Use of Yeast species as the Biocomponent for Priority Environmental Contaminants Biosensor Devices

Saroja Gurazada

Thesis submitted to
Auckland University of Technology
in fulfilment of the degree of
Doctor of Philosophy (PhD)

2008

School of Applied Science

Primary Supervisor: Dr. John Robertson

TABLE OF CONTENTS

ANNEXRE II
Baronian, K.H.R & Gurazada, S. (2007) - Electrochemical detection of wild type *Saccharomyces cerevisiae* responses to estrogens. *Biosensors and Bioelectronics, 22,* 2493-2499.
ANNEXURE III
Baronian, K.H., Gurazada, S and Thomas, A. (2005). Electrochemical detection of yeast responses to catabolizable substances. *Australian Journal of Chemistry, 58,* 270-274.

LIST OF FIGURES

LIST OF TABLES

ATTESTATION OF AUTHORSHIP

I, Saroja Gurazada, hereby declare that this submission is my own work and that to the best of my knowledge and belief it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

ACKNOWLEDGEMENTS

ABSTRACT

Along with an increasing understanding of the harmful effects on the environment of a wide range of pollutants has come the need for more sensitive, faster and less expensive detection methods of identification and quantitation.

Many environmental pollutants occur in low levels and often in complex matrices thus analysis can be difficult, time consuming and costly. Because of the availability and easy cultivation of the microorganisms with potentially high specificity, there is considerable interest in the use of living microorganisms as the analytical component (the biocomponent) of sensors for pollutants.

While a number of biosensors using bacteria have been developed, yeast have been comparatively rarely used as the biocomponent. Yeast are attractive because they are easy to culture and they are eukaryotes which means their biochemistry is in many respects closer to that of higher organisms. This thesis describes the development of whole cell bioassays that use yeast cells as a sensing element and redox mediators to probe the intracellular redox reactions to monitor the catabolic activity of the yeast resulting from the external substrate, steady-state voltammetry is utilised as the electrochemical detection technique.

The isogenic differential enzyme analysis (IDEA) concept of Lincoln Ventures Limited, lead NERF funded research consortium uses bacteria that have been cultured using specific organic pollutants as the carbon source which are the biocomponent in sensors. The use of wild type yeast *Arxula adeninivorans* that has the ability to use a very wide variety of substrates as sources of carbon and nitrogen was used as an alternative to bacteria to validate the "IDEA" concept. Naphthalene and di-butyl phthalate were chosen as model target contaminant molecules. The performance, detection limits and the usefulness of yeast based biosensor applications for environmental analysis are discussed.

This thesis also describes the development and optimisation of a simple, cost effective *in vivo* estrogen bioassay for the detection of estrogens using either genetically modified or a wild type yeast *Saccharomyces cerevisiae*. In this study, catabolic repression by

glucose was exploited to achieve specificity to estrogens in complex environmental samples that eliminates the requirement for conventional sample preparation. This is the first time that the use of wild type yeast to quantify estrogens has been reported. The attractive features of the bioassay are its use of a non-GMO organism, its speed, its high specificity and sensitivity with a detection limit of 10^{-15} M. The similarity of binding affinities for major estrogens to those of human estrogen receptors makes this *in vivo* estrogen bioassay very useful for analytical/screening procedures. The electrochemical detection method also makes it easy to interface with a variety of electronic devices.

ABBREVIATIONS

bp = base pairs

cDNA = complementary deoxyribonucleic acid

DBP = di-butyl phthalate

DNA = deoxyribose nucleic acid

DNA pol = DNA polymerase enzyme

dNTP = deoxyribonucleotide triphosphates

EBP = estrogen binding protein

EBP1 = gene encoding estrogen binding protein in *Candida albicans*

ERE = estrogen responsive elements

EtBr = ethidium bromide

(^{3}H) 17β-estradiol = tritiated hydrogen (radiolabel led) 17ß-estradiol

FBPase = fructose 1,6-bisphosphatase

FADH2 = flavin adenine dinucleotide

FMN = flavin monooxygenases

GFP= green fluorescent protein

hER = human estrogen receptor

HXT = genes encoding glucose transporter proteins

HXT7 = gene encoding high affinity glucose transporter

HXT2 = gene encoding glucose signalling protein

kb = kilobase

Lac Z = gene encoding β-galactosidase enzyme

Lux = gene encoding luciferase enzyme

MIG1 = gene encoding glucose repressor

mRNA = messenger ribonucleic acid

nA = nanoamperes

NAD (P) - nicotine amide adenine dinucleotide phosphate

OD = optical density

ORF = open reading frame

OYE = old yellow enzyme

PAGE = polyacrylamide gel electrophoresis

PCR = polymerase chain reaction.

PDR = pleiotropic drug resístance genes

PDR5 = gene encoding estradiol export protein

PDR5 = estradiol export protein

PbEBP = gene encoding estrogen binding protein in *Paracoccioides brasiliensis*

Δpdr5 = deletion mutant in gene encoding estradiol export protein

rpm = revolutions per minute

ScEBP = gene encoding estrogen binding protein in *Saccharomyces cerevisiae*

SNQ2 = estradiol import protein

SNQ2 = gene encoding estradiol import protein

Δsnq2 = deletion mutant in gene encoding estradiol import protein

Snf = protein kinase

Taq = *Thermus aquaticas*

TBE buffer = trisborate ethidium bromide buffer

TMPD = 2, 3, 5, 6-tetramethyl-1, 4-phenylenediamine

tPMET = Trans-plasma membrane electron transport

URA = uracil

CHAPTER 1

INTRODUCTION

Environmental contaminants are a threat to humans and other life forms and are of increasing concern to both the public and to government agencies. As we develop a better understanding of the complex web of life on earth, more substances are being added to those that are known to be harmful to the environment. Thus, there is an urgent need to develop rapid, reliable and cost effective methods of environmental monitoring.

Increasing numbers of biosensors and bioassays are being developed to meet these demands as they offer several advantages such as cost effectiveness and greater sensitivity over other chemical sensors and instrumental analysis. The goal of this research was to develop yeast-based bioassays that can detect environmental contaminants. For this work the priority environmental contaminants were selected from various EPA (US Environment Protection Agency) lists viz (EPA) National Online Service Library (OCLC), United States of America, naphthalene (EPA/OCLC - 822R03005; Miller & Miller, 1974 ; Dipple, Michejda & Weisburger, 1985), phthalates (EPA/OCLC - 744b-94-001; Juberg, 2000; Colborn & Clement, 1992) and estrogens (EPA/OCLC - 44724044; Juberg, 2000; Colborn & Clement, 1992; Hilscherova et al., 2000). The contaminants were selected based on their interaction with the cell; naphthalene and phthalates are metabolised by the cell, estrogens bind to a cell receptor.

Prior to this study there were no reports of wild type yeast being used for estrogen screening and analysis. This study established that using the glucose catabolite repression property of *S. cerevisiae* to eliminate the catabolism of all other catabolisable molecules in complex environmental matrices. An excellent specificity and sensitivity to estrogens was shown. This is a novel application for *S. cerevisiae* and has significant potential for a sensitive, inexpensive biosensor.

1.1 Biosensors
A biosensor is a device in which a biological component such as an enzyme, antibody, nucleic acid or microorganism (whole-cell) interacts with an analyte, which is then detected by an electronic component (transducer) and transmuted into a measurable signal. Biosensors have been reported which can analyse various environmental

pollutants including phenolics (Wang et al., 1991), ammonia (Hikuma et al., 1980), formaldehyde (Cammann, 1992), polycyclic aromatic hydrocarbons (PAHs) (Vo-Dinh et al., 1987), metals (Uo et al., 1992; Tescione & Belfort, 1993), insecticides (Rogers et al., 1991; Anis et al., 1992) and herbicides (Anis et al., 1993). Biosensors can thus be used for the monitoring of environmental contaminants.

Biosensors are useful not only for measuring various analytes such as gases, ions and organic compounds, but also for the study of microbial metabolic responses to various analytes such as phthalates, polycyclic aromatic compounds (PAHs), phenols, benzene, benzidines/aromatic amines, organophosphates, nitrates/nitrites, which can be used as sole nitrogen and carbon sources (Rogers, 1995). Biosensors have applications in environmental analysis (Rogers, 1995) medical diagnostics (Wise et al., 1991) bioreactor monitoring and controlling, clinical chemistry testing and fermentation control (US patent.no. 5540828).

Sensitive biosensors have been developed using nanotechnology (Vo Dinnh et al., 2001; Haruyama, 2003; Jain, 2003). Nanomaterials are submicron in size and have been employed in the manufacture of devices such as nanosensors, nanoprobes and other nanosystems to enable rapid analysis of multiple substances *in vivo* (Jianrong et al., 2004). A new technique has been developed to rapidly recognise the biological target molecules by using super magnetic nanoparticles and microscope based on a high-transition temperature superconducting quantum interference device (SQUID) (Chemela et al., 2000). Most biological molecules can be labelled electrochemically with metal nanoparticles without losing their biological activities. A new electrochemical method for monitoring biotin-streptavidin interaction has been developed using colloidal gold as an electrochemical label (Gonzalez-Garcia et al., 2000). Biotinylated albumin is adsorbed on the pre-treated surface of a carbon paste electrode immersed in the colloidal gold-sterptavidin labelled solution. Adsorptive voltammetry is used to monitor colloidal gold bound to streptavidin. The analytical signal is highly reproducible and a linear relationship between peak current and streptavidin concentration from 2.5 nM to 25 μM was obtained for a sequential co-operative assay (Gonzalez-Garcia et al., 2000). Sensitive real-time electrically based biosensors were developed (Cui et al., 2001) to monitor chemical and biological components by using sensitive boron-doped silicon nanowires (SiNW's). Biotin-modified SiNW's were used to detect biotin-streptavidin interaction and could detect streptavidin to a picomolar concentration range. Optical

fibres of less than 1 μm diameter were coated with antibodies and used for detection of the toxic substance benzopyrene tetrol (BPT) within mammary carcinoma (human) cells and rat liver epithelial cells (Cullum et al., 2000). Park et al. (2002) reported a new array-based electrical detection of DNA with nanoparticle probe. Alkyl-thiol-modified oligonucleotides were immobilised onto the surface of Silicon dioxide substrate between two gold microelectrodes with 20 μm gaps. Silver deposition facilitated by the gold nanoparticles bridged the gap and is led to readily measurable conductivity changes. This method could detect target DNA at a very low concentration of 500 fM (Park et al., 2002).

Biocomponent technology is normally interfaced with an electronic transducer to convert a biochemical signal into a quantifiable physical response, e.g., an electrical signal. A general diagram of a biosensor depicting its function is shown (Figure 1.1)
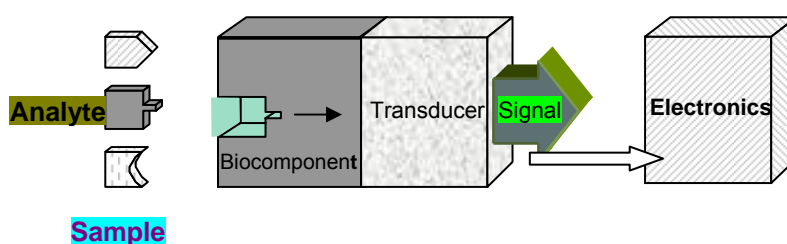
Sensing Element	Transducer	Signal quantitation
Tissue	Optical	
Receptor		
Cell	Electrochemical	
Enzyme	Acoustic	
Antibody		
DNA		

Figure 1.1 A generic biosensor schematic.
(From - Pasco et al., 2001)

The success of the biosensor design depends on various factors some of which are given below (Hall, 1992)

 a. Identification of the analyte and appropriate transduction systems.

 b. Immobilisation and optimisation of recognition elements and transducer technology.

 c. Identification of the appropriate physicochemical method.

 d. Assessment of the range of detection of interfering molecules to obtain specificity.

