, the rate of glucose then also being in decline and the production of ethanol increasing both at 120 and 260 RPM.

### 5.3.6 Degradation of Cytochrome P-450 in Non-Growing *S. cerevisiae* using Anaerobiosis, Chloramphenicol, 2,4-Dinitrophenol and Cycloheximide as Protective Agents

Figure 5.23 shows the degradation of cytochrome P-450 in non-growing *S. cerevisiae* NCYC 240 using anaerobiosis, chloramphenicol, 2,4-dinitrophenol and cycloheximide as protective agents. It can be seen that the enzyme in 0.1 M phosphate buffer alone has the greatest amount of degradation, there being only 2% of the original concentration left after 8 hours. When the enzyme is in 20% glucose with 0.1 M phosphate buffer the degradation is more gradual, indeed after 8 hours 85% of the original concentration of enzyme still remains (the cytochrome P-450 maximum accumulation was 2.4 nmol/g wet weight of yeast). Yet when the enzyme is in 20% glucose with phosphate buffer under aerobic
Percentage survival of cytochrome P-450

Time, in hours, of incubation at 30°C in shaking water bath.

- ▲ 0.1 M-phosphate buffer + 20% glucose under anaerobic conditions.
- ✗ 0.1 M-phosphate buffer + 20% glucose + 1mM cycloheximide.
- ○ 0.1 M-phosphate buffer + 20% glucose + 1mM chloramphenicol.
- △ 0.1 M-phosphate buffer + 20% glucose + 1mM-2,4-dinitrophenol.
- ● 0.1 M-phosphate buffer + 20% glucose
- □ 0.1 M-phosphate buffer

Figure 5.23. Degradation of cytochrome P-450, in whole yeast, S. cerevisiae NCYC 240, in 0.1 M-potassium phosphate buffer, protection by chloramphenicol, cycloheximide, dinitrophenol and anaerobic conditions.

Values are the mean of two determinations.
conditions only 25% of the original enzyme concentration remains after 8 hours. The results indicate that oxygen is required for degradation.

Mitochondrial utilization of oxygen may be involved in the degradative process, although oxygen itself can destroy cytochrome P-450 in the absence of substrate due to formation of membrane lipid peroxides in situ. Glucose and oxygen are normally considered to be needed for the production of a small quantity of ATP by mitochondria. ATP may be required for the biosynthesis of proteinases or for the transport across membranes of the enzyme for degradation into appropriate vacuoles (Murakami et al., 1979).

The protective agents used in Figure 5.23 all inhibit mitochondrial function, either at oxidative phosphorylation as with dinitrophenol, or by lowering of intracellular cyclic AMP concentration as with 20% glucose, or by inhibition of protein synthesis as in the case of chloramphenicol and cycloheximide. Chloramphenicol inhibits mainly mitochondrial protein synthesis and induced protein synthesis in eukaryotes; at low concentration bacteria are very sensitive to its effects. Cycloheximide is frequently used to inhibit cytoplasmic protein synthesis in yeast. Anaerobiosis, chloramphenicol and 20% glucose all promote the biosynthesis of cytochrome P-450 in yeast through prevention of the biosynthesis of cytochrome $a + a_3$ in the yeast mitochondrion (Wiseman, 1980). The similar effect of cycloheximide suggests that protein biosynthesis in the cytosol, in addition to that in the mitochondria, is also required for cytochrome P-450 degradation (or the destruction of the active conformational form).
CHAPTER 6

THE EFFECT OF AGE AND SIZE OF INOCULATION ON CYTOCHROME P-450 ACCUMULATION
6.1 INTRODUCTION

Many workers do not consider inoculum size when discussing yeast growth and enzyme production, those that do use a wide range of inoculum sizes (see Table 6.1). Different inoculation levels produce different growth curves (Thomas, 1979). With small inocula there are longer exponential phases and longer lag phases (Thomas and Rose, 1979). Larger inocula reduce the lag phase but also the exponential phase (Nagodawithana and Steinkraus, 1976) and usually result in a lower yield of biomass (Strehlano et al., 1983).

There is source data in the literature on the effect of inoculation levels on yeast growth but little on the production of specific enzymes such as cytochrome P-450, one of the exceptions being by Wiseman and Woods (1977) on the rapid and commercial production of microsomal cytochrome P-450 in yeast, with which the results presented here were compared.

From previous chapters (2, 4 and 5) it can be seen that the level of cytochrome P-450 accumulation is not always consistent and it was considered that the age of the slope and methods of inoculation were worthy of greater consideration.

6.2 MATERIALS AND METHODS

Conditions for growth are as described in chapter 2 unless described otherwise. The yeast strains used were *S. cerevisiae* NCYC 754 and 240. Yeast was inoculated directly from slopes in these experiments unless described otherwise. For the experiment described in Figure 6.5, the inoculations were prepared by transferring 10 loopfuls of yeast from a slope into 5 ml of 0.1 M phosphate buffer, pH 7 which was then shaken
<table>
<thead>
<tr>
<th>Author</th>
<th>Date of Publication</th>
<th>Inoculation Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blatiak et al.</td>
<td>1980</td>
<td>0.008% (v/v)</td>
</tr>
<tr>
<td>Barford and Hall</td>
<td>1979</td>
<td>0.05% (v/v)</td>
</tr>
<tr>
<td>Yoshida et al.</td>
<td>1974</td>
<td>0.5% (v/v)</td>
</tr>
<tr>
<td>Woods</td>
<td>1979</td>
<td>3.3% (w/v)</td>
</tr>
<tr>
<td>Thomas et al.</td>
<td>1978</td>
<td>1% (v/v)</td>
</tr>
<tr>
<td>Ohta and Hayashida</td>
<td>1983</td>
<td>1% (v/v)</td>
</tr>
<tr>
<td>Skipton et al.</td>
<td>1973</td>
<td>1% (v/v)</td>
</tr>
<tr>
<td>Strehaiano and Goma</td>
<td>1981</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Aiba et al.</td>
<td>1968</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Nagodawithana and Steinkraus</td>
<td>1976</td>
<td>60% (v/v)</td>
</tr>
</tbody>
</table>

Table 6.1

The different inoculation levels used by different authors.
thoroughly. Volumes of this solution up to 600 µl were then used for inoculation of fresh media.

Preparation of the slopes is as described in the text. Cytochrome P-450 was determined as described in chapter 2.

6.3 RESULTS AND DISCUSSION

6.3.1 The Effect of Slope Age on Cytochrome P-450 Accumulation

Figure 6.1 shows the effect of the age of the slope, kept at 8°C, on the biosynthesis of cytochrome P-450 in *S. cerevisiae* NCYC 754. The yeast was grown at 30°C in 20% glucose growth medium and the whole yeast was assayed to determine the level of cytochrome P-450 biosynthesis after 42 hours. It can be seen from Figure 6.1 that there is a gradual decline in the value of cytochrome P-450 biosynthesis, as measured after 42 hours. However after 9 weeks there is a levelling-off in the value of the cytochrome P-450 at about 4 nmol/g wet weight with however greater standard deviation values.

Figure 6.1 shows that the yeast biomass (as measured by dry weight) achieved is roughly constant throughout the period of this experiment. This suggests that the growth achieved at this time is roughly constant despite the falling cytochrome P-450 levels. However, this does not take into account growth rate which may vary at the different slope ages. 42 hours represents the beginning of stationary phase and thus quite variable growth rates might well result in the same biomass after 42 hours. As accumulation of cytochrome P-450 is thought to increase at faster growth rates (see discussion in chapter 5) it is possible that Figure 6.1 might reflect a faster growth rate with fresh slopes than with older ones.
Figure 6.1. The effect of the age of the slope on the accumulation of cytochrome P-450 and biomass in *Saccharomyces cerevisiae*. Enzyme assay taken after 42 hours growth.

NCYC 754 grown at 30°C at 120 RPM shake speed.

Values are the mean of six determinations, bars indicate standard deviations.
The Effect of Different Inoculation Methods on Cytochrome P-450 Accumulation

Figure 6.2 shows the effect on the accumulation of cytochrome P-450 after inoculating flasks with NCYC 240 yeast previously grown in three different conditions. The control flasks (A) were inoculated in the standard way from a Sabouraud-dextrose agar slope. Flasks B were inoculated with 0.1% (w/v) yeast from a flask which was previously grown in 0.5% glucose growth media. Flasks C were inoculated with 0.1% (w/v) yeast previously grown for 48 hours in 20% glucose growth medium.

From Figure 6.2 it can be seen that in flasks A not until 32 hours does the accumulation of cytochrome P-450 reach the values of flasks B and C, this may correspond to a longer lag phase since flasks B and C had the larger inoculation.

There is no significant difference in the maximum concentration of cytochrome P-450 obtained in flasks A (2.6 nmol/g wet weight) or B (2.7 nmol/g wet weight), except the time at which the value was obtained, flask B after 40 hours, flask a after 48 hours. Flask C had a maximum concentration of cytochrome P-450 of only 1.6 nmol/g wet weight after 28 hours, which may be due to the different physiological state of the yeast used for inoculation. It is known that fermenting yeast is required for cytochrome P-450 production.

Wiseman and Woods (1977) reported a rapid and economical method of producing relatively large amounts of cytochrome P-450 using a heavy inoculum of yeast, previously grown to the end of the exponential phase in growth medium containing as little as 0.5% glucose and transferring
Figure 6.2. The effect of different inoculation methods on the accumulation of cytochrome P-450 in S. cerevisiae NCYC 240. Grown in 20% glucose growth media. Values are the mean of four determinations.
Inoculation Level & Dry Weight of Yeast after 42 hours grammes/100 ml
---
2 loops (CONTROL) & 0.74 ± 0.02
0.5% v/v & 0.72 ± 0.02
1.0% v/v & 0.69 ± 0.03
2.0% v/v & 0.66 ± 0.01
5.0% v/v & 0.59 ± 0.03

Table 6.2 The effect of different inoculation levels on the dry weight of yeast

*S. cerevisiae* NCYC 240 grown at 30°C in a shaking water bath.

The values are the mean of four determinations.
the yeast to a relatively small volume of 20% glucose growth medium. Wiseman and Woods found no cytochrome P-450 in 0.5% glucose growth medium but on transferance to 20% glucose growth medium 2.8 nmol/g wet weight was produced in 10 hours. From Figure 6.2 it can be seen that a very similar value of cytochrome P-450 (2.7 nmol/g wet weight) was obtained but only after a much longer time (40 hours), presumably because of a smaller inoculation 0.1% (w/v) compared with the 3.3% (w/v) as used by Wiseman and Woods.

In agreement with results obtained here in chapter 2, Woods (1979) reports that on transferring yeast protoplasts from 0.5% glucose growth medium to 5% glucose growth medium there is a slight increase in cytochrome P-450 level, 3.0 nmol/g wet weight (compared with 2.8 nmol/g wet weight in 20% glucose growth medium) which is achieved in 5 hours (compared with 8 hours with 20% glucose growth media). Using 5% glucose growth media must be more economical, since less glucose is being used, and is faster.

Advantages in using slopes for "dry" inoculation method:
1. High cytochrome P-450 values.
2. Saving time on preparing media since previous growth of yeast not required.
3. Less expensive because less media used.
4. Immediate availability of dry slopes, once prepared.

The results of this experiment were important in work on the 5 litre microprocessor controlled bioreactor (see chapter 8) where high cytochrome P-450 values were required in as short a time scale as possible. It was found that growing yeast in 0.5% glucose for 48 hours and then inoculating into the 5 litre fermenter gave significantly higher
cytochrome P-450 values than previously growing the yeast in 20% glucose.

6.3.3 The Effect of Different Levels of Inoculation on Cytochrome P-450 Accumulation

Figures 6.3 and 6.4 show the result of different levels of inoculation on cytochrome P-450 accumulation. For inoculation values 0.5% to 5% yeast was previously grown for 48 hours in 20% glucose growth medium at 30°C in a shaking water bath, various inoculum quantities were then transferred to 20% glucose growth media. The control was inoculated from a slope. The maximum values of cytochrome P-450 accumulation for the inocula were 3.0 nmol/g wet weight for the control after 42 hours; 2.4 nmol/g wet weight with 1% (v/v) after 24 hours; 2.3 nmol/g wet weight with 0.5% (v/v) after 42 hours; 2.2 nmol/g wet weight with 2% (v/v) after 24 hours and 1.8 nmol/g wet weight with 5% after 24 hours. Suggesting that the smaller inoculations afforded the higher cytochrome P-450 values. Table 6.2 indicates the effect of 0.5% to 5% inoculation levels on yeast dry weight after 42 hours and from the data it would seem that the accumulation of cytochrome P-450 is growth-related as confirmed in chapter 2. The maximum dry weight value being attained by the control which also gave the greatest cytochrome P-450 concentration. 0.5% (v/v) inoculation and 1% (v/v) inoculation gave similarly high dry weight and cytochrome P-450 concentration; the 2% and 5% (v/v) inoculations gave the lowest dry weight and the lowest cytochrome P-450 values, which may be a growth effect. It is possible that with the 1%, 2% and 5% inoculations the cytochrome P-450 accumulation may have peaked prior to the 24 hour reading taken, since the values after 24 hours are descending. The 0.5% and 1% inoculations are increasing in cytochrome P-450 values after 24 hours. Strehaiano
Cytochrome P-450
nmol/g. wet wt.

Control
1% v/v
0.5 v/v

Time in hours

Figure 6.3. The effect of different inoculation levels on the accumulation of cytochrome P-450 in S. cerevisiae NCYC 240 in 2% glucose growth medium at 30°C in shaking water bath. Values are the mean of four determinations.
The effect of different inoculation levels on the accumulation of cytochrome P-450 in *S. cerevisiae* NCYC 240. Grown at 30°C in shaking water bath. Values are the mean of four determinations.
al. (1983) working on *Saccharomyces cerevisiae* 495, *S. sake* and *S. bayanus* also found a cleavage in the biomass yield with increase in inoculum size. Strehaiano *et al.* used high inoculation values 2%, 10%, 20% and 60% (v/v) and found that any inoculation above 2% decreased biomass yield. The results are in broad agreement with those of Strehaiano *et al.* *S. cerevisiae* grown at a high glucose concentration synthesize cytochrome P-450 quickly during the exponential growth phase up to a maximum concentration at the beginning of the stationary phase (see chapter 2). Thus the accumulation of cytochrome P-450 is normally associated with growth, since the enzyme level begins to decline when rapid growth ends (A. Blatiak *et al.*, 1985, see chapter 2).

Different size inocula require different oxygen tensions because a higher inoculum size leads to a more viscous medium, reducing the oxygen tension in that medium (Brown and Zainudeen, 1978). Also as yeast biomass increases the dissolved oxygen tension decreases by a change in water structure around each cell due to its membrane potential (Baburin *et al.*, 1981). Very small changes in oxygen tension are known to affect cytochrome P-450 biosynthesis quite markedly (Rogers and Stewart, 1973). Agitation is known to increase the homogeneity in the flasks so helping to improve the availability of nutrients and the dispersal of metabolites (Blatiak *et al.*, 1985, see chapter 5). It is possible that the higher inocula may require a higher degree of agitation since any oxygen tension limitation in the media may have inhibited cytochrome P-450 biosynthesis. So, different inoculation levels may require different agitation rates and the effect of different inoculation levels on cytochrome P-450 biosynthesis may be related to oxygenation levels.
6.3.4 The Effect of Very Small Inoculation Levels on Cytochrome P-450 Accumulation

Figure 6.5 shows the effect of different very small inoculation levels on the accumulation of cytochrome P-450 in yeast. There is no significant difference between any of the cytochrome P-450 values or of the biomass. It appears as though 100 µl may be an optimum but the standard deviation bars do overlap for all the values of cytochrome P-450 and biomass.

After the experiments drawn in Figures 6.3 and 6.4 it was considered of interest to investigate the lowest inoculation values and the first few experiments confirmed this interest as the 100 µl results appeared optimal. Even after the experiments were repeated and individual graphs drawn it was not immediately apparent that the 100 µl inoculation was not optimal. Only once the results were pooled and the graphs drawn was it appreciated that the standard deviations overlapped.