1.2 Biocomponents that can be used for biosensors
(the use of biocomponents and whole cells as biosensors)

A variety of biological elements can be used as biocomponents in the biosensors. Examples of biological components include enzymes (glucose oxidase, lactate oxidase, oxalate oxidase, urease, cholesterol oxidase, choline oxidase and esterase), nucleic acids (DNA and RNA) proteins (antibodies), tissues e.g., kidney, potato tuber, bovine liver, banana slice and sugar beet (Eggins, 1996) and microbes (bacteria and yeast). A good biosensing system requires reliability, simple operation, portability, specificity to the analyte, real-time analysis capacity and long shelf life (D'Souza, 2001).

1.2.1 Antibodies
Antibodies can bind specifically to a wide variety of natural and synthetic molecules. Antibody technology has been used for a substantial period in medical diagnostic tests to identify diseases for preparative, quantitative analysis and an enzyme assays. The ELISA (enzyme linked immunosorbent assay) has been used for detection and quantitation of biochemicals in medical and other diagnostics (Ren et al., 1998., Engvall & Perlmann, 1972). Immunosensors using antibodies as biocomponents have been used to detect pathogens such as *Salmonella typhimurium* a positive antigen (Jung et al., 2004). Suzuki et al. (2002) developed miniaturised immunosensors based on surface-plasmon resonance (SPR) to detect human immunoglobulin G (IgG). One difficulty in immunosnesor development is dissociation of the strongly bound antigen/antibody complex and a "regenerable" immunosnesor has been developed for this reason (Suzuki et al., 2002).

1.2.2 Cell receptors

Cells use receptors on the surface of the cell membrane for the transmission of responses across the membrane, e.g., neural transmission and hormone regulation. The binding of a ligand to a receptor causes a detectable cellular response that is related to the concentration of the ligand. Cell receptors have an advantage over an antibody/antigen binding in that only the active form of the ligand will bind to the receptor compared to an antibody, which will often bind to active and inactive forms, i.e., more selectivity (Glatz et al., 2006). Glatz et al. (2006) have used G-protein coupled receptors (GPCRs) and their G-proteins to detect various binding parameters in a cell free environment. Routledge & Sumpter (1996) studied the human estrogen receptor (ER) and its ligands interaction by using genetically engineered hER *S. cerevisiae*. The receptor-based biosensors have been developed using acetylcholine and opiate receptors immobilised in a polymeric film in the presence of polymerising agent (glutaraldehyde) and detect the receptor-ligand (anolyte) interaction electrochemically (US. Patent. no. 5192507).

1.2.3 Enzymes

Enzymes are biological catalysts of cells. Purified enzymes can be used as biocomponents because they exhibit high substrate specificities. A range of enzymes with some redox properties such as oxidases, peroxidases and reductases have been used for enzyme sensors using electrochemical detection. The most widespread enzyme biosensor today is the glucose biosensor. Glucose oxidase enzyme does not require a co-factor but some enzymes such as dehydrogenases (except glucose dehydrogenase, which contains a prosthetic group) require a co-factor such as NAD^+. Glucose oxidase (Gox) enzyme catalyses glucose in the presence of oxygen to gluconic acid and hydrogen peroxide. The glucose concentration can be quantitated by measuring either oxygen consumption by using a Clark O_2 electrode (Clark, 1962) or by measuring the dissociation of hydrogen peroxide using the peroxidase enzyme. Consumption of O_2 by Gox will result in a decrease in current or dissociation of H_2O_2 will increase the current.

Other enzyme detection systems in biosensors

Many enzymes from the groups of hydrolases, lyases, oxidoreductases, ligases and transferases are known to effect pH as a consequence of their biocatalytic action. This change in pH occurs when its substrate is completely converted to the end product. Stredansky et al (2000) have developed amperometric biosensors using penicillinase, urease and oxaloacetate decarboxylase enzymes and pH sensitive redox compounds

such as hematein, lauryl gallate, o-phenylenediamine and methylene blue. These were used to qunatitate penicillin, oxaloaceate and urea (Stredansky et al., 2000). For example, conversion of penicillin to penicillolic acid is using the penicillinase or oxaloacetate to pyruvate using oxaloactetate decarboxylase enzyme biosensors result in a lower pH. The conversion of urea by using urease enzyme biosensor, which produce ammonium ion, which results in an increase in pH (Stredansky et al., 2000)

Various non-electrical detection methods are also possible, for example, enzymes that have sufficiently exothermic reactions, can be used as the biomolecule and a micro calorimeter can be used to detect thermal energy (Liu & Lu, 2004). Enzymes that change the conductivity or impedance of a solution can be used as the biomolecule with a suitable detection system. For example, urease can be immobilised on the surface of an interdigitated electrode and the impedance of the solution changes when urea is added in solution (Barhoumi et al., 2006). Where an enzyme produces a gas, a potentiometric gas electrode can be used as the detector (Yamazoe & Miura, 1998).

Advantages of enzymes in biosensors
- They bind specifically to their substrate, i.e., high selectivity.
- They are fast acting therefore have a fast response.
- Their catalytic effect makes detection of a response simpler.

Disadvantages of enzymes in biosensors
- They can be expensive (some).
- Some lose activity quickly (varies).
- Most of them lose activity when immobilised.

1.3 Using whole-cells as biosensors
The advantage of having whole-cells as biocomponent is that they are naturally available and are capable of measuring biologically available forms of environmental pollutants, i.e., bioavailable pollutants (Roger, 1995). Whole-cells as biocomponents consist of all enzymes plus a suite of other membranes and biochemical processes that are advantageous. However, whole-cells as biocomponents have the limitation of slow response times compared to the enzyme-based sensors (Rainina et al., 1996). This problem can be overcome by permeabilising the cells (D'Souza, 2001).

1.3.1 Mediator interactions with redox enzymes of the whole-cells

Glucose can be measured using glucose oxidase enzyme by substituting oxygen which has a limited aqueous permeability that limits the analyte (glucose) linear detection range by using an artificial electron acceptor (mediator) such as ferrocene or potassium ferricyanide which have much (several thousands fold) higher aqueous solubility than oxygen. The mediator in its oxidised form will interact with the reduced substrate molecule and oxide it by reducing itself. The reduced mediator is oxidised at the working electrode and the oxidation of the mediator can be quantified by measuring anodic current represents the concentration of the glucose

1.3 .2 Mediators

Electrochemical techniques have been used for probing a number of processes occurring in living cells. It is possible to observe electrochemically the biochemical reactions inside a cell by measuring bioelectrocatalysis, with electron transfer mediators. Hydrophilic mediators are water-soluble and do not enter the cell while lipophilic mediators cross the membrane and interact with intracellular redox molecules. These reduced molecules can then be quantified electrochemically, usually at a lower anodic potential than oxygen (Kalab & Skladal, 1994). The redox activity of the mediators provides an electrochemical or optical signal. Ramsey and Turner (1988), Richardson, Gardner & Rawson (1991), Ertl et al. (2000a; 2000b), Ertl and Mikkleson (2001), Pasco et al. (2005; 2004; 2000) and Tizzard et al. (2004) have monitored the catabolism in bacteria by replacing oxygen with mediators. An oxidised mediator is reduced in its interaction with the reduced electron transport chain molecules and the reduced mediator is quantified by electrochemical methods such as amperometry, coulommetry or voltammetry.

Zhao et al. (2005) exploited the mediated electrochemical detection technique to evaluate the cytotoxic effects of furfural and furfural alcohol on *S. cerevisiae* and two xylose fermenting yeast, *Pachysolen tannophilus* and *Pichia stipitis*. In their studies, they found that both furfural and furfural alcohol imposed an inhibitory effect on all three yeast species although less in *S. cerevisiae*. Furfural was a more potent inhibitor than furfural alcohol. They have shown that the mediated electrochemical method is likely to be an appropriate tool for cytotoxic evaluation in yeast (Zhao et al., 2005).

As interest in biosensor devices has increased, the interaction of redox mediators with biological molecules has acquired a higher profile and much effort has been invested in their characterisation (Cass et al., 1984). The list of mediator compounds that facilitate redox transfers in biological redox systems and electrodes with their formal potentials and chemical structures was reviewed by several groups and is summarised in Table 1, Appendix - VII (Fultz and Durst, 1982; Wong and Hart, 1992; Gorton et al., 1991). Desirable mediator characteristics have been discussed by Clark (1960) and Szentrimay, Yeh & Kuwan (1977). The characteristic properties of an "ideal" mediator have been listed, which included well-defined electron stoichiometry, known formal potential, fast heterogenous and homogenous electron transfer, ready solubility in aqueous media around pH 7.0, stability in both oxidised and reduced forms and no interaction with the biocomponent in a way that alters its redox potential (Szentrimay, Yeh, & Kuwan, 1977). To ascertain whether the mediator has altered the redox potential of the biocomponent, the use of several different mediators is generally recommended and the biocomponent parameter being measured should not vary with the mediator used (Flutz & Durst, 1982).

Strategies employed to incorporate a redox mediator into an electrochemical system involve either adding the mediator to solution or immobilising it on the surface of the electrode producing modified sensors (Murray, Eving & Durst, 1987; Pandey, 1998). Limoges and Savent (2003) reported that the catalytic response of an immobilised redox enzyme connected to the electrode by a freely diffusing mediator (substrate) might depend on Michaelis-Menten characteristics of the enzyme-redox co-substrate systems. Although the use of a mediator in solution is not a very convenient strategy, it avoids the need to immobilise the mediator, which means the mediator still keeps its original properties and achieves better sensitivities because it is working under optimal conditions (high concentration, free mobility, etc). For these reasons, mediators in solution are sometimes used as a preliminary study of determination of some surface modifiers (Prieto-Simon & Fabregas, 2004). Antiochia et al. (2004) compared the use of redox mediator in solution and of surface modified electrodes in the electrocatalytic oxidation of NADH, and found that an electrode modified with an electrodeposited film derived from 3,4- dihydroxybenzaldehyde offered higher sensitivity, a lower detection limit and a larger linear dynamic range compared to the bare electrode in solution.

1.3.3 Hydrophilic mediators

Hydrophilic mediators interact with cell redox molecules accessible from the environment of the cells and the periplasm (the region near or immediately within the bacterial or other cell wall, outside the plasma membrane). For example, prokaryote respiratory electron transport molecules that are located in the cell membrane and accessible from the periplasm can be oxidised by hydrophilic mediators, such as potassium ferricyanide, ruthenium hexamine and carboxymethylferrocenium. There are several reports in which potassium ferricyanide has been used as a redox mediator to couple with cellular respiration (Hadjipetrou, Gray-Young & Lilly, 1966; Ramsey & Turner, 1988; Gaisford et al., 1991).

Other cellular mechanisms involved in the reduction of hydrophilic mediators are the various trans-plasma membrane electron transport (tPMET) systems. Electrons from internal nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) are transferred by membrane spanning NADH/NADPH dehydrogenase enzymes to potassium ferricyanide.

1.3.4 Lipophilic mediators

Lipophilic mediators are lipid soluble, can cross lipid-by-layer membrane of the cell, and can access internal electron transport chain molecules. Examples of lipophilic mediators are phenazine ethosulphate, dichloroindophenol and neutral red, benzoquinones such as 2-methyl-1,4-naphthoquinone (menadione), benzoquinone, 1,2-naphthoquinone, and benzoamines such as 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD) and N, N-dimethyl-p-phenylenediamine. Heiskanen et al. (2004) compared menadione and sodium bisulphate (MSB) as artificial electron acceptors for their ability to transform internal cellular redox activity into electrode current in the presence and absence of glucose and found that hydrophobic menadione was superior to water-soluble bisulphate for probing intact cells. An elegant demonstration of the interaction of the hydrophilic and lipophilic mediators with bacterial and mammalian cells can be observed by using scanning electron microscopy (Liu et al., 2001; Cai et al., 2002). These studies showed that the electrochemical images of the hydrophilic and lipophilic mediators of the cell were different. Hydrophilic mediators were imaged (observed) at the cell surface whereas lipophilic mediators were found within the cells. Rabinowitz et al. (1998) reported that menadione interacts with all NAD(P)H dependent dehydrogenases as well as with elements of the electron transport chain. Lipophilic mediator interact with the intracellular reduced proteins that are generated during

catabolism and oxidise them by reducing itself. The reduced lipophilic mediator shuttles the electrons to an extracellular hydrophilic mediator such as potassium ferricyanide, which can thus be reduced. Reduced potassium ferricyanide in both the single mediator system comprising hydrophilic mediator and the double mediator system comprising hydrophilic and lipophilic mediators can be quantified by electrochemical techniques such as limiting current amperometry (Catteral et al., 2001) and voltammetry (Baronian et al., 2002; Trosok, Driscoll & Luong., 2001a and b).