6.3.5 The Effect of Slope Preparation on Cytochrome P-450 Accumulation

Figure 6.6 shows how the method of manufacture of the slopes can make a difference in the accumulation of cytochrome P-450. Most of the workers in Dr. Wiseman's laboratory when making up Sabouroud dextrose slopes left the tops slightly loose and incubated for 24 hours at 30°C, then screwed the tops on tightly and then kept the slopes at 8°C until required. I. Qureshi (personal communication) stoppered the tops immediately and kept them at 8°C without the 30°C incubation. I. Qureshi's method of preparing slopes gave higher cytochrome P-450 values with NCYC 239 (Qureshi et al., 1980). Whilst cytochrome P-450 biosynthesis seems generally to be growth-related as biomass increases, an increasing proportion of cells become dormant (Brown and Zainudeen,
Figure 6.5. Graph to show the effect of different very small inoculation levels on the accumulation of cytochrome P-450 in *S. cerevisiae* NCYC 754 in 20% glucose growth media at 30°C in 120 RPM orbital shaker.

Enzyme assay and dry weight determinations taken after 42 hours growth.

Mean of seven determinations, bars indicate standard deviations.
Figure 6.6. Growth of NCYC 239 in 20% glucose media from 2 different slopes. Values are the mean of 3 determinations. Bars indicate standard deviations.
1978). This occurs in the stationary phase and may affect the concentration of cytochrome P-450.

The physiological state of *S. cerevisiae* depends largely on environmental conditions, being a facultative anaerobe whose growth and metabolism varies with the concentration of oxygen present. Therefore, the continued budding by yeasts may affect the SA/V ratio; when the yeasts are unicellular the Sa/V ratio is higher and allows for greater diffusion of O₂ but with increased budding, gaseous exchange and nutrient uptake may be impeded.

Thus it can be seen that the inoculum age, size and previous conditions of growth substantially affect the accumulation of cytochrome P-450 in *S. cerevisiae*. 
CHAPTER 7

THE EFFECT OF HAEM IN CYTOCHROME P-450 ACCUMULATION
INTRODUCTION

Since Keilin's discovery in 1925 of cytochromes in baker's yeast it has been generally agreed that the cytochrome pattern in yeast cells is very dependent on growth conditions. Yeast cells grown aerobically respire actively, contain mitochondria and are characterised by the possession of a cytochrome pattern consisting of cytochrome a, b, c and c\textsubscript{1}. Cells grown anaerobically are poor in respiratory activity, lack typical mitochondria and contain haemoproteins usually called a\textsubscript{1} and b\textsubscript{1}, although the haemoproteins in anaerobic yeast have yet to be fully characterized.

Cyclic molecules such as the tetrapyrrolic macromolecules, with the correct number of alternating double bonds are quite stable, the creation of additional bonding giving strength to the molecule. The haem molecule given the right molecular environment can readily gain or lose electrons and hence its function in electron transfer.

Important regulators in the respiratory metabolism of yeast *S. cerevisiae* appear to be oxygen and glucose (Criddle and Schatz, 1969; Sols *et al.*, 1971). Haem, the prosthetic group of a number of haemoproteins that are directly or indirectly involved in respiratory metabolism has also been shown to have some regulatory function in some cases (Szledziewski *et al.*, 1981). For some haemoproteins only evidence for post-translational control by these factors has been shown (Woodrow and Schatz, 1979). Catalase A, the second catalase protein of *S. cerevisiae* has been shown to be controlled by oxygen (Zimniak, *et al.*, 1976), glucose (Rytka *et al.*, 1978) and haem (Woloszczuk *et al.*, 1980) through the control of mRNA levels (Hortner *et al.*, 1982) although there is also regulation at post-transcriptional level (Laz *et al.*, 1984).
The biogenesis of mitochondria involves the assembly of inner and outer mitochondrial membranes. The components being derived from both mitochondrial and nucleocytoplasmic genetic systems (Schatz and Mason, 1974) (see chapter 3). The actual process of membrane assembly is not yet known (Gopalan et al., 1984). The effects of glucose repression-derepression of mitochondriogenesis in cells of S. cerevisiae allows for the study of mitochondrial membrane assembly. Growing yeast cells in high glucose medium leads to repression of yeast cells characterised by the abolition of new synthesis of mitochondria and breakdown of pre-existing mitochondria (Jayaraman et al., 1974). During repression, most of the respiratory functions are impaired (see chapters 2 and 5). Apart from the mitochondrial respiratory enzymes, a cytosolically localized enzyme α-aminolaevulinic acid (ALA) dehydratase is also subjected to this catabolite repression (Jayaraman et al., 1971). This enzyme is one of the rate-limiting enzymes in haem biosynthesis during the onset of derepression (Jayaraman et al., 1971). Consequently during the repression phase haem levels are thought to decrease.

Reduction of glucose concentration in the medium results in derepression of this enzyme with the restoration of haem synthesis. Gopalan et al., (1984) studied the involvement of haem in the biogenesis of mitochondria. During repression-derepression of yeast cells found that exogenous addition of haemin to repressed cells resulted in the reversal of glucose repression with respect to oxygen uptake by yeast cells. Gopalan et al. (1984) found that the rate of oxygen consumption by repressed cells in the presence of haemin was comparable to that of derepressed cells, furthermore addition of levulinic acid, a competitive inhibitor of haem synthesis (Malamud et al., 1979) to derepressing cells led to a drop in the
7.2.1 Measurement of Haem

The determination of haem in solution is based on characteristic absorption spectra of haem-pyridine complexes (haemochromes).
Differential spectra of pyridine haemochromes are measured in aqueous solution after reduction with dithionite against a sample oxidized with hydrogen peroxide (Falk, 1964).

A suspension of yeast cells in 50% glycerol (1 g wet weight per 5 ml) were used for this assay up. Two portions of the suspension, 1.1 ml each, were placed in two 3 ml cuvettes of 1 cm path length. To each cuvette 0.5 ml pyridine was added carefully in a fume cupboard to overlay the yeast suspension. Then 0.2 ml of 3% hydrogen peroxide was introduced to the reference cuvette and 0.2 ml of water to the sample cuvette; the addition being made slowly to avoid a mixing of the phases. To the sample cuvette a few crystals of sodium dithionite were added. The contents of both cuvettes were made alkaline with 0.2 ml of 4 M sodium hydroxide, stirred, and after 3.7 minutes (the time required to allow pyridine to penetrate into the cell and the chromogenic to occur, as after 7 minutes, the absorption maxima decrease) the spectrum was recorded over the wavelength range 525-650 nm with the Varian Cary 219 spectrophotometer. Absorption of pyridine haemochrome was read between 557 nm and 575 nm. An extinction coefficient of 32.4 mM$^{-1}$cm$^{-1}$ (Omura and Sato, 1964) was used to calculate haem levels. Flasks were made anaerobic as described earlier.

7.2.2. **Addition of Haem to Yeast Cultures**

The haem used was bovine haem from Sigma Chemical Co. Ltd. *Saccharomyces cerevisiae* strain GL7 (erg 12 haem 3) (Gollub et al., 1977) was grown in 20% glucose growth medium, lanosterol was added to the medium as 0.2% solution in Tween 80/ethanol (1:4 w/w) to a final concentration of 10 µg/ml (Gollub et al., 1977). The haem was added to the solution to a final concentration of 2.5 µg/ml.
To assay the yeast, after addition of the haemin to the medium, for cytochrome P-450 accumulation the yeast had to be spun down twice in 40% glucose and then washed in buffer three times to clear the dark haemin which would otherwise have obscured the enzyme assay.

7.3 RESULTS AND DISCUSSION

7.3.1 Measurement of Haem and Cytochrome P-450 Accumulation in Yeast

From Table 7.1 it can be seen that the level of haem is greater in low glucose grown yeast (with a lower cytochrome P-450 accumulation) than in high glucose grown yeast (with a high cytochrome P-450 level of accumulation). The total haem value in aerobic and anaerobic yeast grown in 0.5% glucose concentration is 41.5 nmol/g wet weight which is 37% more than in 20% glucose grown yeast the total haem value, in aerobic and anaerobic yeast, being 30.4 nmol/g wet weight. This may be because of the repression of 5-aminolaevulinic acid dehydratase by the high glucose concentration (Jayaraman et al., 1971). Also as mitochondria are needed for the biosynthesis of the haem moiety (Gudenus et al., 1984) then the derepressed conditions in 0.5% glucose media, where more mitochondria are present within the cell, may explain the higher haem level.

The haem content was greater in 0.5% glucose aerobically, and anaerobically grown, yeast after 24 hours than in 48 hours. This may be due to yeast autolysis which may have begun to occur soon after 24 hours since the carbohydrate energy source may by then have been excluded. The haem values in 20% glucose aerobically and anaerobically grown media did not decline after 24 hours.
<table>
<thead>
<tr>
<th>Yeast grown in growth media containing</th>
<th>Conditions of growth</th>
<th>Time of haem assay</th>
<th>Cytochrome P-450 in nmol/g wet weight</th>
<th>Haem value in nmol/g wet wt. in media</th>
<th>Haem value in yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% Glucose</td>
<td>Aerobic</td>
<td>24</td>
<td>0</td>
<td>0.14</td>
<td>12.8</td>
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<td></td>
<td></td>
<td>42</td>
<td></td>
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<td>11.7</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>24</td>
<td>0</td>
<td>0.12</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42</td>
<td>0</td>
<td>0.17</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td>20% Glucose</td>
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<td>0.19</td>
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</tr>
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<td>0.10</td>
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</tr>
<tr>
<td></td>
<td>Anaerobic</td>
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<td>0.18</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42</td>
<td>6.0</td>
<td>0.18</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>30.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1: Cytochrome P-450 accumulation in *S. cerevisiae* NCYC 754 and haem values when grown under aerobic and anaerobic conditions.

Yeast was grown in a Gallenkamp shaker at 120 RPM at 30°C. Values are the mean of two independent experiments.
From Table 7.1 the yeast cells grown aerobically whether on 0.5% glucose growth medium or on 20% glucose growth medium contained more haem than under conditions of anaerobic growth. At 0.5% glucose under aerobic conditions the total haem assayed for 24 and 42 hours was 24.5 nmol/g wet weight compared to 17.0 nmol/g wet weight of yeast under aerobic conditions. This being a 42.8% increase in haem content with aerobic non-repressed conditions. In 20% glucose growth media the total haem assayed for 24 and 42 hours under aerobic conditions was 17.1 nmol/g wet weight and under anaerobic conditions was 13.3 nmol/g wet weight of yeast. So, there is a 28.5% increase in haem under aerobic conditions under conditions of glucose repression.

Lukaszkiewicz and Bilinski (1979) found that yeast cells grown aerobically on 10% glucose contained more than five times the amount of haem (83.7 nmol/g dry weight) than yeast cell grown anaerobically on glucose concentration not given (15.4 nmol/g dry weight). The values of haem obtained by the workers were considerably greater than those in Table 7.1, the reasons for which include the facts that Lukaszkiewicz and Bilinski used dry weight of yeast, employed a much lower absorption $\text{mM}^{-1}\text{cm}^{-1}$ coefficient value (14 $\text{mM}^{-1}\text{cm}^{-1}$ as opposed to the author's 32.4 $\text{mM}^{-1}\text{cm}^{-1}$), there are also likely to be strain-dependent differences between the yeast strains used by Lukaszkiewicz and Bilinski and in this work. Even lower values were obtained here for the haem content of yeast the greatest difference in haem content between aerobic and anaerobic growth was only 42.8%.

Bilinski et al. (1978) found the haem content in aerobically grown yeast to be 10 times more as opposed to 5 times more (Lukaszkiewicz and Bilinski, 1979) than the amount present in anaerobically grown yeast.
Hence under different conditions the same workers find significant variations in the amounts of haem present under aerobic or anaerobic conditions. From Table 7.1 it can be seen that there is a slight increase in the amount of haem present under aerobic conditions in 0.5% growth media, in agreement with Biliński et al. (1978) and Lukaszkiewicz and Biliński (1979). Strain dependent differences may account for some of the variation.

Haem synthesis may be considered restricted under anaerobic conditions since two late steps in haem synthesis are known to require molecular oxygen (Sano and Granick, 1961; Porra and Falk, 1964) except in yeast (Poulson and Polglase, 1974; Biliński et al., 1978) but even then there may be lower values of haem in yeast when grown anaerobically as Table 7.1 shows. No cytochrome P-450 accumulation was found at 0.5% glucose growth conditions but a considerable amount was found in 20% glucose growth media. Under anaerobic conditions the haem and cytochrome P-450 values were both lower at 24 hours under anaerobic conditions than in aerobic conditions. After 42 hours however, there was slightly more cytochrome P-450 under anaerobic conditions although there was also less haem than under aerobic conditions.

In low glucose growth medium the haem is taken up by the functional mitochondrial cytochromes and no measurement has been made of these enzymes in this experiment. Therefore, it is not possible to determine how much haem is available for incorporation into cytochrome P-450 under these conditions. In high glucose growth medium the cytochrome P-450 levels are greatly increased (as described in chapter 2), however, the total haem levels in the yeast are reduced. This is presumably because haem is not required for mitochondrial cytochromes and other
glucose derepressed proteins such as catalase. Indeed, haem itself has been shown to be directly involved in the regulation of these protein levels (Hortner et al., 1982; Laz et al., 1984). After 42 hours anaerobic growth in 20% glucose growth media it can be seen from Table 7.1 that the levels of cytochrome P-450 and haem are approximately the same, under these conditions most of the cellular haem may well be bound to cytochrome P-450; hence under these conditions haem supply may be limiting cytochrome P-450 accumulation. Results from Table 7.1 may suggest that some regulation of cytochrome P-450 accumulation levels by haem may take place (either directly or indirectly) as has been shown for other yeast haemoproteins (Hortner et al., 1982).

7.3.2 The Measurement of Haem in the Media

From Table 7.1 it can be seen that the levels of haem found were very low. It is possible that the values were near the threshold of sensitivity of the instrument employed. It is doubtful whether the values are significantly different, the differences found are very small indeed. The values of haem may represent more the amount present in the media, perhaps in the yeast extract rather than the quantity of haem being excreted by the yeast. This may explain a decline in haem values from 24 to 42 hours for example, in 20% glucose aerobic growth conditions the 24 hour value being 0.19 nmol/g wet weight but after 42 hours only 0.10 nmol/g wet weight. If the yeast was excreting haem then the values should increase with time such as under 0.5% glucose anaerobic growth conditions where after 24 hours the value was 0.12 nmol/g wet weight which increased to 0.17 nmol/g wet weight. The last set of figures decreases the significance of the results since under anaerobic conditions it is recognised that there may be a restriction in haem synthesis (Sano and Granick, 1961) and consequently it is less likely that more haem
would be excreted under these conditions. Lukaszkiewcz and Bilinski found 1.8 nmol/g haem dry weight of yeast excreted with 10% glucose growth medium under aerobic conditions and 86.0 nmol/g dry weight of yeast excreted with glucose growth medium under anaerobic conditions; the publication does not mention the glucose concentration used under anaerobic conditions, nor the time or growth phase at which the assays were taken. The results follow from Jayaraman et al. (1971), if -aminolaevulinic dehydratase enzyme is rate limiting and is subject to catabolite repression, then it follows that the yeast will not be able to excrete the haem in larger quantities where is is required, as under anaerobic growth.

7.3.3 The Effect of Added Haem on Cytochrome P-450 Accumulation in Haem Deficient Yeast

The haem deficient yeast strain GL7 was grown in a 20% glucose growth medium supplemented with lanosterol and haem.

At no time point could any cytochrome P-450 accumulation be recorded. Various concentrations of glucose under aerobic and anaerobic conditions were tried but no cytochrome P-450 accumulation was found. When used as supplements in aerobic culture media, -aminolaevulinic acid, protoporphyrin IX and haem all induced some cytochrome production in haem mutant strains (Guarente and Mason, 1983). If GL7 is deficient is lanosterol biosynthesis, it may also be deficient in the gene for cytochrome P-450.

It has been shown that haem enhances the synthesis of catalase T in yeast (Hamilton et al., 1982) and furthermore Gopalan et al. (1984) showed that haemin stimulates the incorporation of amino acids into
cytoplasmic proteins. In this chapter it was considered possible that exogenous haemin may well affect the cytochrome P-450 accumulation in yeast cells, however, this could not be clearly demonstrated.
CHAPTER 8

THE USE OF THE BIOREACTOR
IN CYTOCHROME P-450 ACCUMULATION
8.1 INTRODUCTION

8.1.1 The Bioreactor in Biotechnology

The Spinks Report 1980 defined biotechnology as the application of biological organisms, systems or processes to manufacturing and service industries.

In Chapter 1 there was a summary of the applications of cytochrome P-450, because of the importance of this enzyme, many laboratories, all over the world, are attempting to arrive at industrially and medically utilisable quantities.

Much of biotechnology involves the discovery and subsequent optimization of the biological and biochemical processes needed to exploit the source of natural raw material (A. Wiseman, 1983).

The development of a successful large scale production process is the result of accelerating and intensifying an original concept, usually a small scale or laboratory process. Operating a large scale process is not just a matter of carrying out the original procedure with larger quantities of material, but must include new concepts to deal with the problems arising from large scale production (M. Winkler, 1983).

A bioreactor may be defined as a vessel suited for the accumulation of a required product. Bioreactors may be operated as continuous or batch systems. In continuous systems, fresh reagents flow continuously into the bioreactor and the product stream flows out continuously. In batch systems the bioreactor is given the reagents and as the reaction proceeds the conditions in the bioreactor change as reagents are consumed and products formed. Continuous systems can operate on a steady state
whilst batch systems cannot, hence microprocessors are useful in the control of batch systems where the control settings may be changed to different phases of the process. Bioreactors must prevent contamination of the culture from the environment while also preventing release of the culture into the environment, especially with genetically engineered microorganisms. Although it has been reported (Burrows, 1979) that completely aseptic conditions are not required with baker's yeast.

Bioreactors are agitated mechanically to maintain homogeneity, for rapid diffusion of products and heat. The volume of the bioreactor is larger than the required culture by 30-50% (M. Winkler, 1983), leaving a headspace allowing room for forming a disengagement of liquid droplets from the exhaust gas. There is more hydrostatic pressure at the bottom of the flask which enhances gas dissolution so again agitation is important to maintain homogeneity with regard to dissolved oxygen tensions, known to influence cytochrome P-450 accumulation (Blatiak et al., 1985a).

8.1.2 The Bioreactor's Microprocessor Control

Once incubation begins there are relatively few analog loops to be implemented in bioreactors in the control of physical factors. The control actions used were implemented every twenty minutes and operated on a number of controlled variables.

The dissolved oxygen control was effected by speeding up or slowing down the agitator speed. The response time is several minutes and the frequency of control intervention had to be kept low to avoid oscillation which destabilizes the bioreactor operation, by attempting to correct every random variation, which may lead to extreme cyclical changes in
the medium by overcorrection in opposite directions, known as "hunting". Fast response is not necessary because changes in dissolved oxygen tension are due to growth of the organism. Many problems can arise especially if substrates or antifoam reagents were discontinuously added or if there were static heads of fluid which might cause pressure gradients. Dissolved oxygen probes are susceptible to mechanical damage to their membranes and electrodes. In the bioreactor a duplicate probe may have to be considered in the future.

It is known how sensitive cytochrome P-450 accumulation is to changes in oxygen pressure (Blatiaik et al., 1985a) and for this reason dissolved oxygen control was regarded as very important.

Temperature and pH control were next on the list of priorities with regard to optimising cytochrome P-450 accumulation in the bioreactor. The control of these factors was by analog loops. Reliability of pH probes is very good, according to Fox (1984) 98% of all batch starts yielding accurate pH data through their entire course.

Ohashi et al. (1979) have measured the optical density of _S. cerevisiae_ on-line and have thus been able to correlate the results with biomass. Ohashi et al. constructed a submersible colorimeter cell incorporating a gas disentrainment device to separate air bubbles, using the force of the impeller drive to drive the yeast and media through, forcing it through a switchback. The residence time was 1-3 minutes, enough to allow the air bubbles to escape, the degassed media then flowed through the colorimeter chamber and back to the bulk of the media in the reactor. The light path was between a tungsten bulb and a phototransistor. With _S. cerevisiae_ the response was linear up to $3 \times 10^7$ cells/ml with a drop
of insensitivity beyond that point. This system would suit the author's needs very well and with high power light emitting diodes being available the $3 \times 10^7$ limit may be stretched (Fox, 1984).

An alternative to optical density measurement is flow microcalorimetry which can indicate biomass. A microcalorimeter is a fine bore tube contained within a thermostatically-controlled block. Heat generated by the metabolism of the microorganisms in the culture is detected by sensitive temperature measurement (Beezer, 1980). Since this is a flow technique it is suitable for on-line measurement. Its major disadvantage is that as the sample passes through the instrument it quickly becomes depleted of dissolved oxygen, which may therefore suit yeast biomass measurement.

The microprocessor controlled bioreactor can print data in various forms including graphical representations. The computer can display information in analog form to give a rapid assessment of the bioreactors operation, furthermore a digital display can be made available for more greater precision. Information can also be displayed in functional groups representing a particular process within the operation of the bioreactor.

Because of the importance of cytochrome P-450 (see Chapter 1) any method of enhancing its accumulation from \textit{S. cerevisiae} on a larger scale was considered of interest.

Research in biological processes occurs in the following three main areas:

(i) efficient, high yielding strains

(ii) formulation of media
The most efficient, high yielding strains and variations in media components were already considered in Chapter Two. This chapter deals with the application of results discovered in previous work with smaller batch cultures (250 ml flasks). In transferring a laboratory scale process to a production scale there are many scale-up problems. Banks (1979) suggests that there are four stages necessary in scaling up from the laboratory to the production plant;

(i) Shaken flasks (50-1000 cm$^3$)  
(ii) Laboratory stirred fermenter (5-20 dm$^3$)  
(iii) Pilot scale fermenters (50-5000 dm$^3$)  
(iv) Production fermenters (25-1000 m$^3$)

Thus the chapter deals, in part, with the first stage of scale-up.

Normal problems associated with scale-up such as inoculum development, media sterilisation, antifoam toxicity, trace metal pick-up and aeration-agitation have generally been overcome since it is known that the 5 litre bioreactor can produce a high concentration of cytochrome P-450.

The use of 8% glucose medium was reported as being successful in enhancing cytochrome P-450 accumulation in 100 ml media batches in 250 ml flasks (Blatiak et al., 1987, see chapter 2). Using 8% glucose a similar enhancement effect in a 5 litre bioreactor would greatly facilitate purification procedures of cytochrome P-450.

In view of the anticipated enhanced quantity of cytochrome it was considered worthwhile collecting the enzyme by preparing microsomal cytochrome P-450 using the method of Sadler et al. (1983).
8.2 MATERIALS AND METHODS

8.2.1 The 1.4 litre Bioreactor

The 1.4 litre bioreactor (fermenter) see plate 8.1 had a working volume of up to 1.1 litres. It has a polypropylene head plate and backing flange. The head plate penetrations are made via nylon screw-in connectors of 3/8 inch diameter. Culture temperature is regulated by a solid-state controller using a thermometer sensor. Aeration is provided by a diaphragm pump, with front panel indication and needle valve control of the air flow rate. An encapsulated magnetically-coupled follower acts as the impeller. The 1/10 H.P. universally-wound motor drives the impeller at speeds regulated by a thyristor controller and has front-panel adjustment. The solid state dissolved tension meter may be used with any galvanic oxygen probe.

Before calibration the entire bioreactor vessel was autoclaved. To calibrate the oxygen electrode to zero it was placed in saturated sulphite solution. The electrode was then rinsed with distilled water and placed in air saturated water, the span control being adjusted to 99.9%, the temperature having been set at 30°C.

The pH meter range was 0-10 and was standardised by two values at pH 4 and 8 (ensuring greatest accuracy in the required range). There is a solid state pH controller which incorporates a high impedance amplification stage. The controller provides a single switch mains output, suitable for activation of single pump, enabling the controller to activate the titrant pump should the pH in the bioreactor vessel fall below the set point control value.
Plate 8.1

A. Bioreactor vessel with
   pH probe
   Temperature probe
   Oxygen electrode probe
   Magnetic stirrer

B. Controller
   Temperature control
   RPM control
   Airflow indication

C. Controller
   Dissolved oxygen tension control
   pH control
   RPM indicator
   Temperature indicator

D. Peristaltic pump

E. Chart recorder
Figure 8.1 The appearance of the microprocessor controlled bioreactor and associated equipment.
Key to Figure 8.1

A. Temperature meter
B. pH meter
C. Measurement of temperature in the circuit
D. Impeller motor drive
E. Microprocessor interphase
F. Monitor
G. Microprocessor
H. Water cooled agitator bearing
I. pH addition pumps
J. Four litre fermenter
K. pH addition reservoirs
L. Air compressor
M. Air filter
N. Dissolved oxygen tension meter
P. Thermocirculator
Figure 8.2 Details of the bioreactor design.
Key to Figure 8.2

Q. pH electrode
R. Agitator shaft blades and bearings
S. Air sparger
T. Dissolved oxygen tension sensor
U. Glass walled vessel
V. Water heating supply and return
W. Top plate
As can be seen from plate 8.1 two vessels are capable of simultaneous operation enabling carefully controlled experiments to be performed.

8.2.2 The Microprocessor Controlled 5 litre Bioreactor

Figures 8.1 and 8.2 with plates 8.2 and 8.3 show the design and appearance of the bioreactor.

The bioreactor consists of a baffled, almost cylindrical glass body where the diameter is largest at both ends but decreases towards the centre of the light to create additional circulation of the medium. The glass body has rubber seals between two stainless steel plates. The top plate contains the parts for instruments and pH additions as well as the connecting points of the temperature controlled water tubes (see Figure 8), the connecting point of the air tube and the drive shaft of the impeller. Plate 8.2 shows an overall view of the system and plate 8.3 shows the vessel.

Air was provided by an air compressor running at constant speed and its flow rate was measured by a rotameter which was calibrated at 20 ml/min intervals for a range 0 to 600 ml/min. The control was effected by a manual valve which could be adjusted to give specific air flow rates by passing excess air through a by-pass valve. Incoming air was passed through autoclaved glass wool to remove contaminants.

The temperature was controlled by using a thermocirculator which consists of a scaled, transparent perspex cylindrical tank with a heater; a centrifugal pump; a temperature indicator-controller and a coil for cooling the circulating water. The water was circulated continuously to bring the temperature of the bioreactor to the desired set point.
The temperature signal from the temperature controlled water was logged into the Apple II microprocessor and the on/off control was effected by a PID-controller which was capable of constant set-point control. The temperature control was accurate to $\pm 0.05^\circ$C, as indicated by the microprocessor printout read from a thermistor inserted into a port in the vessel.

The pH control system consisted of a combination of a pH electrode with a remote reservoir (Kent Industrial Measurements, Chertsey, Surrey) an EIL pH meter/controller which is calibrated both in pH units and mV, and two possible pumps for alkali and acid additions with their respective reservoirs. The electrolyte used was a KCl solution saturated with AgCl. The electrode was inserted through a port on the top plate. The mV signals from the pH meter were logged into the Apple II microprocessor controller and the control was effected by a PID-controller which monitors the overshoot of the set-point when an addition of acid or alkali was made. This was to avoid continuous fluctuation ("hunting") which otherwise quickly drains the reservoirs. The PID control enabled the pH to be kept within $\pm 0.005$ of the set point.

The impeller speed was determined using a stroboscope with an electronic detection system and a digital output which was logged into the Apple II microprocessor. The control was via a variable resistor power unit with the relay being controlled by an automatic PID-controller. The r.p.m. meter was based on the power input of the motor and was calibrated in 50 r.p.m. steps from 0 to 1500. The r.p.m. could be controlled to within $\pm 5$ r.p.m. in the range 0 to 300 r.p.m.
The oxygen electrode (Uniprobe Instruments Ltd., Cardiff) consisted of a silver cathode and lead anode encased in a 12 mm diameter glass body. The cathode was connected to the anode by a lead containing electrolyte. A gas permeable membrane was placed over the cathode to separate the cathode-anode assembly from the fermentation medium. Diffused oxygen was reduced electrochemically at the cathode by the reaction

\[ \text{O}_2 + 2\text{H}_2\text{O} + 4e^- \rightarrow 4\text{OH}^- \]

causing a current to flow which was proportional to the partial pressure of the gas mixture with which the dissolved oxygen would be in equilibrium. This current was fed into the oxygen meter which was calibrated from 0 to 100, the 100 corresponding to the partial pressure of oxygen in the gas with which the dissolved oxygen would be in equilibrium. The meter was connected to the Apple II microprocessor to monitor the dissolved oxygen tension of the medium.

8.2.3 Preparation of the 5 litre Bioreactor

The bioreactor with all the instruments, was autoclaved empty, except for 1 ml of the antifoam, PPG 2000.

The medium was prepared in two 5 litre flasks there being 2.1 litres of the glucose and 2.1 litres of the growth medium, as for 100 ml batch preparations (see Chapter 2) except that the autoclave timing was for 1 hour (not 15 minutes as with 100 ml batch) at 15 p.s.i. When the medium had cooled it was transferred into the bioreactor using a peristaltic pump by means of interconnecting tubing, half of which was autoclaved with the medium and the other half of the tubing had been autoclaved with the vessel.
As a sterility check a batch had been sterilised in this way and maintained in the bioreactor with filtered air sprayed at 250 ml/min for one week and showed no visible signs of contamination. As a further control two 250 ml flasks each with 100 ml of glucose growth media extracted from the vessel after inoculation were incubated at 30°C in a water bath and the cytochrome P-450 values compared with two more 250 ml flasks with 100 ml of glucose growth media which were inoculated with 2 loops from fresh slopes. Thus the accumulation of cytochrome P-450 in the vessel could be compared with the two 250 ml flasks which, in turn, could be compared with the two flasks inoculated from fresh slopes. As a further sterility check 50 ml of media (prior to inoculation) was taken from the bioreactor and grown in a 250 ml flask at 30°C in a water bath. As an additional precaution a sample was taken before centrifuging the yeast and examined for contamination under the electron microscope.

8.2.4. Sampling from the Bioreactor

40 ml samples were taken for the first 16 hours at four hourly intervals to ensure that there was sufficient biomass for the cytochrome P-450 assay.

The samples were taken using a previously autoclaved pipette through a port on the top plate of the vessel (see Figure 8.3). The remainder of the assay was performed as described in Chapter Two except that the spectrophotometer was a SP1800.

8.2.5. Media for the Bioreactor

The media was made up as prescribed by Salihon et al. (1983) that is 16.9 g yeast extract, 10.4 g mycological peptone and 0.9 g sodium
chloride per litre autoclaved separately from the 140 g per litre of glucose.

All other preparations and solutions are as described in previous chapters.

8.2.6 Preparation of Microsomal Cytochrome P-450 from S. cerevisiae using Polyethylene Glycol Precipitation

Yeast was grown as described in chapter 2. The yeast was then harvested at 3000 RPM for 5 minutes in a M.S.E. bench centrifuge to sediment the cells. The supernatant was discarded and the yeast re-suspended in 0.1 M phosphate buffer, pH 7.2 containing 1 mM EDTA and 1 mM dithiothreitol to give a concentration of 300 g (wet weight) of yeast per litre. The yeast suspension was then passed twice through an APV Manton-Gaulin homogeniser (Manor Royal, Crawley, Sussex) operating at 600 bar. The disruptate was centrifuged at 3000 RPM for 5 minutes to sediment intact cells and cell debris. To the separated supernatant 8% polyethylene glycol (PEG) was added. The solution was placed in a cold room at 8°C and stirred for 1 hour, then centrifuged in a Beckman J6-B at 2300 RPM for 20 minutes. The sediment then contained the cytochrome P-450.

8.3 RESULTS AND DISCUSSION

8.3.1 Cytochrome P-450 Accumulation in S. cerevisiae in the 1.4 litre Bioreactor

The accumulation of cytochrome P-450 in S. cerevisiae NCYC 240 was found to be 2.7 nmol/g weight of yeast after 37 hours using the 1.4 litre bioreactor which was similar to the amounts obtained in 100 ml media in 250 ml flasks. The 1.4 litre bioreactor was only used for a brief period
of time because access to the 5 litre bioreactor was negotiated soon afterwards with the discovery of the NCYC 754 strain.

8.3.2 Accumulation and Degradation of Cytochrome P-450 in *S. cerevisiae* in the 5 litre Bioreactor

Figure 8.3 shows the accumulation and degradation of cytochrome P-450 in *S. cerevisiae* NCYC 754 in the bioreactor. Once the cytochrome P-450 had reached its peak value the yeast was transferred to 8% glucose media.