1.3.5 Mediator interaction in prokaryotes

Using blocking agents, Ramsey and Turner (1998) demonstrated in *Escherichia coli* that 90% of the reduction of the hydrophilic mediator, ferricyanide, arose from electron transport redox molecules. Use of the blocking agent potassium cyanide which is known to inhibit the terminal oxidases of the electron transport chain, resulted in an increase in the potassium reduction whereas the NADH dehydrogenase inhibitor amobarbital which blocks at the beginning of the electron transport chain caused a decrease of potassium ferricyanide reduction and respiration rate in *E. coli* suggesting that the mediator was interacting after NADH dehydrogenase. These results are consistent with mediator interaction studies, which show that electron transport mediated by potassium ferricyanide reduction occurs after NADH dehydrogenase and before the terminal oxidases of *E. coli* (Ramsey & Turner, 1998).

1.3.6 Trans-plasma membrane electron transport (tPMET) systems involved in mediator reduction in prokaryotes and eukaryotes

In prokaryotes, the ferricyanide reduction occurs partly by the trans-plasma membrane electron transport systems such as the ferrireductase system. This enzyme is responsible for reducing environmental Fe (III) to Fe (II) in iron uptake systems (Eide, 2000). A facultative anaerobic bacterium, *Shewanella putreficans,* uses water-insoluble Fe (III) as its electron acceptor (Nelson & Saffani, 1994). *S. putreficans* is known to localise the majority of its membrane-bound cytochromes on its outer membrane (Myers & Myers, 1992), and is electrochemically active (Kim et al., 1999) when grown under anaerobic conditions. Armold, Di Christina & Hoffman. (1986) show that the outer membrane cytochromes were involved in the reduction of water insoluble Fe (III) under anaerobic conditions by using cyanide, the cytochrome oxidase inhibitor.

The plasma membrane of mammalian, yeast and plant cells contains electron transport systems in which NAD (P) H oxidase is considered the key enzyme (Crane & Low,

1976; Kulberg & Christensen, 1979; Goldenberg, 1982). Ferrireductases responsible for the iron uptake in yeast are located in the trans-plasma membrane electron transport systems and are involved in the reduction of potassium ferricyanide (Lesuisse & Labbe, 1993; Crane et al., 1982; Lesuisse & Labbe, 1992). Menadione has been found to be redox mediator for the trans-plasma membrane redox systems; dissolved oxygen and impermeable ferricyanide were reduced effectively in cell suspensions of yeast, mammalian and plant cells (Crane et al., 1985; Yamashoji & Kajimoto, 1986; Yamashoji, Ikeda & Yamashoji, 1989). However, the extracellular reduction of menadione to menadiol has not been demonstrated. NAD (P) H: menadione oxidoreductase was found to be present in the plasma membrane and is responsible for the reduction of dissolved oxygen and ferricyanide was thought to play a role in cell growth (Yamoshoji, Ikeda & Yamashoji, 1991). Various oxidants in rat liver cells and human carcinoma cells were found to be reduced to control cell growth (Clark, Patrick & Patten, 1981; Ellem & Kay, 1983). Merker et al. (2002) reported intracellular redox status of pulmonary arterial endothelial cell trans-plasma membrane electron transport (tPMET) activity, and the tPMET activity is more highly correlated with the poise of the NADH/NAD$^+$ redox pair.

1.3.7 Mediator interactions and origins of the electrochemical responses from single and double mediator systems in yeast

It is important to understand the origins of the electrochemical responses generated from the single hydrophilic mediator such as ferricyanide and the double mediator system comprising hydrophilic mediator coupled with a lipophilic mediator such as 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD)

Origins of single hydrophilic mediator responses in yeast-tPMET- Detection of cell redox events in yeast using a single hydrophilic mediator such as ferricyanide results in relatively small electrochemical responses (Baronian et al., 2002). This is because ferricyanide cannot cross the cell membrane and is thus limited to oxidising cell surface redox molecules such as molecules of the trans-plasma membrane redox systems (tPMET) by using ferrireductase enzyme and discussed earlier (1.3.6).

Origins of double mediator responses in yeast – The double mediator system comprises of both a lipophilic mediator, such as TMPD or menadione and a hydrophilic mediator such as potassium ferricyanide. The lipophilic mediator can cross the lipid bi-

layer membrane of yeast cells and interact with the intracellular proteins involved in redox reactions. They shuttle the electrons to the potassium ferricyanide hydrophilic mediator that can only interact with redox molecules external to or embedded in the cell membrane. Reduced potassium ferricyanide in the single and double mediator systems is detected by limiting current amperometry (Catteral et al., 2001) and voltammetry (Trosok et al., 2001; Baronian et al., 2002)**.**

The origins of double mediated yeast responses were investigated by using specific blockers that block the metabolic pathways (Banerjee & Vishwanathan, 1981). Glycolytic enzymes especially NADH-dependent dehydrogenases in *S. cerevisiae* were sensitive to furfural. Zhao et al. (2005) used double mediator systems comprising 2, 6-dichlorophenol indophenol (DCPIP)/ferricyanide or menadione /ferricyanide to detect changes of function in catabolic enzymes induced by furfural in *S. cerevisiae Pachysolen tannophilus* and *Pichia stipites*. They elucidated the functions of glycolytic enzymes by blocking the electron transport chain (respiration rate) with furfural in *S. cerevisiae, P. tannophilus* and *P. stipitis*. DCPIP/ferricyanide has been frequently used in detecting cellular respiration activity in cells since DCPIP is considered to directly accept electrons from the electron transport chain. Zhao et al. (2005) have shown the decreased average specific reduction rates in *S. cerevisiae* in the menadione/ferricyanide double mediator system in the presence of furfural is due to the direct inhibition of glycolytic enzymes resulting in decreased glycolytic NAD(P)H production. By the additional NADH flux to furfural, they have also demonstrated that the increased average specific reduction rates were increased in *S. cerevisiae* using DCPIP/ferricyanide double mediator system, supporting earlier reports (Soboleva & Golubkov, 1973). Soboleva and Golubkov (1973) reported that furfural decreases the cytochrome content in *S. cerevisiae* suggesting that furfural strongly inhibited the respiration in yeast by decreasing the cytochromes, before which DCPIP interacts and shuttles out the electrons. In all cases the two xylose-fermenting yeast were more sensitive to furfural than *S. cerevisiae.*

Zhao et al. (2005) used iodoacetate (an inhibitor of the glycolytic pathway) and epiandrosterone (inhibitor of the pentose phosphate pathway) to investigate their effects on these two major catabolic pathways in *S. cerevisiae*. The study used menadione/ferricyanide double mediator system to detect the effects of the two inhibitors by detecting the reduced ferrocyanide accumulation after exposing cells to

these two inhibitors by steady-state voltammetry. They demonstrated complete inhibition of the glycolytic pathway not only with exogenous glucose but also endogenous microbial metabolism with 500 µM iodoacetate. In contrast to iodoacetate, the epiandrosterone exhibited no considerable inhibition up to 200 µM and only 4% of overall electrochemical response was inhibited by 300 µM epiandrosterone (Zhao et al., 2005). This suggested that the major metabolic pathway for glucose that operates is the glycolytic pathway while the pentose phosphate pathway operates to the extent of less than 10%, which was consistent with the previous report (Gancedo & Languna, 1973). These results suggest that glycolysis is the major metabolic pathway that operates in *S. cerevisiae* responsible for cytoplasmic metabolic flux and mediator reduction.

1.4 Transducers

The essential requirement of biosensor construction is to match the appropriate biological component and the electronic transducer to produce a relevant signal during analysis. Transducers include electrochemical, optical, acoustic, piezoelectric, calorimetric and electronic (Sethi, 1994).

1.4.1 Electrochemical transducers

Electrochemical transducers such as a potentiometric transducer record changes in voltage when the current is held constant. Amperometric transducers record changes in current when the voltage is held constant. Coulometry quantifies accumulated charge.

1.5 Electrochemical techniques

Coulometry, amperometry and Voltammetry, are three electrochemical techniques used to quantify redox species in solution.

1.5.1 Coulometry

Coulometry can be used as an electroanalytical technique for measuring an unknown concentration of an analyte in solution when it changes from one oxidation state to another during an electrolysis reaction. In this method, a measured amount of charge is delivered using a constant current source. Since concentration polarisation is inevitable in coulometric reactions, most (if not all) of the reaction must occur distant from the working electrode. Otherwise, the potential will need to constantly increase as the reaction progresses to maintain the production of products. Therefore, coulometric reactions usually have an intermediate reagent such as a redox mediator that quantitatively reacts with the analyte to avoid concentration polarisation.

If a mediated cell reaction is allowed to run without the regeneration of the mediator at an electrode, reduced mediator will accumulate. The total reduced mediator can later be oxidised at an electrode and quantified. This quantity will be directly related to the cell-substrate interaction. Typically, an amperometric cell consists of a very large surface area working electrode to facilitate complete oxidation of the reduced mediator.

1.5.2 Amperometry
Amperometry is performed at a fixed potential at the working electrode with respect to the reference electrode. The reduction or oxidation of a redox species at the surface of a working electrode generates the current (rate of flow of electrons) which is measured. Amperometry enables a real time cumulative measure of electron transfer.

1.5.3 Voltammetry
The amounts of oxidised and reduced forms of an electroactive species in solution can be determined by steady-state voltammetry using either linear sweep or cyclic voltammetry. In steady-state voltammetry the potential of a working electrode is swept relative to a reference electrode.

In this study steady-state voltammetry has been used as the detection technique for the following reasons:
- The technique has been used routinely in the laboratory because it provides information about the proportions of each oxidation state of the redox species and the anodic and cathodic plateau currents allow quantitation of each redox form.
- Malfunction is thus more easily detected by noting the shift of the recorded full voltammogram than with techniques that give partial data. In addition, steady-state voltammetry offers quick detection of surface fouling, i.e., gradual loss of electrochemical responses due to the surface adsorption of highly reactive intermediates produced over the course of mediator (ferricyanide) oxidation.

1.6 Microbial biosensors
Microbes as sensing elements (biocomponents) have several advantages over purified enzymes, proteins, etc and provide more information, e.g., in toxicity and genotoxicity testing. Microbes, in contrast to purified enzymes, are capable of responding to a variety of chemical compounds. Microorganisms can also adapt to new and adverse conditions

easily with time and hence, can degrade new molecules (D'souza, 2001). The advantage of whole-cells as a source of intracellular enzymes compared to purified enzymes has been illustrated for industrial applications (Bickerstaff, 1997). Whole-cells not only provide the advantage of preserving the enzyme in its natural environment and protecting it from external toxicants but also provide multi-purpose catalyst applications. Both viable and non-viable cells have been used in whole-cell biosensors. The use of viable cells is gaining more attention than the non-viable cells in the construction of biosensors as the viable cells can metabolise various organic compounds aerobically or anaerobically and produce end products that can be quantified such as ammonia, carbon dioxide and acetic acid (D'Sousaza, 2001). Viable cells are used when overall substrate assimilation capacity of the microorganism is taken into account such as in the measurement of biochemical oxygen demand (BOD) or utilisation of vitamins, sugars, nitrogenous compounds and organic acids as metabolites (Riedel, 1998).

The advantages of microbial whole-cell biosensors are:

1. They are less sensitive to inhibition by solutes and more tolerant of sub-optimal pH and temperature values.
2. They are cheaper because an active enzyme does not have to be isolated.
3. Recognition is usually directed towards the molecules of biological relevance.
4. They potentially have a longer shelf life than enzyme biosensors.