There are considerable differences in the levels of cytochrome P-450 accumulation in the bioreactor, the experiments were carried out with the same batch of mycological peptone, which is known to influence cytochrome P-450 accumulation (Salihon, 1984). The first readings were taken 24 hours after inoculation. It is not known why with time course X there was a higher value of cytochrome P-450 accumulation after 24 hours than 25 or 26 hours. The peak arrived after 28 hours with time courses X and Y but in Z only after 30 hours.

With assays from the bioreactor the cytochrome P-450 peak could be anticipated by the simultaneous drop in wet weight. This is different from the work with the flasks where the wet weight continued to rise a little after the observed cytochrome P-450 peak (see chapter 5). In time course Y there was a significant rapid increase in cytochrome P-450 between 25 and 26 hours where the value rose from 7.7 nmoles/g wet weight to 12.4 nmoles/g wet weight. No similar increases in flasks have ever been recorded by the authors. The reason for this sudden increase in the enzyme cannot be explained.
Figure 8.3 Accumulation of cytochrome P-450 in *S. cerevisiae* NCYC 754 in the bioreactor. Degradation of the enzyme is in 8% glucose media. Results are three separate time courses.
The rate of degradation after maximum cytochrome P-450 accumulation follows a similar gradient for all three time courses for the first two hours, then there are some slight variations.

There are mathematical models which can be used to analyse fermentation data. There are major constraints on the use of models mainly the difficulty in obtaining reliable values for the parameters describing the response of the biological system, such as field coefficients and specific growth rates. As can be seen from Figure 8.3 there is considerable variation in the accumulation of cytochrome P-450, the reasons may include the age of the inoculum slope (see Chapter 6) since the experiments were performed at three week time intervals apart. A predictive mathematical technique reported by Gyllenburg et al. (1969) suggested that the history of previous batch runs in a bioreactor may be used to form an exception of how a current run may proceed. Their basic assumption being that product formation follows a similar pattern in a set of fermentation runs. They suggest that while deviations from a pattern may occur in practice they do so with defined probabilities. So to utilize a model it would be necessary to have many more individual experiments but eventually it may be possible to calculate performance predictions.

8.3.3 The Effect of 8% Glucose Incubation on Cytochrome P-450 Accumulation in S. cerevisiae NCYC 754 previously grown in Optimised Media in 250 ml Flasks

Figure 8.4 shows the effect of 8% glucose media, added after 42 hours, on cytochrome P-450 accumulation in S. cerevisiae NCYC 754 which had previously been grown in optimised and non-optimised media. In Chapter 2 some work was carried out with optimised media which was found to be
Cytochrome P-450
n.mol/g.wet wt.

Previously grown in non-optimised media

Previously grown in optimised media.

Figure 8.4 The effect of 8% glucose media, added after 42 hours, on cytochrome P-450 accumulation in \textit{S. cerevisiae} NCYC 754 which had previously been grown in optimised and non-optimised media.

Results are the mean of three determinations, bars indicate standard deviations.
an improvement on the non-optimised media normally used. Figure 8.1 shows that even with the optimised media there is cytochrome P-450 accumulation, which is not as high as seen previously (see chapter 2). The level of enhancement on incubating the yeast previously grown in optimised media is only a 48% increase whereas with non-optimised media growth the increase was 86%. This may reflect on a more suitable dissolved oxygen concentration present in optimised media which then the 8% glucose solution cannot improve on as much.

8.3.4 The Effect on Yeast Cytochrome P-450 Accumulation when Transferring to 8% Glucose Media in the Bioreactor

Figure 8.5 shows the effect on yeast cytochrome P-450 accumulation when 8% glucose was added to \textit{S. cerevisiae} NCYC 754 in the 5 litre bioreactor at maximum cytochrome P-450 accumulation. Clearly there is little difference between the cytochrome P-450 values with the different media, there being considerable overlap in the standard deviations. One of the reasons why the accumulation of cytochrome P-450 may not have enhanced is that the bioreactor was computer controlled about a set 5.01 pH, the 8% glucose was measured at pH 6.6 consequently after the transferance of the yeast to 8% glucose there was a substantial influx of acid into the bioreactor in order to return the pH to 5.01. The effect of acid on cytochrome P-450 has not been researched but it may well make the enzyme more labile.

In Figure 8.5 at 5 hours no standard deviation is shown, there being only 2 results available. Sometimes during scale-up commercial-grade chemicals, rather than analytical grade were used but in this instance, analytical grade chemicals were used throughout. Had the 5 litre been made of metal then contamination problems could be a possibility but this is not the case since the bioreactor was made of glass.
Figure 8.5  The effect of 8% glucose on cytochrome P-450 accumulation in *S. cerevisiae* NCYC 754 in the *S*. *l*. bioreactor.

Results are the mean of three determinations, bars indicate standard deviations.
Some scale-up problems arise from the different ways in which process parameters are affected by the discussions of the bioreactor, which although of geometrically similar proportions have different volumes; the volume is proportional to the cube of the diameter, but the bioreactor wall area is proportional to the square of the bioreactor diameter, so that the specific surface area, relevant to heat transfer, is proportional to the reciprocal of the vessel diameter (Winkler, 1983). Thus it is almost impossible to reproduce identical environments on different scales. Certain parameters can be maintained at desired values regardless of scale such as media composition, pH and temperature but in bioreactors maintaining the availability of dissolved oxygen is more difficult. It is possible that the 8% glucose may have changed the viscosity of the yeast environment, thereby affecting the rate of dissolved oxygen tension. Previous work has shown how critical the oxygen tension is in cytochrome P-450 accumulation (Blatiak et al., 1985) and degradation (Blatiak et al., 1980).

8.3.5 Recovery of Microsomal Cytochrome P-450 from S. cerevisiae using Polyethylene Glycol Precipitation

The preparation of microsomal cytochrome P-450 has been previously described (Chapter 2). This was quite a time consuming method requiring high speed centrifugation at 108,000 g for 1 hour which may represent a bottleneck in large scale preparations of the enzyme in view of the low volume capacity of centrifuges operating at such speed. The method of Sadler et al. (1983) requires only low speed centrifugation of a polyethylene glycol (PEG) precipitate.

Both methods of microsomal cytochrome P-450 extraction were employed twice to give a comparison of mean values. The PEG method
was found to be rather unsatisfactory and most of the cytochrome P-450 seemed to be lost after low speed centrifugation with PEG. The high speed centrifugation method, used from the same batch of accumulated cytochrome P-450, gave a concentration which was more than four times higher (1.5 nanomoles/ml compared to 0.34 nanomoles/ml).

The cytochrome P-450 seemed to be lost after the addition of 8% PEG which was added at room temperature following the method of Sadler. It is possible that if the separated supernatant be kept in ice buckets and transferred immediately to the 8°C cold room for the addition of PEG there may be less enzyme loss. Whilst stirring for 1 hour at 8°C as described by Sadler there may be further loss of enzyme. 4°C may offer more protection against thermal degradation of cytochrome P-450. Sadler et al. (1983) claims that his PEG method loses only about half the enzyme that might otherwise be recovered in the high speed centrifugation method. The differences between the results found here and those described by Sadler et al., are difficult to explain. Differences in initial enzyme concentration may help to explain certain differences only.

The work on bioreactors has an important significance in school education and the development of an inexpensive micro-processor monitored bioreactor is being considered with the co-operation of Mrs. Wood of Philip Harris.
CHAPTER 9

FINAL DISCUSSION
9.1 FINAL DISCUSSION

Lindemayer and Smith (1964) showed that the cytochrome P-450 they discovered in *S. cerevisiae* could only be detected when the yeast was grown under specific conditions. It was shown here in agreement with Ishidate et al. (1969) that cytochrome P-450 which had accumulated in high quantities in semi-anaerobically grown yeast was rapidly lost on exposure to aerobic conditions. However, if chloramphenicol or a high concentration of glucose was present the loss of cytochrome P-450 can be prevented because of the inhibition of the development of mitochondrial cytochromes (see chapter 4, Blatia et al., 1980). High cytochrome P-450 accumulations have been found with growth by fermentation and repression of mitochondrial cytochromes. It has also been shown here that even under semi-anaerobic conditions no cytochrome P-450 was detected in low glucose (0.5%) concentration growth media (unless supplemented with ethanol, see on). There is a requirement for a relatively high glucose concentration to be present for cytochrome P-450 to accumulate even under semi-anaerobic conditions. (The lowest glucose concentration investigated here with detectable cytochrome P-450 was 5%). Although this does not mean that 5% is the lowest glucose concentration in which cytochrome P-450 can accumulate.

The endogenous role of cytochrome P-450 is in the 14α-demethylation of lanosterol, the first step in the biosynthetic pathway of ergosterol which is the major sterol in yeast membranes (Yoshida and Aoyama, 1980) and has been shown to be required for yeast membrane synthesis in the endoplasmic reticulum (Aoyama et al., 1984). Hata et al. (1981) report a second function for a yeast cytochrome P-450 in the \( \Delta^{22} \)-desaturation of ergosta-5,7-dien-3β-ol to form ergosterol, since mutants lacking a
functional lanosterol demethylase contained significant levels of \( \Delta^{22} \)-desaturation activity (Heta et al., 1983). It has been shown in this thesis that cytochrome P-450 accumulation occurs under conditions of rapid yeast growth which would necessitate the biosynthesis of large amounts of cellular membrane.

It is interesting to consider how ergosterol may be synthesised under conditions of aerobic growth at low glucose concentration, in the apparent absence of spectrally detectable cytochrome P-450. Aoyama et al. (1981) have demonstrated that yeast grown under these conditions is capable of lanosterol 14\( \alpha \)-demethylation, the activity being subject in inhibition by antibodies to purified yeast cytochrome P-450. If the fast rate of growth of yeast under fermentation conditions results in a higher level of lanosterol 14\( \alpha \)-demethylation and a higher level of cytochrome P-450 accumulation (Aoyama et al., 1981) then, conversely, with a low rate of growth such as in low glucose concentrations, there would be a lower level of lanosterol 14\( \alpha \)-demethylation and perhaps an accumulation of cytochrome P-450 which, though present, is not, as yet, possible to detect spectrophotometrically.

The amount of cytochrome P-450 accumulated in yeast during growth on high glucose growth media was found, in this thesis, to be highly strain dependent. The *S. cerevisiae* strain NCYC 240 has been identified as consisting of two morphological variants NCYC 753 and 754. The variant NCYC 754 is the major component (80%) and NCYC is the minor component (20%). These strains were examined for their ability to produce cytochrome P-450, detected spectrally in whole cells (King, Blatiak and Wiseman, 1983b). The major morphological variant NCYC 754 was found to accumulate nearly three times as much cytochrome P-
450 as NCYC and eight times as much as NCYC 753. Since NCYC 240 contains 80% of NCYC 754 the difference in the level of cytochrome P-450 accumulation between the two strains is difficult to explain (King, Blatiak and Wiseman, 1983b).

Petite mutant forms of *S. cerevisiae* were also investigated for their ability to accumulate cytochrome P-450 and from these experiments electron micrographs revealed unusual features in the external morphology.

Triton X-100, which was used for its stabilization effect, was found to bind to yeast cytochrome P-450 perhaps in the same way as a reverse type II binding compound and modulated the spin state to its low spin form (Azari, 1984). This resulted in lower mid-point redox potential and therefore the reduction of yeast cytochrome P-450 by sodium dithionite was found to be time dependent.

The possibility of interconversion of cytochrome P-420 to P-450 was investigated and whilst it is possible to infer such an occurrence in whole yeast and possibly yeast microsomal fraction it seems most unlikely to occur from purified yeast cytochrome P-450.

Another factor which may be involved in the accumulation of cytochrome P-450 is the amount of oxygen present during the growth of yeast. Rogers and Stewart (1973) demonstrated that cytochrome P-450 did not accumulate in yeast when grown without oxygen. It has been demonstrated in this thesis that *S. cerevisiae* grown in 20% glucose growth medium accumulates cytochrome P-450 up to a maximum level at the end of the exponential growth phase. The accumulation of
cytochrome P-450 being strongly associated with rapid growth, the stationary phase exhibiting a decline in cytochrome P-450 accumulation.

This thesis describes the role of oxygen as a substrate inducer of cytochrome P-450 in *S. cerevisiae*. When a yeast culture, which is growing exponentially under aerobic conditions, is made semi-anaerobic the cytochrome P-450 accumulation is inhibited with an increase in cytochrome P-450 less than 10% of the aerobic control. This effect is not due to an altered growth rate, since similar growth rates observed under both sets of conditions, with sufficient glucose still present to ensure actively fermenting yeast cells under glucose repression. In this instances, one effect of oxygen may be in acting as a substrate inducer of yeast cytochrome P-450. This may also explain why cytochrome P-450 cannot be detected in yeast grown under strictly anaerobic conditions (Rogers and Stewart, 1973). Since the endogenous role of cytochrome P-450 in *S. cerevisiae* in the 14a-demethylation of lanosterol in the biosynthetic pathway of ergosterol (Yoshida and Aoyama, 1980) when yeast is grown under strictly anaerobic conditions, ergosterol needs to be added to the growth medium as a supplement because oxygen is required for ergosterol biosynthesis.

This thesis examined the effect of culture shake speed and local oxygenation on the observed maximal accumulation of *S. cerevisiae* NCYC 754. A clear maximum cytochrome P-450 accumulation is observed at 120 rev/min with the enzyme level per gram of yeast decreasing at both slower and faster shake speeds. The maximum cytochrome P-450 accumulation occurs at the same shake speed as the maximum growth rate and also the maximum yield of yeast biomass. This reinforced the association between high cytochrome P-450
accumulation, biomass, and high rate of growth (King, Blatiak, Wiseman and Winkler, 1985). The optimum shake speed is considered to reflect the optimum degree of agitation of the culture, for cytochrome P-450 accumulation, in giving enhanced transfer of solutes between the microbial biomass, the bulk medium and the associated gas phase. Transfer rates generally increase with increasing agitation and an optimal agitation intensity may be expected to correspond to the optimal level of a particular solute. Agitation improves the availability of nutrients and the dispersal of metabolites in the microbial microenvironment, whilst increasing the dissolution rate of a sparingly soluble gas such as oxygen by replacing saturated liquid elements at the gas-liquid interface with elements from the bulk liquid. The appearance of an optimum agitation intensity would suggest that there is a corresponding optimum solute concentration and in this system the relevant solute is probably oxygen (Blatiak et al., 1985).

The culture shake speed experiments also showed that the level of oxygenation had an effect on the degradation of cytochrome P-450. The level of oxygenation when correlated with shake speed distinctly showed less degradation at higher and lower oxygen levels than at the oxygen level required for maximal cytochrome accumulation.

Karenlampi et al. (1981) studied the different levels of cytochrome P-450 accumulation in S. cerevisiae on a range of sugars. These workers found that high levels of cytochrome P-450 accumulated during growth of yeast at high concentrations of strongly fermentable sugars such as glucose, fructose or sucrose. There were lower levels of cytochrome P-450 accumulation with galactose or maltose, with simultaneous fermentation and respiration. When, however, a non-fermentable carbon
source was used for growth such as glycerol or lactate, no cytochrome P-450 accumulation occurred. These workers concluded that cytochrome P-450 accumulation occurred under conditions of rapid growth and fermentation but not necessarily with mitochondrial repression. This may be because the required haem is synthesised during both fermentative and respirative conditions (Labbe-Bois and Volland, 1977) it may be that the apoprotein synthesis is regulated in these different conditions.

Hortner et al. (1982) showed that the production of yeast haemoproteins iso-1-cytochrome c, catalase A and catalase T are coordinately controlled by glucose, oxygen and haem through the control of mRNA levels; there is also regulation at the post-transcriptional level (Laz et al., 1984). The levels of iso-1-cytochrome c and iso-2-cytochrome c are reduced in yeast grown anaerobically or under glucose repression, whereas the amount of apoprotein of each of cytochrome b, cytochrome c peroxidase and cytochrome c, is not reduced (Ros and Schatz, 1976). The production and addition of the haem is therefore the crucial controlling step in these systems, although it should be noted that the biosynthesis of the haem moiety occurs in mitochondria (Gudenus et al., 1984). Different haemoproteins respond differently to glucose, oxygen, haem and ethanol and this thesis has offered a contribution to the understanding of the mechanisms involved in the coordinate control of the production of eukaryotic proteins. However, it has not been shown here that haem concentration affects yeast cytochrome P-450 accumulation.