However, there are some disadvantages reported when whole-cells have been used as the sensing element (biocomponent).

1. Some have longer response times than enzyme electrodes.
2. They take a longer time to return to the base line signal after use.
3. Selectivity may be a problem as the cells contain many enzymes. Selectivity is somewhat concentration dependent but pre-treatment should reduce this. Care must be taken to ensure specificity/selectivity is achieved if required.
4. The cells have to be kept viable.

Selection of the appropriate microorganism is important for biosensor application, because the microorganism selected should be able to utilise the range of compounds present in the sample. Adaptation of the microbe to a particular compound by pre-conditioning the cells and inducing the metabolic pathway specific to the substrate has been reported (Fleschin et al., 1998; Baronian, 2004). Mixed microbial consortia sometimes offer advantages over single pure cultures in biochemical degradation of

complex organic substrates as each of them might possess different degradative enzymes that enable them to degrade the complex organic substrates (Joshi & D'Souza, 1999). Some reduction of slow response times and non-specificity of whole-cell biosensors can be achieved using permeabilisation techniques (Felix, 1982; D'Sousza, 1989a).

1.6.1 Advantages of yeast based whole-cell biosensors compared to bacterial based whole-cell biosensors

Most of the whole-cell biosensors that have been reported to date are based on bacterial cells. The use of yeast species in the detection and evaluation of carcinogens has been reviewed (Parry, 1999). He found that yeast cells are more robust than bacteria with superior pH, temperature and osmolarity/ionic strength tolerance. In addition, Walmsley and Keenan (2000) also discussed the use of yeast in whole-cell biosensors and gave two further advantages:

1. Yeast share with bacteria rapid growth and ease of manipulation as well as growth on a broad range of substrates.

2. Yeast are eukaryotes and can sometimes provide information of direct relevance to other eukaryotes such as humans in the genotoxicity tests that prokaryote cells (bacteria) cannot.

Although these advantages have been demonstrated for yeast species as a biocomponent in biosensors, unfortunately the use of yeast in biosensors is small compared to the number of bacterial cell types that have been exploited.

1.7 Oxygen consumption as a measure of the catabolism of the organism

The catabolic rate of any aerobically respiring organism can be measured by the consumption of oxygen. Measuring the oxygen consumption by microorganisms in wastewater over a period of five days is known as the biochemical oxygen demand (BOD-5). BOD-5 is the international standard for measuring biodegradable organic levels in wastewater by microorganisms (Eaton, Clesceri & Greenberg, 1995). However, this is time consuming and is associated with several practical difficulties, e.g., measurement depends on temperature, pH, oxygen concentration, presence of toxins and the type of microorganisms. Rapid biochemical oxygen demand (BOD) techniques have been developed with a combination of microorganisms and oxygen electrodes to measure the changes in dissolved oxygen concentrations at the sensor surface (Slama et al., 1996), but availability of oxygen has been shown as the limiting factor for microbial catabolism (Reshtilov et al., 1998). Approaches to increase the

oxygen concentration are also problematic because of the low solubility of oxygen in water (Logan & Wagenseller, 1993). Recently, catabolism of an organism has been monitored by an alternative approach by excluding oxygen and the inclusion of redox mediators that capture electrons from the redox molecules, which are associated with the catabolic processes (Baronian et al., 2002; Pasco et al., 2000).

1.8 Monitoring catabolism in yeast using the double mediated electrochemical detection technique

The metabolic processes of living cells are dependent on electron transfer through a series of intermediate redox molecules to a terminal electron acceptor. Catabolism is the oxidation of the substrate molecule, which releases energy in the form of ATP. This process involves a series of redox reactions that produce NADH, NADPH and FADH2 (flavin adenine dinucleotide).

A commonly used method of monitoring catabolism is the measurement of oxygen consumption. This is applicable to a broad spectrum of organisms such as bacteria, yeast, protozoa, algae, plant and animal tissues. An oxygen probe can be used to measure the change in oxygen concentration over a 5-day period in biochemical oxygen demand (BOD-5) assessment or real-time analysis of oxygen consumption in rapid biosensors (Tanaka et al., 1994; Chan et al., 2000). An alternative technique to detect oxygen consumption by metabolising cells using immobilised oxygen sensitive fluorescent material based on luminescent ruthenium complexes was described by Preininger, Kilmant & Wofbels (1994). Towe, Fleschsig & Spaulding (1996) constructed an immersible oxygen sensor by circulating small quantities of oxygen sensitive fluorescent dye ruthenium tris- (2, 2-bipyridyl) II dichloride through oxygen permeable silicone tubing immersed in test medium. This avoided the problems associated with the degradation of the dye owing to leaching of the dye through membranes by immobilisation (Towe, Fleschsig & Spaulding, 1996).

The consumption of oxygen was found to be linearly related to the catabolic rate of the cells (Pasco et al. 2000). Because of the low solubility of oxygen, the detection of oxygen consumption as a measure of the cells catabolism can only provide a limited linear response range to extracellular catabolites, limiting the sensitivity of assay method such as BOD. This problem can be overcome by the use of a highly soluble hydrophilic mediator such as potassium ferricyanide, which can be present in the sensing element at much higher concentrations than oxygen (Baronian et al., 2002;

Pasco et al., 2000). The "MICREDOX®" (developed by 'Lincoln Technology') technique in bacteria was developed by using hydrophilic mediator potassium ferricyanide as a substitute for oxygen for BOD estimation (Pasco, Hay & Webber, 2001).

Mediated electrochemical detection of catabolism in yeast can be performed with a single hydrophilic mediator potassium ferricyanide or with a double mediator system comprising a lipophilic and hydrophilic mediator by adapting the "MICREDOX®" technique. The lipophilic mediator menadione or 2,3,5,6-tetramethyl1,4-phenylenediamine (TMPD) interact with the intracellular proteins involved in redox reactions by crossing the lipid bilayer-membrane of eukaryotic cells. They shuttle the electrons to the potassium ferricyanide hydrophilic mediator, which can only interact with redox molecules external to, or embedded in the cell membrane. Reduced potassium ferricyanide in the single and the double mediator systems is detected by electrochemical techniques.

Mediated detection of catabolism appears to offer some advantages over the oxygen consumption method; it can be used in deoxygenated samples and with respiratory as well as fermentative organisms. Mediators are highly soluble in their respective cellular environments which allows a large number of cells to be used and this should permit detection of a substrate over a greater range of concentrations (Baronian et al., 2007, personal communication) A double mediator system comprising of the hydrophilic mediator ferricyanide and lipophilic mediator menadione or TMPD can be used to investigate the substrate dependent catabolic responses in yeast cells using steady-state voltammetry technique. The current measure represents the yeast substrate dependent catabolic response.

1.9 Biotechnological relevance/significance of using *Arxula adeninivorans* as a biocomponent

The new genus *A. adeninivorans* is described as ascomycetous yeast (Middlehoven et al., 1984). Strains of this species have been isolated from widely different geographical locations such as in the Netherlands, South Africa and Siberia in maize silage, soil and wood hydrolysates respectively (Kunze & Kunze, 1994). *A. adeninivorans* is soil-borne and non-pathogenic. *A. adeninivorans* can tolerate high salt concentrations up to 10-20% NaCl. The salt tolerance property of *A. adeninivorans* was exploited in the development of a novel BOD sensor for monitoring wastewater in coastal and island

locations in Germany and Hong Kong (Chan et al., 1999; *A. adeninivorans* to et al., 1999). The *A. adeninivorans* based sensor offers a good correlation between BOD and BOD-5 for both domestic and industrial wastewater with a different salinity. In addition, this novel sensor is also suitable for rapid monitoring of different types of wastewater from coastal and island regions without pre-treatment. The *A. adeninivorans* based BOD sensor is the best sensor known to date for wastewater treatment in coastal and island regions (Lehmann et al., 1999).

Industrially important properties such as growth up to 48°C, utilisation of n-alkanes and good starch degradation makes this yeast interesting for biotechnological research (Gienow et al., 1990; Middlehoven, de Jong & de Winter, 1991). The uncommon ability of *A. adeninivorans* to utilise a wide variety of mono, di and poly saccharides, polyalcohols, organic acids, purines and amines as their sole carbon energy and nitrogen source was reported (Middlehoven, de Jong & de Winter, 1991). This indicates that *A. adeninivorans* possess many degradative enzymes (which are selectively induced under specific growth conditions) which makes it very useful as a biocomponent in the biosensor devices in the detection of environmental contaminants.

1.9.1 Molecular characterisation of *A. adeninivorans*

Middlehoven, Hoogkamer-Te Niet & Kreger-van Rij (1984) isolated and designated this yeast as *Trichosporon adeninivorans*, renamed in 1990 as *A. adeninivorans* (van der Walt, Smith & Yamada, 1990). Since *A. adeninivorans* strains have been isolated from different geographical origins, it was important to verify its genetic variations by DNA finger printing and studying chromosomal patterns to confirm its authenticity. (Kunze & Kunze, 1994). They found some similarities and some differences and concluded that chromosomal mutations could be the reason for some of the differences found in the different *Arxula* strains.

The authenticity of the *A. adeninivorans* Ls3 strain used in the current study as sensing element requires the verification of the presence of the unique self-splicing 411 bp insertion within the 25S rDNA between 2901 and 3319 nucleotides (Rosel and Kunze, 1994).

1.9.2 Immobilisation of the biocomponent

Biomaterials can be immobilised on membranes either through adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques. The choice of support and technique for the preparation of membranes depends on achieving low diffusional resistance of the membrane (Jakel et al., 1998). Cross-linking has been used for stabilisation of enzymes (Tyagi, Batra & Gupta, 1999). Entrapment and adsorption techniques are most commonly used with cells. Microbial cells have been immobilised by entrapment in a variety of synthetic or natural polymeric gels for use in biosensor applications including polyurethane-based hydrogels (Koenig, Zaborosch & Spencer, 1997a) and polyvinyl alcohol (Tag et al., 2000). Polyvinyl alcohol (PVAL) is one of the most widely studied polymers for immobilising microbial cells, as it can form membranes, fibres or beads. Enzymes and cells have been immobilised in these membranes either by entrapment, covalent binding, cross-linking or freezing and thawing (D'Souza, 2001). Albumin-poly (ethylene glycol) hydrogels may be useful in *in vivo* implantable biosensors (D'Urso & Fortier, 1996).

1.9.3 Immobilisation of *A. adeninivorans* cells in 'Lentikats®' and use of immobilised cells as the biocomponent in biosensor devices

Polyvinyl-alcohol (PVAL) lenticels were used to immobilise *A. adeninivorans* cells in 'Lentikats'® developed by Department of Technology, Federal Agricultural Research Centre, Germany ("Genialab Biotechnology"). In this method the 'Lentikats' are formed by gelation of PVAL hydrogel (10% w/w PVAL and 90% w/w water) at room temperature and are subsequently stabilised by 'Lentikat stabilisation solution' supplied by Genialab Biotechnology. These 'Lentikats' are lens-shaped with short diffusion distances (height) and maximum thickness of approximately 0.2 to 0.4 mm (Figure 1.2) offering advantage of reduced rate limitation by diffusion (Jakel et al., 1998).
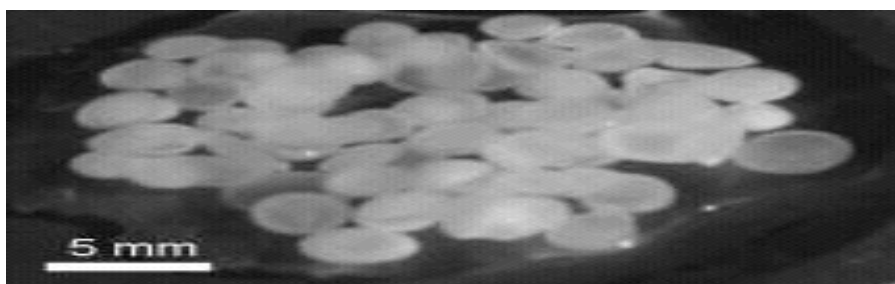
Figure 1.2 Immobilised *A. adeninivorans* cells in Lens-shaped 'Lentikats®' with a diameter of 5 mm and thickness of 0.2 – 0.4 mm.
(From - "Genialab Biotechnology", German website).

Processes with immobilised cells offer several advantages compared to free cells (Dervakos & Webb, 1991). Firstly, immobilised cells can be reused because the biocatalytic properties of the cells are relatively well preserved by the immobilisation medium's protective environment, e.g., from effects such as pH and temperature variations (Dervakos & Webb, 1991). The second advantage is that the immobilised cells can be used in non-sterile conditions (Dervakos & Webb, 1991). The cells are loaded into the immobilisation medium under sterile conditions to prevent contamination. Highly elastic and stable hydrogels like PVAL provide a favourable environment to cells. The gelation of the PVAL molecule occurs by the formation of hydrogen bonds between the OH-groups of this molecule at room temperature. In the system used the particles that are obtained are lens shaped ('Lentikats') with characteristic short diffusion distances (height) and are easy to separate. Jakel et al. (1998) reported little loss in activity by using this 'Lentikat' immobilisation technique to entrap nitrifying bacteria which are temperature sensitive (Jakel et al., 1998). Chan et al. (1999) in part-I and Lehmann et al. (1999) in Part-II reported using polycarbamyl sulfonate (PCS) to immobilise salt-tolerant *A. adeninivorans*, which they have successfully used as a biocomponent in the construction and application of a novel biosensor to measure the BOD of real sample from coastal regions where commercial BOD sensors are not sensitive (Chan et al., 1999; Lehmann et al., 1999).

Free and immobilised *A. adeninivorans* cells were used to detect glucose dependent catabolic responses using a double mediated electrochemical detection system. Immobilised *A. adeninivorans* has been used as biocomponent in an amperometric oxygen biosensor and mediated biosensor, the efficiencies of which for the analysis of wastewater have been compared (Baronian et al., 2007, personal communication). This study show that the oxygen sensor had a short detection time of 70 sec per sample plus 5 min rest time between samples compared to 60 min incubation time and 3 min analysis time per sample in the mediator sensor. However, both systems have advantages for the analysis of wastewater and selection of the system depends on the cost of the analytes of the system, nature and number of the samples to be analysed.

1.10 Use of yeast based biosensors to detect priority environmental contaminants

There are several reports on the use of wild type fungal cells and yeast to detect toxicity. *S. cerevisiae* was used as a sensing element to assess the toxicity of seven cholonic acids and a toxicity degree/scale was constructed from their results (Campanella et al., 1996). Valani et al. (2001) reported *Candida aquatextoris* utilising 4-(1-nonyl) phenol as a sole carbon and energy source. Deshpande, Rale & Lynch (1992) discussed the use of *Aureobasidium pullulans* to remove oil spill contamination occurring in marine and in fresh waters. *A. pullulans* also actively degrades dioxins and organochlorine compounds present in the bleaching effluents from Kraft pulping, which involves chemical treatment to get fibre out of tress. Ternan and McMullan (2002) have reported *Kluyveromyces marxians* utilising the xenobiotic molecules iminodiacetate and nitrilodiacetate as a solenitrogen source. *Penicillium stecki* is the first fungus demonstrated to degrade the herbicide Simazine.

1.10.1 The concept of "IDEA" (Isogenic Differential Enzyme Analysis) to develop an *A. adeninivorans* based biosensor to detect priority environmental contaminants

The "IDEA" concept emerged from the ability of bacteria to recognise and degrade or detoxify specific contaminants (Lloyd-Jones, 2002). The concept relies on using bacterial cells containing enzymes that are able to metabolise a target chemical rapidly altering their respiratory/catabolic rate in the presence of this chemical, compared to that of otherwise identical cells lacking these enzymes. The resulting pairs provide a differential respiration response to the presence of the target chemical, thus providing the basis for developing a biosensor for its detection. "IDEA" involves measuring the difference in respiration/catabolic rates between pairs of yeast that are grown in the presence of contaminant (pre-conditioned) and in the absence of contaminant (control). Pre-conditioned cells are grown in the presence of a specific contaminant molecule as a sole carbon source to induce the catabolic pathways associated with the degradation of the molecule. In this study, a double mediator system comprising potassium ferricyanide hydrophilic mediator coupled with 2,3,5,6 tetramethyl 1,4-phenylenediamine (TMPD) as a lipophilic mediator, was used to monitor *A. adeninivorans* catabolic response to the contaminant (substrate) and steady-state voltammetry as the electrochemical detection technique. This is a convenient method for determining the amounts of oxidised and reduced forms of an electroactive species in the solution.

1.10.2 Model environmental contaminants: Gallic acid, naphthalene and di-butyl phthalate

Gallic acid is a known environmental contaminant (Benitez et al., 2005). Naphthalene and di-butyl phthalate, which are known environmental contaminants were selected from a list of priority contaminants prepared by the "IDEA" biosensors group (Lloyd-Jones, 2002). The rationale for selection of these contaminants was that they should fulfil the following criteria:

1.   Have some solubility in water.

2.   Can be metabolised.

3.   Have a relatively high detection limit.

4.   High cost of current test (see Appendix – VIII for naphthalene and di-butyl phthalate).

1.10.3 Gallic acid

Gallic acid is a major pollutant present in wastewater generated in wood processing as well as in other wastewater from food manufacturing industries (Benitez et al., 2005). Gallic acid (3, 4, 5-trihydroxybenzoic acid) is a colourless or slightly yellow crystalline compound obtained from plant products such as nutgalls. It is a strong natural antioxidant. It is obtained by the hydrolysis of tannic acid with sulphuric acid or an enzyme. Gallic acid at very low concentrations is harmful to aquatic life. It has been shown that tannins in aquatic environments have harmful effects on aquatic life, as they are inhibitory to microbial growth, respiration and metabolism (Mahadevan & Muthukumar, 1980). Gallic acid is found in wastewater treatment plants in the wood industry. Boiling processes in the veneer industry generate large amounts of wood extract containing phenolic acids including gallic acid, vanillic acid and ellagic acid (Mayer, Koch & Puls, 2007). Gallic acid is found in olive mill wastewater, which is readily degradable under anaerobic conditions even at concentrations as high as 1000 mg L^{-1}(Kouroutzidou et al., 2005). It is used in photographic developers, azo dyes, ink dyes and in paper manufacturing. Gallic acid is a strong chelating agent and forms very stable complexes with iron (III). It has been shown to have phytotoxicity and antifungal activity (Dowd, Duvick & Rood, 1997).

Middlehoven, de Jong & de Winter (1991) reported that *A. adeninivorans* exhibits an uncommon ability to utilise several aromatic compounds as a sole carbon source. Among them gallic acid proved to be a suitable source of carbon for *A. adeninivorans*. These and other organic solvents and aromatic compounds, which are potentially toxic,

were tested by using a simple slant test (Middlehoven, de Jong & de Winter, 1991). Growth was observed after 2 days. They also reported that *A. adeninivorans* growth on many aromatic compounds occurred in slant culture but not in liquid media if added at once at 1 g/litre while inoculating yeast (Middlehoven, de Jong & de Winter, 1991). If however the substrate was added little by little, these compounds are assimilated by *A. adeninivorans* liquid cultures.

1.10.4 Polynuclear aromatic hydrocarbons (Naphthalene)
Naphthalene is a fused-ring bicyclic aromatic hydrocarbon, which is the simplest member of the large class of environmentally prevalent complex polycyclic aromatic hydrocarbons (PAHs). Because of naphthalene's simplicity, it has been served as a model molecule for understanding the degradation of PAHs. Naphthalene and its substituted derivatives are commonly found in crude oil and oil products. Certain PAHs are strong human carcinogens and exhibit cytotoxic, carcinogenic and mutagenic effects in laboratory animals (Miller & Miller, 1974) and hence the microbial metabolism of these compounds is worth investigating. PAHs are hazardous environmental contaminants (Dipple, Michejda & Weisburger, 1985) mainly derived from anthropogenic pyrolysis of organic matter such as fossil fuel consumption and the coal gas refining process. Naphthalene is the most reported polycyclic aromatic hydrocarbon contaminant in hazardous waste.

Naphthalene needs molecular oxygen to break down the aromatic ring. The microbial metabolism of naphthalene has been studied over a period of many years and reviews have summarised these earlier investigations (Heitkamp & Cerniglia, 1989). These studies have indicated that naphthalene is rapidly mineralised by pure bacterial cultures and microbial consortia in sediments and aquatic ecosystems. Eighty six species of fungi belonging to thirteen classes and distributed among five major fungal taxa were examined for their ability to metabolise naphthalene (Cerniglia et al., 1978; Cerniglia & Gibson, 1978). There is evidence that some fungal species metabolise aromatic hydrocarbons to metabolites that are identical to those formed by mammalian enzymes (Auret et al., 1971; Ferris et al., 1976, 1973; Smith & Rosazza, 1974).

Fungal metabolism of aromatic hydrocarbons has been studied extensively in both whole-cell and cell-free cytoplasmic preparations (Cerniglia, 1997, 1981). Fungi metabolise aromatic hydrocarbons by cytochrome P-450 monooxygenase and epoxy hydrolase enzyme systems through a sequence of reactions similar to those of

mammalian systems (Cerniglia 1981; Cerniglia; et al., 1978; Cerniglia & Gibson, 1978, 1977). P-450 monooxygenases are also utilised for the initial oxidation step in the biodegradation of aromatic hydrocarbons in fungi (Cerniglia et al., 1978). A single amino acid substitution in cytochrome P-450cam at the single Y96 residue results in a one to two orders of magnitude increase in the degradation rate and thus plays an important role in determining substrate specificity of the P-450 enzyme (Paul et al., 1998).

Although several groups have investigated the fungal metabolism of aromatic hydrocarbons, little is known about their ability to form sulphate and glucuronide conjugates of aromatic hydrocarbons. Conjugation reactions in mammals are important as detoxification pathways in the removal of toxic substances and in the activation of highly reactive N-hydroxylated aryl amines to form highly reactive compounds which can bind to critical cellular macromolecules leading to the induction of biological effects (Miller & Miller, 1974). Cerniglia (1981) reported for the first time the presence of glucuronide and sulphate conjugates formed from the metabolism of naphthalene by the filamentous fungus *Cunninghamella elegans,* and suggested that these reactions are important in detoxification and elimination of xenobiotics in fungi.

Cerniglia et al. (1978) reported that *Cunninghamella elegans* oxidises naphthalene and the metabolic pathway is depicted in the next page (Figure 1.3).
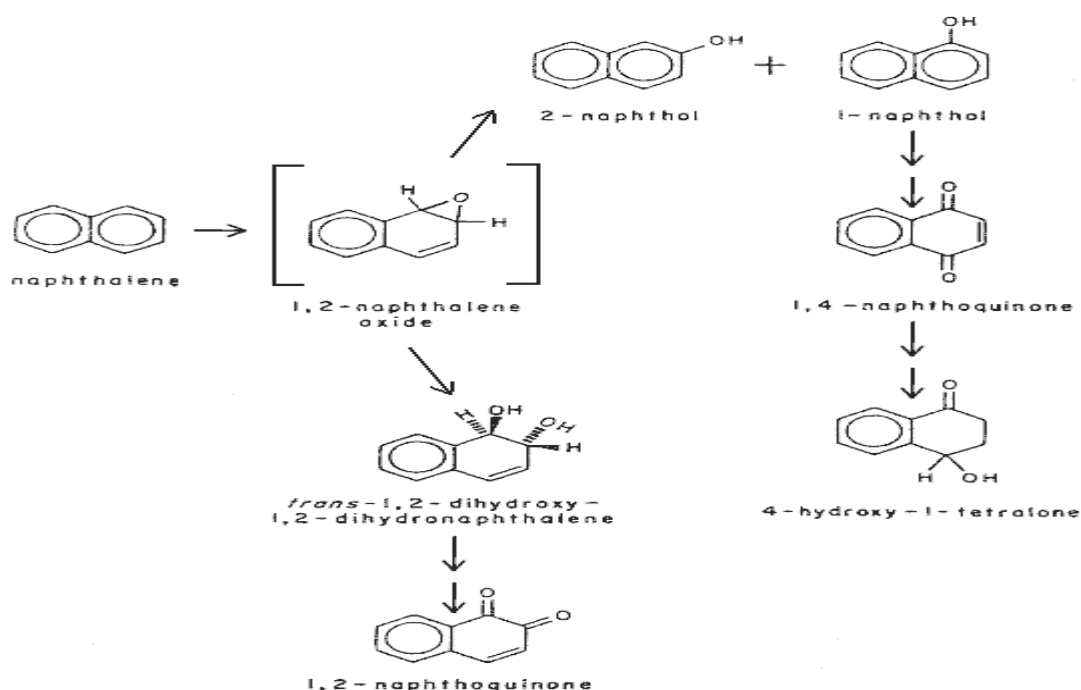
Figure 1.3 Metabolic Pathways of Naphthalene.
(From Cerniglia et al., 1978)

Microsomal preparations from *C. elegans* contain a cytochrome P-450 enzyme that oxidise naphthalene to 1-naphthol, 2-naphthol, trans1,2-dihydroxy-1,2-dihydronaphthalene and 1,2-napahthaquinone. The predominant metabolite of naphthalene is 1-naphthol. There are greater similarities between the enzyme systems used by mammals and fungi compared to those used by bacteria for the initial oxidation of aromatic hydrocarbons (Cerniglia et al., 1978).