The degradation of cytochrome P-450 was found to require the presence of oxygen as much less degradation occurred under semi-anaerobic
conditions (Blatiak et al., 1980). Induced enzymes such as α-glucosidase are readily lost under conditions of de-adaptation (Wiseman and Lim, 1974). Little is known about the mechanism of destruction of enzymes under such conditions in yeast although an obligatory requirement for glucose as an energy source is usually stated. No glucose requirements for cytochrome P-450 degradation was found, although the yeast was grown in 20% glucose, but high glucose concentrations gave a marked protective effect against cytochrome P-450 (Blatiak et al., 1980). Mitochondrial utilization of the oxygen is probably involved in the degradative process, although oxygen itself can destroy cytochrome P-450 in the absence of substrates, due to formation of membrane lipid peroxides in situ. Glucose and oxygen are normally considered to be required for the production of a small quantity of ATP by mitochondria. In general, ATP may be required for biosynthesis of proteinases or for the transport across membranes of the degradative enzymes into appropriate vacuoles (Murakami et al., 1979).

Anaerobiosis, chloramphenicol, dinitrophenol and 20% glucose all inhibit mitochondrial function either at oxidative phosphorylation (dinitrophenol) or by lowering of intracellular cyclic AMP concentration (20% glucose) or by inhibition of protein synthesis (chloramphenicol); anaerobiosis, chloramphenicol and 20% glucose all promote the accumulation of cytochrome P-450 in yeast through prevention of the biosynthesis of cytochrome a + a3 in the yeast mitochondrion (Wiseman, 1980). The similar effect of cycloheximide, however, suggests that protein synthesis in the cytosol, in addition to that in mitochondria, is also required for cytochrome P-450 degradation (Blatiak et al., 1980).
Until recently, it was thought that yeast accumulated ethanol intracellularly to levels higher than that in the medium during fermentation (Beaver et al., 1982; Loureiro and Ferreira, 1983). However, recent work suggests that this is not the case and that *S. cerevisiae* does not accumulate ethanol against a concentration gradient but that the yeast membrane is freely permeable to ethanol (Guijarro and Lagunas, 1984). Ethanol and other alkanols are known to have many deleterious effects on yeast at higher concentrations of ethanol, including an inhibitory effect on yeast growth rate, fermentation rate and yeast viability (Brown et al., 1981; Jones and Greenfield, 1985). The mechanisms of these effects are unknown, although contributory factors might be the reduction in the uptake of solutes such as glucose (Leao and Van Uden, 1982) and amino acids (Leao and Van Uden, 1984a), passive proton influx across the plasma membrane (Leao and Van Uden, 1984b) and thermal death (Leao and Van Uden, 1982). Little is known about the effects of ethanol on the synthesis and degradation of enzymes in stationary phase yeast, although it should not be overlooked that ethanol causes a decrease in water activity and in dielectric constant of the growth medium. The decrease in viability caused by ethanol may be initiated by the interaction of ethanol with lipid components of cell membranes (Thomas et al., 1978). Thomas et al. (1978) demonstrated that yeast cells with added ergosterol were more resistant to ethanol. Casey et al. (1983) have shown that supplementation with ergosterol and tween 80 enabled yeast to grow in much higher levels of ethanol. Yeast cytochrome P-450 is known to be a key enzyme required for ergosterol biosynthesis (Aoyama et al., 1984), so if ethanol were affecting sterol biosynthesis through an effect on cytochrome P-450, then ergosterol supplementation would indeed be expected to overcome this. However, recent work has suggested that
supplementation with sterols including ergosterol could not protect yeast from ethanol inhibition of yeast growth (Walker-Caprioglio et al., 1985). Nevertheless, yeast is particularly sensitive to inhibition of growth and fermentation by ethanol under anaerobic condition (Aguilera and Benitez, 1985), presumably when cytochrome P-450 is absent.

The effect of ethanol and other alkanols on cytochrome P-450 was examined in this thesis. When reduced and oxidised ethanol was added to late exponential phase yeast cells in control, phosphate buffer, incubations the cytochrome P-450 level degraded rapidly. However when ethanol was added little, change in degradation was seen at first and only later did some degradation occur but less than in the control. This may reflect an altered degradation rate with additional de novo synthesis of ethanol induced cytochrome P-450 (Blatiak et al., 1987).

When yeast cells were incubated in buffer and alkanol, the cytochrome P-450 degradation was found to accelerate in the alkanols except ethanol. The increasing chain length of the alkanol resulted in a faster degradation of cytochrome P-450. This reflects the lipid-solubility of the alkanols; the more lipid-soluble the alkanol, the faster the degradation caused by its addition. The lipid-solubility of alkanols also correlates with many other effects of these compounds on yeast such as glucose transport and fermentation (Leao and Van Uden, 1985), ammonium transport (Leao and Van Uden, 1983) and thermal death (Leao and Van Uden, 1982). This suggests that the alkanols interfere with hydrophobic membrane regions, including the endoplasmic reticulum in which cytochrome P-450 is located. Ethanol however, seems exceptional in its lack of destructive effect on this enzyme (Blatiak et al., 1987).
High levels of glucose in the growth medium cause the accumulation of cytochrome P-450 in growing yeast. In this thesis, the effect of incubating the yeast after the transfer to non-growth conditions in phosphate buffer pH 7.0 with different concentrations of glucose was examined. With 8% (w/v) glucose a clearly marked increase in an already high existing level of cytochrome P-450 to 150% of the original level after 2-5 hours is seen. At 1% (w/v) glucose this effect is not seen, whereas with 5% (w/v) glucose some accumulation of P-450 occurs. At higher concentrations, 12% to 20% (w/v) glucose, less accumulation of P-450 was found, so that at 20% (w/v) glucose the results were similar to the 12% glucose. Under these conditions, fermentation of the glucose occurs, and it is possible that at 8% to 12% (w/v) glucose, fermentation may result in an optimum level of ethanol to further induce P-450. No such further accumulation of cytochrome P-450 could be observed using the 5 l. micro-processor controlled fermenter. However, here the oxygenation may be high enough to destroy the enzyme or, alternatively, to prevent the optimum level of ethanol for induction occurring.

The large accumulation of P-450 in yeast during rapid fermentative growth on high glucose-containing media has been difficult to explain when it seems that sufficient P-450 for lanosterol-14α-demethylation can be made even aerobically in low glucose medium (Aoyama et al., 1981). Although growth at high glucose concentration is very much faster than that at low glucose concentration, it is doubtful whether all of the extra cytochrome P-450 produced would be needed for the production of sufficient ergosterol at this faster rate of growth. The role of this high-glucose induced cytochrome P-450 is not understood therefore. The induction observed in high glucose might be related to the level of ethanol produced rather than the glucose level. In mammalian systems
Ethanol is known to induce a specific form of P-450 which has a very high activity towards the oxidation of ethanol to acetaldehyde (Coon et al., 1984), and is capable of oxidising a range of other substrates by a free-radical mediated mechanism (Ingelman-Sundberg and Hagbjork, 1982). It is likely that a comparable system is present in S. cerevisiae, and which is also capable of oxidising some xenobiotics such as benzo(a)pyrene. It is obvious that a complex mechanism of regulation of P-450 is present in S. cerevisiae. Low concentrations of oxygen induce P-450 whereas high levels repress or destroy the enzyme (Blatiak et al., 1985b). It is possible that haem made in mitochondria is also involved in regulating cytochrome P-450 levels as has been shown for iso-1-cytochrome c, (Guarente and Mason, 1983), so that further roles for oxygen are likely. This thesis has shown that ethanol, as well as glucose, is involved in the control of cytochrome P-450 accumulation.

Ethanol was also found here to be important in the regulation of cytochrome P-450 in S. cerevisiae. Morita and Mifuchi (1984) discovered enhanced cytochrome P-450 accumulation when the 2% glucose growth medium was supplemented with 1.5% or 3% ethanol. However, when cells were grown in glucose medium supplemented with 6% ethanol or in media with ethanol as the sole carbon source, no enhancement was seen. Del Carratore et al. (1984) found that the addition of 2% ethanol to 0.5% glucose growth media induced some cytochrome P-450 accumulation.

The addition of 1% ethanol into 0.5% glucose growth medium was found here to induce cytochrome P-450 (Blatiak et al., 1985, and 1987). It is not known, however, whether the isoenzyme induced by supplementing with ethanol is the same as that induced during growth at high glucose concentration, when the ethanol emanates from inside the yeast cell.
Ethanol is a known inducer of a mammalian cytochrome P-450, in hepatic tissue, which operates via a free radical mediated mechanism (Ingelman-Sundberg and Hagbjork, 1982). The ethanol induced yeast cytochrome P-450 isoenzyme may bear some relationship to the mammalian ethanol induced isoenzyme and may also operate via a free radical mechanism. The mammalian ethanol induced cytochrome P-450 isoenzyme has a very high activity for the oxidation of ethanol to acetaldehyde (Coon et al., 1984), the yeast isoenzyme may be involved in similar activity during fermentation at high glucose concentration during which time large amounts of ethanol are produced. It would be most worthwhile to determine whether ethanol induced yeast cytochrome P-450 isoenzyme was different in any way from that induced normally by glucose since ethanol tolerance in yeast may be then better understood and this would be of significant benefit to the brewing industry. Ethanol is, of course, present in yeast during fermentative growth, under which conditions cytochrome P-450 is produced in large quantities. It is likely therefore that under these conditions ethanol-induced cytochrome P-450 is synthesised in the endoplasmic reticulum of the yeast cell, and that the main role of this isoenzyme may be to oxidise ethanol. The removal of ethanol would be expected to protect the protein biosynthesis system, however, in this thesis, using various agents to remove ethanol did not have a clear effect on cytochrome P-450 accumulation or degradation.

It is possible that a different isoenzyme of cytochrome P-450 may be induced with ethanol, the production of a specific isoenzyme may occur at the expense of another less specific form. If these effects were to occur at transcriptional level then ethanol may be responsible for switching off one gene and switching on another through binding to a
repressor protein causing its activation or inhibition. This may be a positive and negative control exerted by the ethanol (which may thus be acting as an inducer) over the different operators responsible for the functioning of the structural genes for the synthesis of the corresponding forms of mRNA. Oxygen could possibly operate in a similar way to ethanol in inducing cytochrome P-450.
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APPENDIX
APPENDIX 1

Computer Program for Mitochondrial Cytochrome Calculation

OK  BASIC
> BASIC V REV 18.2.9
> LIST
SOLVE 1
90  PRINT "A" PROGRAM TO SOLVE A SYSTEM OF FOUR LINEAR EQUATIONS
100  DIM A (4,4) B (4,4) C (4,1) X (4,1)
110  DATA 21, 10, 3, -3, 14, 63
120  DATA 6.61, 18.10, 4, 57, 91
130  DATA -1, 18, 94, 16, 4.326
140  DATA -.22, -.58, 0, 14.
150  MAT READ A
160  MAT B = INV (A)
170  PRINT "TYPE IN DATA POINTS, ONE NUMBER PER ROW"
180  MAT INPUT C
190  MAT X = B * C
200  PRINT "THE SOLUTIONS, IN THE ORDER C, C1, B, A, ARE"
210  MAT PRINT X
220  END.
> RUN
SOLVE 1.

To get hard copy:
30
LOGIN BCH019
OK, COMO filename (create a name)
OK, BASICV
> OLD  SOLVE1
> RUN

> QUIT
OK, COMO -END
OK, PRINT filename, COM1
OK (spooled) LOGOUT
PUBLICATIONS
Mechanism of degradation of cytochrome P-450 in non-growing Saccharomyces cerevisiae: anaerobiosis, chloramphenicol; dinitrophenol and cycloheximide and protective agents.
Andrew A. Blatiak, Javid A. Gondal and Alan Wiseman

Cytochrome P-450 production and benzo(a)pyrene hydroxylase activity and inducibility in Saccharomyces cerevisiae NCYC 240 and its morphological variants, NCTC 753 and 754.
David J. King, Andrew Blatiak and Alan Wiseman

The role of oxygen as a substrate inducer of cytochrome P-450 in Saccharomyces cerevisiae, and its opposing influence through enzyme degradation.
Andrew Blatiak, David J. King and Alan Wiseman

Effect of culture shake speed and local oxygenation on the observed maximal accumulation of cytochrome P-450 in Saccharomyces cerevisiae at the fastest growing rate.
David J. King, Andrew Blatiak, Alan Wiseman and Michael A. Winkler

Effect of alcohols and of glucose on the level of cytochrome P-450 in Saccharomyces cerevisiae after resuspension in buffer.
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Enzyme induction by oxygen in the accumulation of cytochrome P-450 during batch fermentations in 20% D-glucose with Saccharomyces cerevisiae.
A. Blatiak, D.J. King, A. Wiseman, J. Salihon and M.A. Winkler
Enzyme Microbiology Technology (1985) 7, 553-556.

Regulation of the presence of cytochromes P-450 in Saccharomyces cerevisiae: effects of glucose, ethanol and oxygen concentrations.
A. Blatiak, A. Wiseman and M.A. Winkler
Mechanism of degradation of cytochrome P-450 in non-growing Saccharomyces cerevisiae: anaerobiosis, chloramphenicol, dinitrophenol and cycloheximide as protective agents

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We have reported previously the conditions of glucose repression needed for the biosynthesis of Saccharomyces cerevisiae of a cytochrome P-450 with benzofaipyrene activity (Woods & Wiseman, 1980), and the partial repression of this enzyme in growing protoplasts by added cyclic AMP (Wiseman et al., 1978). Protoplasts used glucose for growth equally well in the presence or absence of cyclic AMP, suggesting adequate transport of glucose in the presence of cyclic AMP. Rapid degradation of cytochrome P-450 in the absence of cyclic AMP was observed after the 6 h incubation period when growth ceases in the protoplasts. This resembles the rapid loss of this enzyme observed in yeast cultures once the stationary phase of growth is reached and is a generally observed phenomenon for enzymes in stationary cultures of bacteria (St. John et al., 1979).

We now report the investigation of this phenomenon in whole yeast and its prevention by shaking incubation under anaerobic conditions, and to a lesser extent by chloramphenicol, cycloheximide, dinitrophenol or 20% glucose, all in non-growth media containing 0.1 M phosphate buffer, pH 7.

Saccharomyces cerevisiae (N.C.Y.C. no. 240) was grown at 30°C in shake flasks in 20% glucose-containing complex medium as described previously (Wiseman et al., 1978). Spectrophotometric assay of cytochrome P-450 in whole yeast suspensions was described also by these authors. De-adaptation studies on whole yeast were performed at 30°C by aerobic shaking incubation in 0.1 M phosphate buffer at pH 7.0 alone, or in the presence of additives (see Fig. 1). Samples were taken at 2, 4, 6 and 8 h for spectrophotometric assay of the cytochrome P-450 (only the active form of the enzyme displays the peak at 430 nm).

Induced enzymes especially, such as α-glucosidase, are readily lost under conditions of de-adaptation, where aerobic shaking incubation for 3 h in 3% glucose is usually used (Wiseman & Lim, 1974). Little is known about the mechanism of destruction of enzymes under such conditions in yeast, although an obligatory requirement for glucose as an energy source is usually stated. We have found no requirement for glucose (but the yeast was grown in 20% glucose medium), readily lost under conditions of de-adaptation, where aerobic shaking incubation for 3 h in 3% glucose is usually used (Wiseman & Lim, 1974). Little is known about the mechanism of destruction of enzymes under such conditions in yeast, although an obligatory requirement for glucose as an energy source is usually stated. We have found no requirement for glucose (but the yeast was grown in 20% glucose medium), either at oxidative phosphorylation (dinitrophenol), or by lowering of intracellular cyclic AMP concentration (20% glucose) or by inhibition of protein biosynthesis in the case of chloramphenicol. Anaerobiosis, chloramphenicol and 20% glucose all promote the biosynthesis of cytochrome P-450 in yeast through prevention of the biosynthesis of cytochrome d + α, in the yeast mitochondrion (Wiseman, 1980). The similar effect of cycloheximide, however, suggests that protein biosynthesis in the cytosol, in addition to that in mitochondria, is also required for cytochrome P-450 degradation (or destruction of the active conformational form). Interest is growing in the inter-relationship between the mitochondrion, cytosol and endoplasmic reticulum of cells. Recently cytochrome c has been shown to be degraded in rat liver mitochondria (Dugue-Magalhaes & Menezes Ferreira, 1980) and there is a stimulation of yeast mitochondria protein biosynthesis by a postpolyribosomal supernatant from yeast, rat liver, or Escherichia coli (Everett et al., 1980). Conversely, a chloramphenicol-sensitive labelling of protein in the microsomal fraction of Neurospora crassa has been reported (Macklin et al., 1977).