Many bacteria that degrade PAHs contain the *nahAc* gene that encodes a component of multimeric naphthalene dioxygenase. As the *nahAc* gene is highly conserved, this gene serves as a potential biomarker for PAH degradation activity (Park & Crowley, 2006). The entire *nahAc* gene has been cloned and sequenced in NAH7 plasmid of *Pseudomonas putida* PpG7 stain (Grund & Gunsalus, 1983).

Organic pollutants with low levels of water solubility often have a significant gas-vapour partitioning co-efficient, which in principle makes measurement possible in the gaseous phase rather than the aqueous phase. A genetically modified bioluminescent

Pseudomonas putida carrying the plasmid with a lux AB reporter gene fusion, which in turn carries a naphthalene promoter, has been constructed (Werlen et al., 2004). They show the sensitivity limit of naphthalene detection in the gas phase is 10 fold lower, i.e., 50 nM compared with that of an aqueous phase sensor (500 nM). However, 50 nM is still above the environmentally significant 4 - 40 nM levels.

1.10.5 Phthalates
Phthalates are commonly used as plasticisers and are produced in large quantities. Three phthalates, di-methyl phthalate (DMP), di-n-butyl phthalate (DBP) and di-2 -ethylhexyl phthalate (DEHP) are known priority environmental pollutants. Di- butyl phthalate is used mainly as a coalescing aid in latex adhesives, as a plasticiser in cellulose plastics and as a solvent for dyes. Release of di- butyl phthalate DBP into the environment can occur during its production and during the incorporation of the phthalates into plastics, adhesives, or dyes. Because di- butyl phthalate is not bound to the final product, it can be leached during the use or disposal of the product. Although plasticisers have low solubility in the aqueous phase, the presence of surfactants and emulsifiers has been shown to increase their transport in this phase. Other factors, such as temperature or exposure to microwaves also affect the leaching and migration of these plasticisers (Begley & Hollifield, 1990). Phthalates that are released can be deposited or taken up by crops, which are intended for consumption by humans or livestock and can enter the food supply (National Toxicology Program, U.S. Department of Health and Human Services, October 2000). High levels of leached plasticisers from blood bags made of PVC were found to accumulate in the lungs, liver, and spleens of blood transfused patients (Faouzi et al., 1994). It has been estimated that an average of 8.2 mg/person of plasticiser is ingested each day because of migration into food products (Loftus et al., 1993).

Considerable attention has been directed to the toxicity of phthalates, and in recent years, there have been several reports on the biodegradation of these chemicals. These reports have established that phthalate esters are easily biodegradable in the laboratory with pure bacterial cultures, activated-sludge digestion and hydrosols (Engelhardt, Wallnofer & Hultzinger, 1975). Biological transformation of phthalates in soil, fresh-water and marine ecosystems appear to be carried out by bacteria and fungi. Although there have been several reports of the complete biodegradability of phthalate esters (Keyser et al., 1976), little is known about pathways by which organisms metabolise these compounds. Several microorganisms are able to utilise DBP and related dialkyl

phthalates as carbon sources, and phthalates are suggested as an intermediate in the biodegradation of phthalate esters to protocatechute (Engelhardt, Wallnofer & Hultzinger., 1975).

Eaton and Ribbson (1982) proposed a complex pathway for the metabolism of di-butyl phthalate and phthalate by *Micrococcus* sp. They proposed the following degradative pathway: di-butyl phthalate → monobutylphthalate → phthalate → 3,4,-dihydroxyphthalate → protocatechute. Protocatechuate is metabolised both by the meta-cleavage pathway through 4-carboxy-2-hydroxymuconic semialdehyde and 4-carboxy-2-hydroxymuconate to pyruvate and oxaloacetate and by an ortho-cleavage pathway through β-ketoadipate. Cells grown on di-butyl phthalate and phthalate readily oxidised di-butylphthalate, phthalate, 3,4-dihydroxypthalate and protocatechuate the pyruvate and oxaloactetate enters the central metabolic pathway through the TCA cycle (Figure 4.4). All metabolic enzymes required for di-butyl phthalate metabolism were found in cells grown in di-buytyl phthalate (Eaton & Ribbson, 1982). *Rhodotorula rubra* was shown to completely break down the original plasticiser DOP, at concentrations up to 2 g/L in 100 hours. This yeast was shown to produce esterase, which could catalyse the initial hydrolysis of the phthalate ester to phthalic acid (Katayama-Hirayama, Tobita & Hirayama, 1992). Unlike other lipases, cutinase is a hydrolytic enzyme that hydrolyses esters. A potential use of cutinase enzyme is the degradation of esters and polyester plastics. The fungal cutinase and yeast esterase enzymes are both involved in the degradation of benzyl butyl phthalate (BBP) (Kim et al., 2002).

1.11 Xenobiotics /Endocrine disruptors

There is increasing concern about the ubiquity of environmental contaminants that mimic the biological activities of the hormones or any other metabolically important substances, these are known as xenobiotic compounds. It is reported that these compounds can cause some kind of cancer, damage to reproductive systems and developmental problems in both humans and wild life (Juberg, 2000; Colborn & Clement, 1992). A wide range of chemicals, both natural and synthetic have now been found to be weakly estrogenic. These include natural and synthetic substances such as phytoestrogens, mycoestrogens, certain pesticides and herbicides, some polychlorinated biphenyls (PCB), plasticisers (phthalates) and some breakdown products of surfactants (Cernigilla, 1981). The xenobiotic compounds mimic the female hormone estrogen in

the body, thereby disturbing normal endocrine functions, e.g., growth, reproduction, secondary sexual characters and homeostasis (Jobling et al., 1995). It is now generally accepted that the synthetic compounds are susceptible to microbial degradation if their chemical structure is similar to the naturally occurring compounds as they enter living systems. Endocrine disrupting compounds, both natural and synthetic, have been listed in Appendix -IX (Hilscherova et al., 2000).

1.12 Estrogens

Estrogens are female sex hormones. The increasing incidence of some cancers is causing concern about the ubiquity of compounds with estrogenic activity in the environment (Raloff, 1994). Both natural and synthetic estrogens are excreted in an inactive conjugated form but are de-conjugated in environment and regain their estrogenic activity (Peterson et al., 2005). Some of these molecules can exert their effect at very low concentrations and thus very sensitive assays are required for detection.

In recent years, there has been increasing interest in chemicals that can modulate the endocrine system. Such chemicals have the potential to disrupt normal reproduction or developmental processes, which can lead to adverse health effects such as compromised reproductive capacity, breast and testicular cancer, reproductive dysfunction such as feminisation or demasculinisation of males and other adverse effects. A wide range of compounds including natural products, pharmaceuticals and industrial products has been shown to be estrogen mimics. As observed in recent years (Sohoni & Soto, 1998) some hormone-mimicking chemicals can elicit multiple endocrine disrupting activities that are mediated by various mechanisms of action, some of which may be active only during certain stages of development. Estrogenic compounds are characterised by their ability to bind and activate the estrogen receptor (ER), which is a transcription factor belonging to the steroid receptor family, but some compounds can disrupt hormone functions at different levels of the endocrine system, without directly interacting with the receptor (Hilscherova et al., 2000).

1.12.1 Genetically modified yeast sensors

The use of recombinant yeast as the sensing element in biosensors is being reported with increasing frequency. Some of the sensors function as model eukaryotic cells to detect toxicity/genotoxicity in either single specific molecules or mixtures of molecules such as environmental samples (Baronian, 2004). Many of the current yeast sensors are based on modified *Saccharomyces cerevisiae*. Other recombinant yeast sensors have

been constructed to detect specific molecules or group of molecules. *S. cerevisiae* mutant was used to increase the sensitivity of this organism to mutagens and carcinogens present in environmental samples (Terizyska et al., 2000). *Aureobasidium pullulans* was transformed with a vector containing the green fluorescent protein (GFP) reporter gene and the level of fluorescence was directly related to the number of viable cells (Webb et al., 2001). A number of recombinant yeast based estrogen assays have been developed for use in various research fields; these include studies into the mechanism of hormone binding and transcriptional activation using site-directed mutagenesis and also as a screen to determine blood plasma levels of estradiol. More recently, genetically modified yeast cells and human cell lines have been used for measuring estrogen activity by transcription activation of the reporter genes. Until now, yeast estrogen bioassays have been based on an extra-chromosomal reporter construct with β-galactosidase as a substrate based reporter protein. Alternative reporter genes are those encoding luciferase, which has been used as a highly sensitive reporter in animal cells and GFP (Bovee et al., 2004 a, 2004b).

1.12.2 Current estrogen bioassays

Currently wide varieties of different bioassays exist for the determination of estrogenic activity in environmental samples or individual compounds. These assays can be classified as *in vitro* or *in vivo* tests (Fang et al., 2000; Parrott et al., 2001). *In vivo* tests are substantially more costly and time-consuming. For this reason *in vitro* assays, such as E-Screen, a growth induction test with a human breast cancer cell line in yeast, (Soto et al., 1992), YES (yeast estrogen screen) assay (Routledge and Sumpter, 1996) or cell free binding assays using estrogen receptors α and β have gained popularity (Baker et al., 1999; Beresford et al., 2000; Bolger et al., 1998; Gaido et al., 1997; Kuiper et al., 1997; Payne et al., 2000; Rehamann, Schramm & Kettrup, 1999). Tests that are based on mammalian cell lines can be more sensitive than yeast based assays (Legler et al., 2002) and are considered more relevant for human health but the responsiveness of bioassays to estrogens and endocrine disrupters varies between different types of mammalian cell lines as the cellular environment including the composition of cofactors and receptor expression alters. This variation complicates the interpretations of the results obtained with mammalian cell lines, reducing their predictive value (Zacharewski, 1997; Gutendorf & Westendorf, 2001).

The limits of detection and determination of an analytical system determines the sensitivity of an assay system. The limit of detection is the lowest value for an analyte that can be statistically distinguished from the blank at which quantifying is just feasible to lead to detection and may not be relied upon for analytical measurements. The limit of determination (quantitation) is the value at which a given analytical procedure will be sufficiently precise to yield a satisfactory quantitative estimate of the analyte. The limits of detection and determination of the estrogen bioassays reported are summarised in Table 4.1.