Fig. 1. Survival of cytochrome P-450 when shaken aerobically at 30°C in 0.1 M-potassium phosphate buffer: protection by chloramphenicol, cycloheximide, dinitrophenol and semi-anaerobic conditions

Symbols: ■, 0.1 M-potassium phosphate buffer + 20% glucose + 1 mM-chloramphenicol; △, 0.1 M-potassium phosphate buffer + 20% glucose + 1 mM-2,4-dinitrophenol; ○, 0.1 M-potassium phosphate buffer + 20% glucose; ●, 0.1 M-potassium phosphate buffer; ×, 0.1 M-potassium phosphate buffer + 20% glucose + 1 mM-cycloheximide; ▲, semi-anaerobic conditions.
The yeast *Saccharomyces cerevisiae* produces cytochrome P-450 when grown under fermentative conditions, such as when grown at a high glucose concentration (Wiseman, 1980). This enzyme from *Saccharomyces cerevisiae* N.C.Y.C. 240 has been shown to be capable of catalysing benzo(a)pyrene hydroxylation (Wiseman & Woods, 1979). This activity is inducible by the addition of benzo(a)pyrene and several other compounds to the yeast growth medium, resulting in an improvement in enzyme efficiency yet only a small overall increase in total cytochrome P-450 amounts (King et al., 1982). The yeast strain N.C.Y.C. 240 has recently been identified as consisting of two morphological variants, N.C.Y.C. 753 and N.C.Y.C. 754 (B. Kirsop, personal communication). The variant N.C.Y.C. 754 is the major (80%) component and N.C.Y.C. 753 is the minor component (20%). We have examined these strains individually for their cytochrome P-450 contents and for their hydroxylation activity towards benzo(a)pyrene and compared these results with those for N.C.Y.C. 240.

Strains of *Saccharomyces cerevisiae* were grown under glucose repression at 30°C as previously described (Wiseman et al., 1978). Cytochrome P-450 was measured by the spectrophotometric method of Omura & Sato (1964). Benzo(a)pyrene induction experiments were carried out at 95 μM benzo(a)pyrene as previously described (King & Wiseman, 1983). Benzo(a)pyrene hydroxylase activity was measured by a modification of the method of Dehnen et al. (1973) as described by Woods & Wiseman (1980).

Fig. 1 shows the time course of cytochrome P-450 production in the *S. cerevisiae* strains N.C.Y.C. 240, 753 and 754. The major morphological variant of N.C.Y.C. 240 (754) produces a far higher value of this enzyme than does strain 240. Little difference is seen in the time taken to reach this peak value, as all of these yeasts grow at approximately the same rate, with the peak in cytochrome P-450 content occurring at the end of the exponential growth phase in each case. The minor variant (753) produces only a very small amount of cytochrome P-450. This provides a new way of distinguishing between these two yeasts.

As expected, N.C.Y.C. 240 produces an intermediate amount of cytochrome P-450. However, as N.C.Y.C. 240 consists of approx. 80% of N.C.Y.C. 754, the marked difference between these two yeasts is still maintained.

The ability of the cytochrome P-450 produced by these yeasts to catalyse benzo(a)pyrene hydroxylation was the same when calculated per nmol of cytochrome P-450, with very similar values for $K_m$ (111 μM (240)), 109 μM (753) and 102 μM (754), and for $V_{max}$ (μmol of 3-hydroxybenzo(a)pyrene/h per nmol of cytochrome P-450) 167 (240), 175 (753) and 222 (754). However, there is much less cytochrome P-450 in N.C.Y.C. 753 and therefore this probably does not contribute much to the metabolism seen in N.C.Y.C. 240.

After growth in the presence of benzo(a)pyrene, the benzo(a)pyrene hydroxylase specific activity of N.C.Y.C. 240 is greatly increased, but total cytochrome P-450 amounts remain similar to those in the uninduced yeast (King et al., 1982; King & Wiseman, 1983). The strains N.C.Y.C. 753 and 754 were also tested for their ability to be induced by growth for 44 h in the presence of 95 μM benzo(a)pyrene. The major form (754) was found to be induced in a similar manner to N.C.Y.C. 240, i.e. with little change in cytochrome P-450 content but a large decrease in $K_m$ to 40 μM and an increase in $V_{max}$ to 476 pmol of 3-hydroxybenzo(a)pyrene/h per nmol of P-450. However, N.C.Y.C. 753 showed very little induction of its benzo(a)pyrene hydroxylase under these conditions. The reasons for this lack of induction in N.C.Y.C. 753 are unknown, but must reflect 'strain' differences between these yeasts.

We thank Dr. Barbara Kirsop Curator of the National Collection of Yeast Cultures, A.R.C. Food Research Institute, Norwich, for drawing our attention to the morphological variants of N.C.Y.C. 240 and for gifts of the strains N.C.Y.C. 753 and N.C.Y.C. 754.

King, D. J. & Wiseman, A. (1983) *Biochem. Soc. Trans.* 11, 000-000
Oxygen is thought to play an important role in the biosynthesis of cytochrome P-450 in *Saccharomyces cerevisiae*. This yeast produces cytochrome P-450 only under fermentative conditions such as when grown at a high glucose concentration or under semi-anaerobic conditions (Wiseman, 1980). Under these conditions, repression of mitochondrial cytochromes is achieved and the intracellular concentration of cyclic AMP is low. The production of yeast cytochrome P-450 is thought to be regulated by the concentration of intracellular cyclic AMP by repression; thus, when the cyclic AMP concentration is low, biosynthesis of cytochrome P-450 can occur (Wiseman et al., 1978). Cytochrome P-450 is produced rapidly during the exponential phase of growth, but is lost during the stationary phase. Previous studies revealed that mitochondrial utilization of oxygen is required for cytochrome P-450 degradation in yeast, and therefore anaerobic conditions exert a protective effect on the enzyme (Blatiak et al., 1980).

*Saccharomyces cerevisiae* (N.C.Y.C 240) was grown aerobically in shake culture as previously described (Wiseman et al., 1978). To achieve anaerobic conditions, flasks were bubbled through with nitrogen for 10 min, filled completely and sealed under nitrogen. Cytochrome P-450 was measured by the method of Omura & Sato (1964).

Fig. 1 shows the change in cytochrome P-450 production in aerobically growing cultures of *S. cerevisiae* when made anaerobic during the exponential growth phase (24 h growth) and during the stationary phase (48 h growth). When a yeast culture growing exponentially is made anaerobic, the biosynthesis of cytochrome P-450 is inhibited, with an increase in cytochrome P-450 between 24 and 48 h of less than 10% of that in the aerobic control. This effect is not due to an altered growth rate, as very similar growth rates were observed under both sets of conditions, with sufficient glucose still present (at 24 h) to ensure actively fermenting yeast cells under glucose repression, as required for optimal cytochrome P-450 biosynthesis (Wiseman et al., 1978). One effect of oxygen may be in acting as a substrate inducer of yeast cytochrome P-450. This may also explain why cytochrome P-450 cannot be detected in yeast grown under strictly anaerobic conditions (Rogers & Stewart, 1973).

When cultures of *S. cerevisiae* were grown anaerobically to the beginning of stationary phase (48 h growth), and then made anaerobic and incubated further at 30°C, cytochrome P-450 did not appear to be lost as quickly. At stationary phase, the yeast cytochrome P-450 concentration is already falling and thus removal of oxygen at this time is unlikely to effect cytochrome P-450 biosynthesis. The slowing of cytochrome P-450 loss under anaerobic conditions is due to protection from degradation, as previously described for yeast in a 20% (w/v) glucose buffer under anaerobic conditions (Blatiak et al., 1980). Trinn et al. (1981) working with yeast in continuous culture, have shown that without glucose repression it is not possible to achieve cytochrome P-450 production by oxygen limitation. T. K. Lim & A. Wiseman (unpublished work) have demonstrated that in steady-state continuous culture of *S. cerevisiae* cytochrome P-450 production is optimal at a dissolved oxygen concentration between 16 and 30% of saturation, in a medium containing 20% glucose (dilution rate 0.1 h⁻¹).

The endogenous role of cytochrome P-450 in *S. cerevisiae* is thought to be in the 14α-demethylation of lanosterol, which is the first step in the biosynthetic pathway of ergosterol, the major sterol in yeast membranes (Yoshida & Aoyama, 1980). When yeast is grown under strictly anaerobic conditions, ergosterol needs to be added to the growth medium as a supplement, because oxygen is required for ergosterol biosynthesis. Therefore, during growth under these conditions cytochrome P-450 is not required for sterol synthesis and does not appear to be produced (Rogers & Stewart, 1973). When oxygen is introduced in small quantities, cytochrome P-450 is produced, presumably for use in sterol biosynthesis. Oxygen may therefore be acting as a substrate inducer of cytochrome P-450 in this system.

**Fig. 1.** Production of cytochrome P-450 in aerobically growing yeast made anaerobic after 24 or 48 h, and growth continued

Aerobic control (**●**) is shown along with the production of cytochrome P-450 after making the yeast anaerobic at 24 h (**○**) and at 48 h (**■**). Growth was in 20% glucose media.

Effect of culture shake speed and local oxygenation on the observed maximal accumulation of cytochrome P-450 in \textit{Saccharomyces cerevisiae} at the fastest growth rate

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The yeast \textit{Saccharomyces cerevisiae} produces a cytochrome P-450 enzyme which is thought to catalyse the 14α-demethylation of lanosterol, a key step in the biosynthesis of ergosterol, the major sterol of yeast membranes (Aoyama et al., 1984). When grown under conditions of fermentative growth, e.g., at a high glucose concentration, this yeast accumulates large quantities of cytochrome P-450 (Wiseman, 1980). Several factors are important in the regulation of cytochrome P-450 biosynthesis in yeast. A single nuclear gene is responsible for controlling cytochrome P-450 biosynthesis with a second gene modulating the amount of the enzyme produced (King et al., 1983). No cytochrome P-450 is produced in yeast grown strictly anaerobically (Rogers & Stewart, 1973) yet several groups have routinely used semi-anaerobic conditions to enhance the level of cytochrome P-450 in their yeast (Ishidate et al., 1969; Yoshida & Aoyama, 1984). Thus oxygen may have an important role in regulating cytochrome P-450 biosynthesis.

\textit{S. cerevisiae} (NCYC 754) was grown at 30°C in an orbital shaker at a series of different shake speeds. The growth medium consisted of 20% (w/v) glucose, 2% (w/v) mycological peptone, 1% (w/v) yeast extract and 0.35% (w/v) sodium chloride. Yeast was inoculated with a wire loop into 100 ml of medium in 250 ml flasks. Dry weight estimates were obtained by removing samples from the culture, washing the yeast with distilled water and drying at 70°C to constant weight. Cytochrome P-450 was determined directly in whole cells using its reduced carbon monoxide difference spectrum (Omura & Sato, 1964).

Fig. 1 shows the effect of growing \textit{S. cerevisiae} in batch culture at a series of shake speeds on the accumulation of cytochrome P-450 and the growth of yeast. A clear maximum cytochrome P-450 level is observed at 120 rev./min, with the enzyme level per g of yeast decreasing at both slower and faster shake speeds. The maximum yield of cytochrome P-450 occurs at the same shake speed as the maximum growth rate and also the maximum yield of yeast biomass. This suggests a close link between a high yield of cytochrome P-450 and a high growth rate.

These interesting findings with the orbital shaker are related to the known effect of agitation in giving enhanced transfer of solutes between the microbial biomass, the bulk medium and the associated gas phase. Transfer rates generally increase with increasing agitation, and an optimal agitation intensity may be expected to correspond to the optimal level of a particular solute. In parallel work, using stirred-tank fermentation (Salihon et al., 1985), cytochrome P-450 yield from \textit{S. cerevisiae} NCYC 754 showed a clearly defined optimum for agitation. With growth conditions optimized using air as oxygen source, growth under identical conditions, but using air mixed with either nitrogen or oxygen, gave severely reduced enzyme yields, indicating that the agitation optimum found in this work indeed corresponds to optimal dissolved oxygen availability.

Blatiak et al. (1983) have previously reported that small amounts of oxygen may induce cytochrome P-450 synthesis. When exponentially growing cultures of \textit{S. cerevisiae} were subsequently made anaerobic, the level of cytochrome P-450 finally produced was less than 10% of that in the control flasks with oxygen available. This effect was not due to an altered growth rate. Also Rogers & Stewart (1973) have shown that \textit{S. cerevisiae} does not produce cytochrome P-450 when grown under strictly anaerobic conditions, and that a low oxygen level in the growth medium is required for maximum cytochrome P-450 production.

The endogenous role of cytochrome P-450 in \textit{S. cerevisiae} is thought to be in ergosterol biosynthesis. When yeast is grown anaerobically, ergosterol and a fatty acid source need to be added to the growth medium because oxygen is required for their biosynthesis. Therefore during growth under anaerobic conditions cytochrome P-450 is not needed for sterol synthesis and does not appear to be produced. When oxygen is introduced in small quantities, cytochrome P-450 is produced, presumably for use in sterol biosynthesis.

Effect of alcohols and of glucose on the level of cytochrome P-450 in Saccharomyces cerevisiae after resuspension in buffer

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There is much interest in the ethanol tolerance of yeast and in commercial production of ethanol by fermentation. The factors contributing to ethanol tolerance of yeast are however not well understood. Cytochrome P-450 is an important enzyme in the biosynthesis of ergosterol, the major yeast membrane sterol (Aoyama et al., 1984). Therefore any effects on this enzyme may be important in explaining the effects of ethanol and higher alcohols in yeast growth, fermentation and yeast viability (Brown et al., 1981). Glucose is known to protect against cytochrome P-450 degradation (Blatiak et al., 1980) but has not alone been previously reported to enhance cytochrome P-450 levels during stationary phase.

Saccharomyces cerevisiae (N.C.Y.C. No. 754) was grown in a medium containing glucose (2%) w/v, mycological peptone (1% w/v), yeast extract (1% w/v) and NaCl (0.5% w/v) in 100 ml batches in an orbital incubator at 30°C (140 rev./min). After 40 h growth, the yeast was harvested by centrifugation and incubated in 0.1 M-phosphate-buffer, pH 7.0, alone, with buffer and 8% (w/v) glucose and also buffer with 6% (v/v) n-alkanols. Cytochrome P-450 levels were determined by the method of Omura et al., (1965).

In S. cerevisiae the cytochrome P-450 level is known to fall rapidly at the end of the exponential growth phase (Wiseman et al., 1978). This has been partially attributed to the decline in the glucose concentration in the growth medium (Wiseman et al., 1975). One reason for the rapid loss of cytochrome P-450 may be the rise in ethanol concentration to approx. 6% at the end of exponential growth. There is also a rise in the concentration of the higher alkanols (fusel-oil).

The deleterious action of ethanol on yeast growth rate, yeast viability and fermentation rate is of unknown cause, although reduced growth rate may be due to a reduction in the uptake of solutes such as glucose (Leao & van Uden, 1982) and of amino acids (Leao & van Uden, 1984). However, little is known about the effect of ethanol on the biosynthesis and degradation of enzymes present in stationary phase yeast, especially in relation to viability and growth rate upon re-inoculation into growth media.

Our incubation (with whole yeast) results show only a small increase in the rate of degradation of cytochrome P-450 in the presence of 6% (v/v) ethanol, when compared with incubation in phosphate buffer alone. Moreover, after 5 h, the cytochrome P-450 level in 6% (v/v) ethanol became higher than the phosphate buffer control. This may be a result of the use of ethanol as an energy source under these aerobic conditions, much like in the enhancement of cytochrome P-450 level upon incubation in glucose.