1.12.3 Yeast estrogen screen (YES) to detect estrogens using the genetically modified human estrogen receptor (hER) *S. cerevisiae*

Yeast tests can be useful compared to tests using mammalian cell lines in the first stage of screening, as they are easy and inexpensive to perform and avoid the need for laborious and costly cell culturing (Leskinen et al., 2005). Furthermore, yeast cells are more resistant to environmental contaminants than mammalian cells, which is an important advantage in screening complex environmental samples. The yeast estrogen screen especially, is often used because of its simplicity. This assay is based on a recombinant *S. cerevisiae* carrying human estrogen receptor alpha (hERα) gene and an expression plasmid harbouring *lac Z* reporter gene (Routledge & Sumpter, 1996). The *lac Z* is activated by estrogens binding to the estrogen receptor and hydrolyses the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) resulting in a colour change from yellow to red which takes 72 hours. The steps involved in the optical system are described in the next page (Figure 1.4).
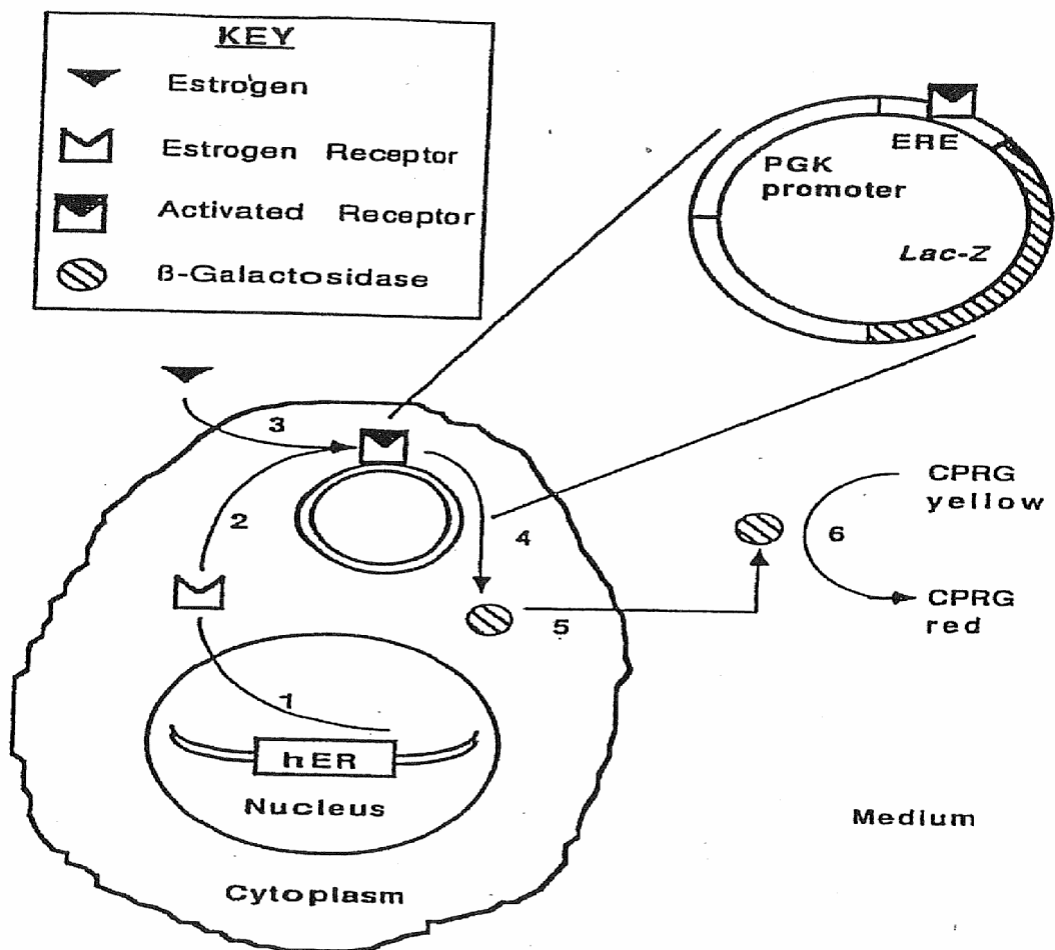
Figure 1.4 Schematic diagram of the estrogen-inducible expression system in yeast. The human estrogen receptor gene is integrated into the main genome and is expressed.
(From - Routledge & Sumpter, 1996).

Sequential steps in estrogen binding and subsequent colour production in the optical detection system (Figure 1.4).

1. When estrogen binds to the estrogen receptor, the receptor is activated.

2. The activated receptor attains a form capable of binding to estrogen response elements (ERE) within a hybrid promoter on the expression plasmid.

3. Binding of the ligand causes induction of the reporter gene *lac- Z*.

4. The production of β-galactosidase enzyme and this enzyme is secreted out into the medium.

5. β-galactosidase enzyme metabolizes the chromogenic substrate CPRG (normally yellow) into red product.

6. The colour change can be measured by absorbance.

The presence of the *lac Z* in the genetic modification system raised the question of whether the *lac Z* construct should result in the degradation of lactose. The chlorophenol red-β-D-galactopyranoside (CPRG) of the optical system was replaced with lactose in this study to investigate the use of a double mediator system as an alternative detection method. β-Galactosidase that is produced due to the presence of estrogens in cell environment resulting in the induction of the *Lac Z* reporter gene, should cleave lactose to glucose and galactose to result in an increase in the concentration of glucose in the cell and a consequent increase in catabolism.

In this study, a second response of hER *S. cerevisiae* to estrogen even without lactose was observed which suggested that there could be an underlying native estrogen receptor and estrogen binding mechanism. This observation led to the investigation of the wild type *S. cerevisiae* responses to estrogens

1.13 Wild type S. *cerevisiae* responses to estrogens

Wild type *S. cerevisiae* is of great interest because of its extensive use for industrial and commercial purposes. In addition to its commercial use *S. cerevisiae* has been extensively studied at molecular level and the entire genome of this organism has been sequenced. Estrogen Binding Protein (EBP) and its endogenous ligand was first isolated in *S. cerevisiae* (Feldman et al., 1982). The ligand was shown to be 17β-estradiol and these authors hypothesised that EBP may represent a primitive hormone receptor system for estrogen like molecules. Although some of the hormones present in fungi are well characterised, very little is known about the hormone-receptor systems that mediate these hormone actions. EBP also exhibits high affinity for mammalian hormones and it has a stereospecific, highly selective binding site for estrogenic molecules (Burshell et al., 1984). *S. cerevisiae* EBP was characterized by Burshell et al. (1984) and shown to be protein in nature and loses its binding affinity under certain conditions, such as changes in salt concentrations, temperature (37ºC binding optimum) and pH (optimum 6 - 6.5). In addition, the binding protein does not withstand freezing and thawing. The estimated molecular size of the protein is in the range of 60 - 70 Kda (Burshell et al., 1984). They characterised structural and binding properties of the EBP in *S. cerevisiae* and compared with mammalian estrogen receptors from the rat uterus. In *S. cerevisiae* the unique binding site for 17β-estradiol labelled with (^{3}H) was shown by competitive binding assays with various steroids, which is unlike other mammalian receptors or plasma-binding proteins (Burshell et al., 1984). The competition binding profiles of a rat uterus estrogen receptor was compared with those of the EBP of *S. cerevisiae*, using

(^{3}H) 17β-estradiol as a radioprobe (Burshell et al., 1984). In this study 17β-estradiol, 17α-estradiol, estrone, estriol, progesterone, tamoxifen, nafoxidine, diethylstilbestrol (DES), zeralanol, zeralenone and promegestone were used to compare these systems. 17β-estradiol is an excellent competitor in both systems. Whereas, 17α-estradiol has about 5% of the activity of 17β-estradiol in both the systems. The most significant differences between the two systems are the high potency binding of DES (a synthetic non-steroidal molecule) from the rat uterus, which is a poor competitor in the yeast systems and the finding that progesterone is a much better competitor in yeast compared to the rat uterus system. Very interestingly, fungal products with estrogenic activity, such as zearalenones, compete more strongly for the rat uterine estrogen receptor than they do for the EBP of *S. cerevisiae*. Estriol and estrone competed almost identically in both systems. The anti-estrogen nafoxidine and tamoxifen were less potent in yeast than in rat uterine systems. The *S. cerevisiae* EBP also differs from the sex steroid-binding globulin and α-fetoprotein. (^{3}H) testosterone does not have a binding site on *S. cerevisiae* EBP and does not compete for the (^{3}H) estradiol binding site (Burshell et al., 1984). 17β-Estradiol has been shown to influence the growth of *S. cerevisiae* by arresting growing cells a process known as growth arrest (GO arrest) stage (Tanaka et al., 1989). This growth arrest occurs as intracellular cAMP (cyclic adenosine monophosphate) levels increase and prevents the cells entering into growth phase (Tanaka et al., 1989).

1.13.1 Estrogen binding affinities of human estrogen receptors

Humans and higher organisms have at least two estrogen receptors (ER) α and β. Gutendrof and Westendorf (2001) assessed the estrogenic/antiestrogenic potency of 11 pure compounds including 17β-estradiol, estrone, estriol, ethynylestradiol, diethylstilbestrol, coumesterol, β -sistosterol, genistein, 4-nonylphenol, 4-octylphenol and bisphenol and complex environmental samples. They have used an array of *in vitro* test systems using human cell lines and estrogen receptors:

a. Two luciferase reporter gene assays using transgenic human cell lines with MCF-7 cells and HeLa cells.
b. Competitive binding assays with recombinant estrogen receptors (ER) α & β.
c. A proliferation assay using MCF-7 cells (E- Screen).

They tested in their studies that the relative potencies of all 11 compounds and found that the cell free binding assays were one to two orders of magnitude higher than the

cell culture assays. The relative potencies of the compounds varied within five orders of magnitude (Gutendrof & Westendorf, 2001). The most potent are 17β-estradiol, diethylstilbestrol and ethynylestradiol. The next potent estrogens are estrone and estriol. The phyto and xenoestrogens and tamoxifen are 10^4-10^5 less potent than 17β-estradiol (Gutendrof & Westendorf, 2001).

1.13.2 Estrogen binding protein (EBP) of *Paracoccidioides brasiliensis* and *Candida albicans*

The EBP from *Paracoccidioides brasiliensis* was isolated and shown to have a higher affinity to 17β-estradiol than estrone, estriol, progesterone, dihydrotestosterone and testosterone (Loose et al., 1983).

C. albicans has both an EBP, which has high affinity for estradiol, and a corticosteroid binding protein, which exhibits high affinity for corticosterone and progesterone (Feldman et al., 1982; Burshell et al., 1984). Skowronski and Feldman (1989) have shown that the abundance of estrogen binding protein is 4-10 times higher during the early logarithmic growth phase than in the stationary phase, and the molecular weight of the EBP determined by Sephacryl S-200 gel exclusion chromatography is 46 Kda.

EBP from *Candida albicans* was isolated and characterised (Madani et al., 1994). This study by competitive binding assays with (^{3}H) 17β-estradiol show that 17β-estradiol binds to EBP with greater affinity than estrone, 17α-estrdiol, progesterone, diethylstilbestrol, testosterone, corticosterone, dexamethasone, aldosterone and ergosterol. *C. albicans* EBP exhibited similar competitive binding profiles to those of *S. cerevisiae* (Madani et al., 1994). The competitive profile shows that 17β-estradiol is the best competitor to displace (^{3}H) estradiol from EBP binding site the next most potent steroid is estrone. Estriol and 17α-estradiol are about 10-fold less potent than 17β-estradiol. The synthetic estrogen diethylstilbestrol (DES), corticosterone, progesterone and anti-estrogen tamoxifen are ineffective competitors for the 17β (^{3}H)-estradiol binding site in EBP (Madani et al., 1994).

The gene encoding estrogen binding protein (*EBP1*) from *C. albicans* was cloned in the yeast expression vector pG5 (pG5-*EBP1*) and over expressed in *S. cerevisiae* (Madani et al., 1994). The oxidoreductase activity was measured by monitoring the oxidation of NADPH. A Southern blot of genomic DNA isolated from *C. albicans* and probed with *EBP1*gene has shown one intense band and two less intense bands. The intense band

represents the *EBP1* gene from the genomic DNA; the less intense bands could be *EBP1* related genes, which could possibly be *OYE* (old yellow enzyme) genes in *C. albicans* (Madani et al., 1994). *OYE1* was characterised in *S. carlsbergensis* (Saito et al, 1991) and *OYE2 OYE3* from *S. cerevisiae,* which are very similar (more than 95%) to each other (Nino et al., 1995). Madani et al. (1994) have identified a section of EBP amino acid sequence (residues 183-233) which is highly conserved among a number of flavoproteins including OYEs.