In our studies of incubation of whole yeast with the first six members of the n-alkanols, the half-life of cytochrome P-450 decreased progressively (except for ethanol) as the chain length of the alkanol was increased. Thus, with an initial cytochrome P-450 level of 3.8 nmol/g wet weight of yeast we find that in 6% (v/v) methanol in buffer the enzyme half-life was 130 min 6% (v/v) ethanol 510 min 6% (v/v) propanol 100 min 6% (v/v) butanol 20 min 6% (v/v) pentanol less than 4 min (phosphate buffer alone 420 min). It is likely that the degradative effect of alkanols on cytochrome P-450 can be correlated with lipid solubility of the alkanol (i.e. with the chain length of the alkanol). This is presumably due to limitation of interaction between the alkanol and the cytochrome P-450 in its location in the membranes of the yeast endoplasmic reticulum. Ethanol itself however is remarkably non-destructive in relation to the cytochrome P-450 present, although it may exhibit many other deleterious effects.

We have reported previously the protection of cytochrome P-450 in whole yeast from degradation by 20% (w/v) glucose (Blatiak et al., 1980). Our results show that incubation of yeast with 8% (w/v) glucose not only protects the enzyme from degradation but also increases the level of cytochrome P-450 by 25% after 4 h, despite the absence of growth. The cause of this enhancement is being investigated in relation to the control of glucose repression and glycolysis in yeast. This finding is important in relation to yield of enzyme in our large-scale batch isolation work.

It is of interest that we in addition observe induction of cytochrome P-450 by ethanol in growth medium containing 0.5% glucose and 1% ethanol in growth under aerobic conditions. 1.5 nmol of cytochrome P-450/g wet weight of yeast was found, whereas without the ethanol no enzyme was observed at this low concentration of glucose. Similar observations have been reported by Del Carratore et al. (1984) and Morita & Mifuchi (1984). This induced cytochrome P-450 may be comparable with that induced in liver of the rabbit, after ethanol ingestion (Ingleman-Sudber & Hagbjork, 1982). This form of cytochrome P-450 is unusual in working through a free-radical mediated mechanism, to oxidise the ethanol.


1985
Enzyme induction by oxygen in the accumulation of cytochrome P-450 during batch fermentations in 20% D-glucose with Saccharomyces cerevisiae

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Production of cytochrome P-450 [RH, reduced-flavoprotein: oxygen oxidoreductase (RH-hydroxylating), EC 1.14.14.1] by Saccharomyces cerevisiae NCYC 754, grown in batch culture on 20% D-glucose medium, was markedly affected by the speed of the orbital shaker. Oxygenation rather than agitation was confirmed as the likely cause of this effect using an optimized system in a microprocessor-controlled 4 litre batch fermenter. Oxygen may be acting as a substrate inducer of the cytochrome P-450 in this yeast.

Keywords: Yeast, cytochrome P-450, oxygen, batch fermentation

Introduction

Cytochrome P-450 [RH, reduced-flavoprotein: oxygen oxidoreductase (RH-hydroxylating), EC 1.14.14.1] designates a group of monoxygenase enzymes, the best known of which are found in the mammalian liver, where they catalyse the monoxygenation of many xenobiotics including drugs, carcinogens and pesticides. Cytochrome P-450 enzymes are also involved in the monoxygenation of many endogenous substrates such as fatty acids and steroids. These enzymes are widespread throughout nature and have been demonstrated to exist in organisms from all animal and most plant phyla and also in many microorganisms.

We are engaged in the large-scale production and isolation of this enzyme from Saccharomyces cerevisiae, which employs a microprocessor-controlled fermenter. It is important to establish the role of oxygen and to optimize this aspect of the process.

In S. cerevisiae, cytochrome P-450 is thought to be involved in the 14a-demethylation of lanosterol, a key step in the biosynthetic pathway to ergosterol, the major yeast membrane sterol. A different yeast cytochrome P-450 enzyme may also be involved in ergosterol biosynthesis, catalysing the Δ22-desaturation of ergosta-5,7-dien-3β-ol. Cytochrome P-450 from S. cerevisiae can also catalyse the monoxygenation of several xenobiotics including benzoylpyrene, aminopyrine, p-nitroanisole and caffeine. Yeast cytochrome P-450 can also metabolize many promutagens in their active mutagenic products, which provides an important in situ yeast test for mutagens and some carcinogens. Despite the endogenous role of cytochrome P-450 in the biosynthesis of ergosterol, the enzyme has only been demonstrated spectrally in yeast grown under conditions of fermentative growth, such as with high D-glucose concentration in the growth medium. Aoyama et al. have shown that S. cerevisiae grown aerobically in 1% D-glucose media contains a very low level of cytochrome P-450, not detectable spectrophotometrically, but capable of catalysing lanosterol 14α-demethylation and subject to inhibition by antibodies to purified yeast cytochrome P-450 from yeast grown by fermentation.

The production of cytochrome P-450 in S. cerevisiae is controlled by a single nuclear gene, and at least one modifier gene is involved in the modulation of the amount of cytochrome P-450 produced, although the identity of these genes is so far unknown. Cyclic AMP may be involved in regulating cytochrome P-450 biosynthesis in S. cerevisiae. During growth at high D-glucose concentration, such that cytochrome P-450 is produced in large quantities, the cyclic AMP level is low. Also the addition of cyclic AMP to yeast protoplasts greatly reduces the production of cytochrome P-450 during subsequent growth in 5% D-glucose medium, whereas 5′-AMP and 2′-(3′)-AMP have no effect, suggesting that cyclic AMP may control cytochrome P-450 production by a form of repression.

Oxygen is important in the regulation of cytochrome P-450 biosynthesis in S. cerevisiae. Several groups have routinely used semianaerobic growth conditions to enhance the level of cytochrome P-450 in their yeast, yet when S. cerevisiae is grown strictly anaerobically, cytochrome P-450 cannot be detected. In this study we have attempted to define more closely the role of oxygen in relation to agitation in the accumulation of cytochrome P-450 in S. cerevisiae. Agitation is known to increase the homogeneity in a system and enhances the transfer of solutes between biomass and the bulk medium and between the liquid medium and the gas phase with which it is in contact. It thus improves the availability of nutrients and the dispersal of metabolites in the microbial microenvironment.
general, transfer rates increase as the intensity of agitation increases, with the effect being most pronounced in the case of sparingly soluble gaseous solutes. Their concentration in the bulk medium is the result of a dynamic balance between the rate of their dissolution or excretion into the medium and the rate of their uptake or desorption from it. Agitation increases the dissolution rate of a sparingly soluble gas such as oxygen by replacing saturated liquid elements at the gas—liquid interface with elements from the bulk liquid. This maintains a high activity driving force for gas dissolution between the gas and liquid phases. The rate of interface element renewal increases as the intensity of agitation increases. A similar mechanism describes the desorption of gaseous metabolites. The appearance of an optimum agitation intensity would suggest that there is a corresponding optimum solute concentration, and in this system the relevant solute is likely to be oxygen. Part of this work has been reported briefly in a preliminary communication.15

Materials and methods

*S. cerevisiae* strains NCYC 240 and 754 were obtained from the National Collection of Yeast Cultures, AFRC, Norwich, and were maintained on slopes of Sabouraud-dextrose agar. Yeast was grown from wire loop inoculations at 30°C in a Gallenkamp orbital incubator in 100 ml medium consisting of (% w/v): D-glucose, 20; mycological peptone, 2; yeast extract, 1; and sodium chloride, 0.5, in a 250 ml conical flask. Parallel work was carried out with a 4 litre microprocessor-controlled fermenter, under optimized conditions for cytochrome P-450 production [25.1°C, pH 5.04, impeller speed 253 rev min⁻¹, gas flow rate 150 ml min⁻¹ in a medium consisting of (% w/v): D-glucose, 14.05; mycological peptone, 1.04; yeast extract, 1.69, and sodium chloride, 0.09]. Mycological peptone was from London Analytical and Bacteriological Media Limited and yeast extract from Oxoid, Basingstoke.

For anaerobic experiments, flasks were bubbled through with oxygen-free nitrogen for 10 min and sealed under nitrogen.

Cytochrome P-450 was determined directly on whole yeast cells using the reduced carbon monoxide difference spectrum method of Omura and Sato.16 An absorption coefficient of 911 mmol⁻¹ cm⁻¹ was used and the results expressed as nmol cytochrome P-450 g⁻¹ (dry weight) yeast.

Results and discussion

*S. cerevisiae* grown at a high D-glucose concentration produces cytochrome P-450 rapidly during the exponential growth phase, up to a maximum level at about the end of the exponential phase and the beginning of the stationary phase (Figure 1). Thus the accumulation of cytochrome P-450 in *S. cerevisiae* is associated with growth, and the enzyme level begins to decline when rapid growth ends. We are investigating the cause of this sharp decline.

Figure 2 shows the effect of growing *S. cerevisiae* (NCYC 754), in batch culture at a series of shake speeds in an orbital shaker, on the accumulation of cytochrome P-450 and the growth of yeast (all in 20% D-glucose media).

A clear optimum in cytochrome P-450 level is observed at 120 rev min⁻¹, with the enzyme level g⁻¹ yeast being lower at both faster and slower shake speeds. The maximum yield of cytochrome P-450 occurs in this orbital shaker work at the same point as the maximum growth rate (and therefore the maximum yield of yeast biomass).

Although other factors such as homogeneity may be important, the peak in cytochrome P-450 level may well reflect the degree of aeration of the culture, with an optimum aeration level being required to produce the maximum cytochrome P-450 level. This hypothesis is supported by parallel work using a 4 litre fermenter under optimized conditions (see below).
Several groups of workers have previously used rather ill-defined, semianaerobic conditions to enhance the level of cytochrome P-450 in S. cerevisiae. Ishidate et al.\textsuperscript{17} reported that high levels of cytochrome P-450 were produced in yeast grown semianaerobically in 4% (w/v) D-glucose medium, whereas the group of Yoshida et al.\textsuperscript{18} and also similar conditions with 3% (w/v) D-glucose media.\textsuperscript{19} Rogers and Stewart\textsuperscript{14} found that when S. cerevisiae was grown on a medium containing 4% (w/v) D-galactose as carbon and energy source cytochrome P-450 production was optimum at a dissolved oxygen concentration of 0.25—0.5 μmol l\textsuperscript{-1}. Also, Trinn et al.\textsuperscript{20} working with S. cerevisiae in continuous culture in a medium containing 3% (w/v) D-glucose, found that a low aeration rate is required for the production of a large amount of cytochrome P-450. Thus, there is agreement in the literature that a relatively low dissolved oxygen concentration is required for optimum cytochrome P-450 production. However, under strictly anaerobic conditions, no detectable cytochrome P-450 is produced.\textsuperscript{14} The optimum dissolved oxygen level found (0.25—0.5 μmol l\textsuperscript{-1})\textsuperscript{14} is well below the sensitivity level of most commercially available dissolved oxygen sensors.

In parallel work using the microprocessor-controlled 4 litre stirred tank fermenter, agitator speed and air flow rate into the culture had been found to affect the yield of cytochrome P-450 from S. cerevisiae NCYC 754 very strongly, being significant at a 99% confidence level.\textsuperscript{23} Culture conditions had then been optimized with respect to a number of operational parameters, including agitator speed and air flow rate, producing an enzyme yield of 126 nmol g\textsuperscript{-1} (dry weight) yeast, more than 2.5 times the yield obtained with the best previous shake flask results. In our present investigation of the effects of agitator speed and gas flow rate, two fermentations were run using the optimum conditions, except that instead of using the optimum air flow rate of 9 litres h\textsuperscript{-1}, a gas flow comprising 6 litres h\textsuperscript{-1} air mixed with 3 litres h\textsuperscript{-1} oxygen was used. Fermentation conditions, including the agitation effect of gas flow, were thus the same as the optimum conditions, except for an increased dissolved oxygen tension (DOT). The enzyme yield was reduced to an average 34.5 nmol g\textsuperscript{-1} (dry weight) yeast, although the biomass yield was about the same as under optimum conditions. Two further runs were performed under optimum conditions except that the gas flow comprised 6 litres h\textsuperscript{-1} air mixed with 3 litres h\textsuperscript{-1} oxygen-free nitrogen. The enzyme yield was again reduced, this time to an average of 30.35 nmol g\textsuperscript{-1} (dry weight) yeast, but with a similar biomass yield to the optimum and increased DOT conditions.\textsuperscript{23,24} This showed that when using the optimum aeration and agitation conditions determined, their principal effect was to maintain optimum dissolved oxygen availability, which supports the conclusion that throughout this work the optimum orbital agitation conditions found correspond to an optimum dissolved oxygen availability.

*Figure 3* shows the change in cytochrome P-450 production in aerobically growing cultures of S. cerevisiae in 20% D-glucose medium, when made anaerobic during the exponential growth phase (24 h growth) and the stationary phase (48 h growth). Results shown are for the lower yielding strain of S. cerevisiae NCYC 240,\textsuperscript{21} although very similar results were obtained with NCYC 754. When a yeast culture growing exponentially is made anaerobic, the biosynthesis of cytochrome P-450 is inhibited (with increase in cytochrome P-450 level between 24 and 48 h of <10% of that in the aerobic control). This effect is not due to an altered growth rate, as very similar growth rates were observed under both sets of conditions. One role of oxygen may be as a substrate inducer of yeast cytochrome P-450, the removal of oxygen thus removing the induction of this enzyme.

When cultures of S. cerevisiae were made anaerobic during the stationary phase (after 48 h growth) and incubated further at 30°C, cytochrome P-450 did not seem to be lost as quickly. At stationary phase the yeast cytochrome P-450 level is already falling, and thus removal of oxygen at this time is unlikely to effect cytochrome P-450 biosynthesis. The slowing of cytochrome P-450 loss under anaerobic conditions is due to protection from degradation as previously described for yeast in a 20% (w/v) D-glucose buffer under anaerobic conditions.\textsuperscript{22}

As mentioned above, the endogenous role of cytochrome P-450 in S. cerevisiae is thought to be in ergosterol biosynthesis. When yeast is grown under strictly anaerobic conditions, ergosterol and a fatty acid source need to be added to the growth medium, as oxygen is required for their biosynthesis. Therefore, under these anaerobic conditions of growth cytochrome P-450 is not needed for sterol synthesis and does not appear to be produced.\textsuperscript{14} When oxygen is introduced in small quantities, cytochrome P-450 is produced, presumably for use in sterol biosynthesis. Oxygen may therefore be acting as a substrate inducer of cytochrome P-450 in this system. A role for oxygen as an inducer of cytochrome P-450 is also interesting from an evolutionary viewpoint. Wickramsinghe and Vilee\textsuperscript{25} have postulated that cytochrome P-450 may initially have evolved for the removal of toxic oxygen species in primitive organisms. Wilkinson\textsuperscript{26} has supported the idea that cytochrome P-450 has a remote monophyletic origin, possibly originally involved in oxygen detoxification, and that it has evolved throughout the plant and animal kingdoms to fulfil a variety of roles.

**References**


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**Figure 3** Production of cytochrome P-450 in 20% D-glucose medium at 30°C. Aerobically growing yeast was made anaerobic after 24 or 48 h and growth continued. •, Aerobic control; •, yeast made anaerobic after 24 h; •, yeast made anaerobic after 48 h. Values are the mean of six determinations, bars indicate standard deviation.
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REGULATION OF THE PRESENCE OF CYTOCHROMES P-450
IN SACCHAROMYCES CEREVISIAE: EFFECTS OF
GLUCOSE, ETHANOL AND OXYGEN

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Running Title: Cytochromes P-450:
Saccharomyces cerevisiae

Section: Biochemistry

Index: Cytochromes P-450
Saccharomyces cerevisiae
Glucose effects: on yeast
Ethanol effects: on yeast
Oxygen effects: on yeast
Summary:

The accumulation of cytochromes P-450 in *S. cerevisiae* is under complex regulatory control. We have shown previously that low levels of oxygen induce cytochrome P-450 in yeast (Blatiak et al., 1985) and we now report that ethanol can induce this enzyme under conditions where cytochrome P-450 is not normally detectable. Glucose in the concentration range 5-20% is well known to cause the accumulation of cytochrome P-450 in growing yeast under conditions therefore of mitochondrial repression, and this finding has been extended to non-growing yeast at a limited range of glucose concentration, where similarly, ethanol is produced by fermentation perhaps at optimal concentration for induction. Added alkanols, other than ethanol, cause rapid degradation of cytochrome P-450 in non-growing yeast, where the rate of loss is directly related to the lipid solubility of the alkanol. Ethanol has an unexpected ability therefore to favour the accumulation of cytochrome P-450 in yeast, and this may be related to an important putative role of one of the isoenzymes present, in the removal by oxidation, of ethanol from the endoplasmic reticulum region of the yeast cell. Increased levels of oxygen, rather than ethanol, in stationary phase culture may trigger the rapid disappearance of cytochrome P-450 observed at this time.
Introduction:

Cytochrome P-450 enzymes (RH, reduced-flavoprotein: oxygen oxidoreductase (RH-hydroxylating), EC 1.14.14.1) are a group of haemoproteins which catalyse the monooxygenation of both endogenous and exogenous substrates. In Saccharomyces cerevisiae, cytochrome P-450 is involved in the 14α-demethylation of lanosterol during ergosterol biosynthesis, which is required for yeast membrane synthesis in the endoplasmic reticulum (Aoyama et al., 1984). Cytochrome P-450 from S. cerevisiae can also catalyse the monooxygenation of some xenobiotic compounds such as benzo(a)pyrene (Wiseman and Woods, 1979; King et al., 1984), aminopyrine, p-nitroanisole and caffeine (Sauer et al., 1982), and activate many promutagens to their active mutagenic products (Callen et al., 1980; Kelly and Parry, 1983).

During growth under strictly fermentation conditions at glucose concentrations in the range 5-20%, S. cerevisiae accumulated large amounts of cytochromes P-450, more than is apparently required for its endogenous role in lanosterol 14α-demethylation (Aoyama et al., 1981). The reasons for this accumulation of cytochromes P-450 are not well understood although factors such as glucose, oxygen and ethanol are believed to be important (Blatiak et al., 1985; Wiseman et al., 1978; Morita and Mifuchi, 1984; Del Carratore et al., 1984; Karenlampi et al., 1981). Also, under these conditions, glucose repression of mitochondrial enzymes, such as cytochrome a+a3 occurs.

The production of cytochrome P-450 in S. cerevisiae was shown by tetrad analysis to be controlled by a single nuclear gene which was not the structural gene, with at least one modifier gene being involved in the modulation of the amount of cytochrome P-450 produced (King et al., 1983). The identity of these genes is so far unknown. Cyclic AMP may be involved in regulating cytochrome P-450 production in S. cerevisiae by some form of negative control (Wiseman et al., 1978). In addition, cytochrome P-450 may be destroyed in stationary phase of culture by a cyclic AMP-dependent covalent modification as
reported for rabbit liver enzyme (Muller et al., 1985), which could be oxygen-concentration dependent.

It has been shown that the production of the yeast haemoproteins iso-1-cytochrome c, catalase T and catalase A are coordinately controlled by glucose, oxygen and haem through the control of mRNA levels (Hortner et al., 1982), although there is also regulation at the post-transcriptional level (Hortner et al., 1982; Laz et al., 1984). The levels of iso-1-cytochrome c and iso-2-cytochrome c are reduced in yeast grown anaerobically or under glucose repression, whereas the amount of apoprotein of each of cytochrome b, cytochrome c peroxidase and cytochrome c, is not reduced (Ross and Schatz, 1976; Ciejan et al., 1980). The production and addition of the haem is therefore the crucial controlling step. Cytochrome P-450 itself is only detectable spectrally when yeast is grown semi-anaerobically or under glucose repression. Different haemoproteins respond differently to glucose, oxygen, haem and ethanol, therefore studies on the regulation of these systems should contribute to our understanding of the mechanisms involved in the coordinate control of the production of eukaryotic proteins.

We have recently shown that cytochrome P-450 is induced in *S. cerevisiae* by small amounts of oxygen but that at high oxygen levels the cytochrome P-450 level is reduced (Blatiak et al., 1985). In this study we attempt to examine the roles of ethanol and glucose in cytochrome P-450 accumulation, and to relate this to oxygenation, both during growth and in stationary phase culture.
Methods:

*Saccharomyces* strain NCYC 754 was maintained on slopes of Sabourand-dextrose agar. Liquid cultures were grown from wire loop inoculations at 30°C in 100 ml of medium of (% w/v): D-glucose, 20; mycological peptone, 2; yeast extract, 1; and sodium chloride, 0.5 in an orbital shaker set at 120 revs/min, shaken aerobically.

Cytochrome P-450 was determined directly on whole yeast cells using the reduced carbon monoxide difference spectrum method of Omura and Sato (1964), using an extinction coefficient of 91 mM⁻¹ cm⁻¹.

Ethanol was measured enzymically by using alcohol dehydrogenase and measuring the increase in absorbance at 340 nm of NADH, using a kit from BCL Boehringer Corporation, London. Glucose was measured by the glucose oxidase method also using a kit from BCL Boehringer Corporation, London. Some experiments, as noted later, were done in a 4 litre micro-processor controlled fermenter under optimal conditions for cytochromes P-450 production. These were as follows: 25.1°C, pH 5.04, impeller speed 253 rev. min⁻¹, gas flow rate (air) 150 ml min⁻¹ in a medium consisting of (% w/v) glucose 14.05, mycological peptone 1.04, yeast extract 1.69 and sodium chloride 0.09 (Blatiak et al., 1985).
Results and Discussion:

Cytochrome P-450 was produced in NCYC 754 to a maximum level at the end of exponential growth and was rapidly lost during stationary phase (Figure 1). The accumulation of cytochrome P-450 seems closely linked to growth. As expected, the level of glucose declines rapidly with a concurrent increase in ethanol level which later falls, probably due to ethanol being used as a carbon source under these aerobic conditions. The rapid fall of cytochrome P-450 level in stationary phase has been partially attributed to the decline in the glucose concentration in the growth medium (Wiseman et al., 1975). Another factor might be the rise in ethanol concentration to approximately 6% at the end of exponential growth. Our results (see on) do not substantiate this view, but favour the view that loss of cytochrome P-450 is due to build up of oxygen in the medium after growth ceases, in association with oxygen-mediated destruction of the enzyme.

Until recently it was thought that yeast accumulated ethanol intracellularly to levels higher than that in the medium during fermentation (Beaver et al., 1982; Loureiro and Ferreira, 1983). However, recent work suggests that this is not the case and that S. cerevisiae does not accumulate ethanol against a concentration gradient but that the yeast membrane is freely permeable to ethanol (Guijarro and Lagunas, 1984). Ethanol (and other alkanols) are known to have many deleterious effects on yeast at higher concentrations of ethanol including an inhibitory effect on yeast growth rate, fermentation rate and yeast viability (Brown et al., 1981; Jones and Greenfield, 1985). The mechanisms of these effects are unknown although contributory factors might be the reduction in the uptake of solutes such as glucose (Leao and Van Uden, 1982) and amino acids (Leao and Van Uden, 1984), passive proton influx across the plasma membrane (Leao and Van Uden, 1984) and thermal death (Leao and Van Uden, 1982). Little is known about the effects of ethanol on the synthesis and degradation of enzymes in stationary phase yeast. The decrease in viability
caused by ethanol may be initiated by the interaction of ethanol with lipid components of cell membranes (Thomas et al., 1978). Thomas et al. (1978) demonstrated that yeast cells with added ergosterol were more resistant to ethanol. Casey et al. (1983) have shown that supplementation with ergosterol and tween 80 enabled yeast to grow in much higher levels of ethanol. Yeast cytochrome P-450 is known to be a key enzyme required for ergosterol biosynthesis (Aoyama et al., 1984), so if ethanol were affecting sterol biosynthesis through an effect on cytochrome P-450, then ergosterol supplementation would indeed be expected to overcome this. However, recent work has suggested that supplementation with sterols including ergosterol could not protect yeast from ethanol inhibition of yeast growth (Walker-Caprioglio et al., 1985). Nevertheless, yeast is particularly sensitive to inhibition of growth and fermentation by ethanol under conditions of anaerobiosis (Aguilera and Benitez, 1985), presumably when cytochrome P-450 is absent.

The effect of adding ethanol to late exponential phase yeast cells was examined for its effect on the cytochrome P-450 level. Cells harvested in late exponential phase, washed and resuspended in phosphate buffer. In control incubations in phosphate buffer the yeast cytochrome P-450 level declined to approximately 15% of its original level in 12 hours (Figure 2). When ethanol was added little change in degradation rate was seen at first, but later the cytochrome P-450 level was stabilized to approximately 40% of the original level after 12 hours. This may reflect an altered degradation rate after 5-6 hours or some new synthesis of ethanol-induced cytochrome P-450. This effect is also reflected in Table 1 which shows an increased half-life for cytochrome P-450 when yeast cells were incubated with ethanol compared to the control in buffer alone. This effect was not seen with alkanols other than ethanol, and these other alkanols all accelerated degradation. An increase in chain length of the alkanol used resulted in faster loss of cytochrome P-450 (Table 1). This finding reflects the lipid-solubility of the alkanols; the more
lipid-soluble the alkanol, the faster the degradation caused by its addition. The lipid-solubility of alkanols also correlates with many other effects of these compounds on yeast such as glucose transport and fermentation (Leao and Van Uden, 1985), ammonium transport (Leao and Van Uden, 1983) and thermal death (Leao and Van Uden, 1982). This suggests that the alkanols interfere with hydrophobic membrane regions, including the endoplasmic reticulum in which cytochrome P-450 is located. Ethanol however seems exceptional in its lack of destructive effect on this enzyme.

When grown in 0.5% glucose media we have found that we can detect no yeast cytochrome P-450 by spectral means (King, 1982), and this finding has been supported by other groups (Aoyama et al., 1981; Karenlampi et al., 1981). To further examine a possible induction effect by ethanol, cultures of yeast in 0.5% glucose media were supplemented with 1% ethanol. This resulted in the induction of spectrally detectable cytochrome P-450, between 20 and 50 hours of incubation, as shown in Figure 3.

Ethanol is a well known inducer of cytochrome P-450 in mammalian liver where it induces a form of P-450 which operates via a free-radical mediated mechanism to oxidise the ethanol (Ingelman-Sundberg and Hagbjork, 1982). Ethanol induction of cytochrome P-450 in S. cerevisiae has also been observed by Del Carratore et al. (1984) and Morita and Mifuchi (1984). This induced form of cytochrome P-450 may be similar to the mammalian form induced by ethanol. The metabolism of 8-methoxypsoralen by S. cerevisiae is also induced by ethanol (Prognon et al., 1984), and this may be mediated by a cytochrome P-450 enzyme system. Ethanol is, of course, present in yeast during fermentative growth, under which conditions cytochrome P-450 (cytochrome P-448 isoenzyme) is produced in large quantities. It is likely therefore that under these conditions ethanol-induced cytochrome P-450 is made, and that the main role of this isoenzyme is to oxidise ethanol. Free-radical mediated forms of cytochrome P-448 would be sensitive to inhibition by free-radical trapping agents such as the dithiothreitol often used to stabilise cytochromes P-450.
High levels of glucose in the growth medium, cause an increase in the level of P-450 in growing yeast. In Figure 4 the effect of incubating yeast under non-growth conditions with different concentrations of glucose is shown. With 8% glucose a marked increase in cytochrome P-450 level to 150% of the original level after 5 hours is seen, which later falls. At 1% glucose this effect is not seen, whereas with 5% glucose some accumulation of P-450 occurs. At higher concentrations, 12% to 20% glucose, progressively less accumulation of P-450 is seen. Under these conditions, fermentation of the glucose does occur and it is likely that at 8%/glucose, fermentation may result in an optimum level of ethanol to induce P-450. No such accumulation of cytochrome P-450 could be observed using the 4 litre micro-processor controlled fermenter. However, here the oxygenation may be high enough to prevent the optimum level of ethanol for induction occurring. In earlier work we found that there was protection from degradation of P-450 by anaerobiosis and other agents that inhibited protein biosynthesis in yeast mitochondria. These would also stop the aerobic metabolism of ethanol (Blatiak et al., 1980). If the yeast is put into 8% glucose-containing growth media then the accumulation of P-450 seen with 8% glucose in buffer is not seen after 5 hours (Figure 5), although normal growth occurs and the P-450 level eventually rises to a maximum at the end of exponential growth, as is usual.

The large accumulation of P-450 in yeast during rapid fermentative growth on high glucose-containing media has long been a puzzle when it seems that sufficient P-450 for lanosterol-14α-demethylation can be made even aerobically in low glucose media (Aoyama et al., 1981). Although growth at high glucose concentration is faster than that at low glucose concentration, it is doubtful whether all of the extra P-450 produced would be needed for the production of sufficient ergosterol at this faster rate of growth. The role of high glucose induced P-450 is not understood. The induction observed in high-glucose might be related to the level of ethanol produced rather than the glucose level. In
mammalian systems ethanol is known to induce a specific form of P-450 which has a very high activity towards the oxidation of ethanol to acetaldehyde (Coon et al., 1984), and is also capable of oxidising a range of other substrates by a free-radical mediated mechanism (Ingelman-Sundberg and Hagbjork, 1982). It is likely that a comparable system is present in S. cerevisiae, and which is also capable of oxidising some xenobiotics such as benzo(a)pyrene.

It is obvious that a complex mechanism of regulation of P-450 is present in S. cerevisiae. Low concentrations of oxygen induce P-450 whereas high levels repress or destroy the enzyme (Blatiak et al., 1985). We have now shown that ethanol, as well as glucose, are also involved in control of P-450 levels. It is likely that haem made in mitochondria is also involved in regulating P-450 levels as has been shown for iso-1-cytochrome c (Guarente and Mason, 1983), so that further roles for oxygen are inevitable. The complexity of the regulatory system is perhaps not surprising as several forms of cytochrome P-450 are now known to exist in S. cerevisiae (King et al., 1982). Part of this work has been reported briefly in a preliminary communication (Blatiak et al., 1985).
Table 1

Half-Life of the Cytochrome P-450 Peak when Whole Yeast (shaken aerobically) is Incubated in the Presence of the First Five Members of the Alkanol Series

<table>
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<tr>
<th>Solution</th>
<th>Time $t_{\frac{1}{2}}$ (mins.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control phosphate buffer alone</td>
<td>420</td>
</tr>
<tr>
<td>Methanol</td>
<td>130</td>
</tr>
<tr>
<td>Ethanol</td>
<td>510</td>
</tr>
<tr>
<td>Propanol</td>
<td>100</td>
</tr>
<tr>
<td>Butanol</td>
<td>20</td>
</tr>
<tr>
<td>Pentanol</td>
<td>22</td>
</tr>
</tbody>
</table>
References:


41. Wiseman, A., Lim, T.K. and McCloud, C. (1975) Biochem. Soc. Trans. 3 276-278. Relationship of cytochrome P-450 to growth phase of Brewer's yeast in 1% or 20% glucose medium.

Cytochrome P-450 n.mol/g.wet wt.

Incubation time (h)
Legends to Figures

Figure 1  The production of cytochrome P-450 during yeast growth in 20% (w/v) glucose medium correlated with the rise in ethanol concentration and loss of glucose in the growth medium. **cytochrome P-450, nmol/g wet weight of yeast** □ glucose concentration, % w/v □ ethanol concentration, % v/v. Values are the means of 6 determinations, error bars indicate standard deviation.

Figure 2  The effect of ethanol on cytochrome P-450 loss (nmol/g wet weight of yeast) during the incubation of yeast resuspended in phosphate buffer ▲ yeast incubated aerobically with shaking in 100 mM phosphate buffer pH 7.0; □ yeast incubated in 100 mM phosphate buffer pH 7.0 containing 6% (v/v) ethanol. Values are the means of 6 determinations, error bars indicate standard deviation.

Figure 3  The production of cytochrome P-450 (nmol/g wet weight of yeast) during yeast growth in 0.5% (w/v) glucose medium containing 1% (v/v) ethanol. In the absence of ethanol no cytochrome P-450 could be detected. Values are the means of 6 determinations, error bars indicate standard deviation.

Figure 4  The effect of added glucose on the cytochrome P-450 level (nmol/g wet weight of yeast) in yeast incubated aerobically with shaking in phosphate buffer ▲ yeast incubated in 100 mM phosphate buffer pH 7.0; □ yeast incubated in buffer containing 1% (w/v) glucose; ○ yeast incubated in buffer containing 12% (w/v) glucose; ○ yeast incubated in buffer containing 8% (w/v) glucose. Values are the means of 6 determinations, error bars indicate standard deviation.