1.13.3 Possible mechanism of EBP mediated oxidoreductase activity

The EBP1 gene over expression in *S. cerevisiae* suggested that EBP has NADH oxidase activity, which is inhibited by the presence of 17β-estradiol. The 17β-estradiol probably modifies the enzyme activity of EBP allosterically, as previously demonstrated for glutamic dehydrogenase, an oxidoreductase from mammalian liver (Yielding & Tomkins, 1960). Estrogens have shown to increase hyphal formation in *C. albicans*. The inhibition of enzyme may be involved in the metabolic changes involved in yeast to mycelial conversion or block the mycelial to yeast conversion. In both the yeast species *C. albicans* and *P. brasiliensis,* EBP-mediated oxidoreductase enzyme activity is involved in pathogenicity. For *P. brasiliensis,* where infection is more prevalent in males than in females, the effect of a low level estradiol on the protein expression during morphogenesis promotes the predominance of the yeast form. (Clemons, Feldman & Stevens, 1989). These authors show not only the effect of temperature shift from 25°C to 37°C but also that estradiol by binding EBP induced the expression of several proteins involved in the morphogenesis from mycelial form to yeast form in *P. brasiliensis.* Their results strongly suggest that the EBP via binding to estradiol is mediating the regulation of protein expression, which is analogous to mammalian steroid receptors. They hypothesised that EBP could represent a primitive yeast hormone receptor system, which may be evolutionarily related to the estrogen receptor of higher organisms (Skowronski & Feldman, 1989).

1.13.4 The role of ABC transport cassette proteins in estradiol transport in *S. cerevisiae*

The ABC superfamily of proteins in *S. cerevisiae* shares homology with multi-drug resisitance (*MDR*) genes, which encode for multidrug resistance in man and mouse (Decottignies al., 1995). The multidrug transporters PDR5 and SNQ2 belong to the ATP binding ABC transport cassette proteins. PDR5 confers resistance to several unrelated drugs in *S. cerevisiae.* PDR5 resembles the p-glycoprotein of MDR type in mammalian cells. PDR5 and SNQ2 in *S. cerevisiae* are responsible for export and uptake of

estradiol respectively. The roles of these genes in estradiol uptake and intracellular accumulation were studied by creating deletion mutants in *pdr5* and *snq2* genes (Mahe, Lemoine, & Kuchler, 1996). The roles of these genes in estradiol uptake and intracellular accumulation were studied by creating deletion mutants in *PDR5* and *SNQ2* genes (Mahe, Lemoine, & Kuchler, 1996). These authors studied the effect of single and double mutants in estradiol accumulation. A yeast strain whose growth was mainly dependent on estradiol in a medium lacking uracil was used. In this study, 3ERE-URA 3 was used as a reporter gene construct. Uracil reporter gene transcription was induced in an estradiol dependent manner via estrogen responsive elements (3 ERE). The single *Δpdr5* mutant was able to grow at 0.5 nM estradiol, while the single *Δsnq2* mutant failed to grow at this estradiol concentration. Double mutants *Δpdr5 Δsnq2* were able to grow at a very low 0.1 nM estradiol concentration, showing that *PDR5* plays a key role in export of estradiol and disrupting the gene had a major effect on estradiol accumulation. The double mutant accumulated estradiol 30 times more efficiently than the isogenic wild type strain. The role of these ABC cassette transport proteins in the transport of anti-estrogen molecules was further characterised by studying these *Δpdr5* and Δsnq2 mutants using a hER/Lac Z reporter gene construct. These studies confirm that the *Δsnq2* and *Δpdr5* accumulated 65.5% and 44.5% more estradiol compared to the wild type (Tran et al., 1997).

1.14 Glucose catabolic repression

Glucose repression is an energy saving mechanism that is found in most free-living microorganisms (Ronne, 1995)**.** It acts to shut off genes that are used for the metabolism of alternative carbon sources in the presence of the preferred carbon source glucose. Many species of yeast including *S. cerevisiae* may thrive on a variety of carbon sources, but glucose and fructose are preferred. When glucose is present, the enzymes required for the utilisation of alternative carbon sources are synthesised at a low rate or are not synthesised. This phenomenon is known as catabolite repression (Gancedo, 1998).

1.14.1 Negative control of gene expression by the yeast glucose repression pathway

In yeast, glucose repression is mediated by a signalling pathway involving the *SNF1* protein kinase. *SNF1* is active in the absence of glucose and causes the derepression of glucose repressed genes by inhibiting the nuclear localisation of the glucose repressor *MIG1*. In the presence of glucose, *SNF1* is inactivated, and *MIG1* is free to repress its target genes. All the genes involved in the glucose repression cascade have been well characterised at the molecular level. Glucose induction and the level of proteins

expressed during glucose induction are also well known. The expression of several *HXT* genes encoding glucose transporters, are induced by glucose. Different *HXT* genes are induced at different levels of glucose. *HXT7* is a gene encoding major component of the high affinity transport activity of yeast cells at low glucose concentration. The trafficking of the HXT7 protein in the cell during its lifetime was monitored by using a chimera of *HXT7* and green fluorescent protein (GFP). The GFP fusion protein has also been used to determine the catalytic centre of activity of the transporters. Glucose repression thus involves two distinct mechanisms: an initial rapid response is mediated through any kinase, whereas, long-term repression specifically requires *HXK2* (Winde, 1996). Recent data suggest that HXK2 protein may have a more direct role in signalling glucose availability to the repression machinery by interacting with transcriptional factors, such as *MIG1*, that control the expression of glucose repressed genes (Rolland, Windericks & Thevelein, 2001).

Another glucose activated signalling pathway in yeast with a dramatic influence on many aspects of cellular physiology, is the adenylate cyclase-protein kinase pathway. Addition of glucose to glucose deprived cells causes a rapid spike in the cyclic adenosine mono phosphate (cAMP) level, triggering a protein phosphorylation cascade that affects a variety of targets at both the post-translational and transcriptional levels.

Several proteins whose expression is repressed by glucose are also rapidly inactivated at the post-translational level upon addition of glucose, for example fructose-1, 6-bisphosphatase and maltase permease (Holder, 1984; Jiang, 2000).

Glucose may affect enzyme levels by causing a decrease in the concentration of the corresponding mRNAs (messenger RNA), a decrease in their translation rate or an increase in the degradation rate of the protein. In turn, mRNA levels depend both on the rate of transcription of the gene and on the stability of the mRNA. The main responsive effect of glucose takes place at the transcriptional level. Examples include mitochondrial enzymes, fructose-1,6,bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase. These effects combine to prevent catabolism of monosaccharides and consequently di and polysaccharides and the absence and/or repression of mitochondria in these cells removes the pathways required for non-conventional substrates such as pentoses, sugar alcohols, organic acids, aliphatic alcohols, hydrocarbons and aromatic compounds (Walker, 1998). By glycolysis, lipids

are ultimately degraded in the mitochondria and via the glyoxalate cycle amino acids are degraded to ammonium and glutamate which are either catabolised via fermentation or the TCA cycle (Walker, 1998). The catabolism of lipids and amino acids could be effectively blocked by blocking glycolytic pathways and inhibiting mitochondrial function by addition of glucose (glucose catabolite repression).

Genes that are specifically regulated by glucose repression process (Gancedo, 1998)

Genes	Enzymes
GAL	Galactose catabolic enzymes
SUC-2	Invertase
FBP-1	Gluconeogenesis enzyme FBPase (fructose 1,6-bisphophatase)
ADH-2	Alcohol dehydrogenase
CYC-1	Iso-1-cytochrome C

The Hap 2/3/4/5/ transcription complex is required for expression of many mitochondrial proteins that function in electron transport and the Tri-carboxylic acid (TCA) cycle (Winde, 1996). Liu and Burow (1999) have shown that four TCA cycle genes; *CIT1*, ACO*1*, *ADH1* and *ADH2,* that are transcriptionally up-regulated by the Hap 2/3/4/5/ transcription complex, reduces or eliminate yeast cell respiratory function.

1.14.2 Exploiting glucose catabolite repression to achieve specificity in double mediated electrochemical yeast estrogen assays in non-extracted samples

Monitoring catabolic rate as a measure of a specific cellular activity may be a problem because other molecules in the environmental sample can also be catabolised resulting in the production of NADH/NADPH. The increased level of NADH/NADPH leads to increased reduction of lipophilic mediator TMPD and augments the electrochemical responses. In order to get specificity for the single analyte, e.g., estrogen, the competing analytes in the sample would need to be removed or controlled. There are methods to achieve specificity to estrogens. One approach is to extract the samples with solvents to

give semi-purified estrogens (Schwizerb et al., 1998; Colucci, Bork & Topp, 2001). Further solid phase extraction of the semi-purified estrogens should prevent the possibility of the additional catabolism (Korner et al., 1999).

In this study, catabolic responses to other catabolites that are present in non-extracted samples were prevented by exploiting the glucose catabolite repression phenomenon that occurs in *S. cerevisiae*.

1.15 Detection of estrogenic molecules in environmental samples

There are several groups working worldwide developing sensitive bioassays to detect estrogen molecules. Sewage samples from different regions tested by different assays are summarised in Table 4.3. Activated sludge treatment processes have proven to be effective in removing estrogenic compounds (Baronti et al., 2000). Agricultural waste and diary farm effluent contain high levels of estrogenic molecules and the endocrine disruption effect of these molecules in the waste samples is unknown (Sarmah et al., 2006). They reported that dairy farm effluent in the Waikato region of New Zealand has shown very high levels of estradiol (19 -1360 ng/L) expressed as estradiol equivalent estrogenicity concentration (EEQ) and the estradiol breakdown product estrone (41-3123 ng/L). The combined concentration of the estrogenic molecules in this area varied from 60 - 4000 ng/ L. The total estrogenic molecules in sewage treatment plant (STP) effluents in the Waikato region of New Zealand was reported as 109 ng/L (Sarmah et al., 2006).

An estrogen bioassay, which was developed in this study using wild type *S. cerevisiae* (Baronian and Gurazada, 2007) was tested to quantitate estrogenic molecules in the environmental samples. This is the first report to our knowledge, which has used wild type *S. cerevisiae* for estrogen screens.

1.16 Summary

There is an urgent need to develop rapid, sensitive cost effective detection systems to meet the increasing demand for environmental pollution monitoring. Biosensors especially whole-cell biosensors, offer advantages over other sensors available in-terms of cost and responses relevant to living systems. Although bacterial biosensors are commonly used as biocomponent, this research has exploited the advantages of using yeast as the biocomponent because of its direct relevance to higher organisms in toxicity

screenings. The unusual ability of the wild type yeast *A. adeninivorans* to utilise a wide variety of organic compounds as a sole carbon source was exploited in developing a biosensor which could detect gallic acid, naphthalene and di-butyl phthalate, listed priority environmental contaminants. Since the detection limits obtained were not as low as environmentally significant concentrations and interference from other catabolisable molecules in the sample occurred, the application of these biosensors for environmental application is limited.

Estrogens and estrogenic molecules are a major threat to human and aquatic life. There is a need to develop a sensitive, reliable and cost-effective biosensor to detect these very low levels of estrogenic compounds present in environment. There are several assays based on genetically modified organisms, human cell lines and other mammalian systems which are either expensive, time consuming or require special facilities. This research has led to the development of an estrogen bioassay based on wild type *S. cerevisiae* which is sensitive and rapid and overcomes the problems associated with the use of genetically modified yeast estrogen assays. The use of wild type *S. cerevisiae* avoids the use of genetically modified organisms and mammalian based cell lines to detect estrogens.

1.17 The specific aims of this study

    1.1 Exploit yeast properties in order to use it as a biocomponent in whole-cell biosensors and bioassays.

    1.2 Use the double mediated electrochemical detection system to detect yeast catabolic responses specific to the contaminant molecule.

    1.3 Develop a yeast based biosensor to detect environmental contaminants.

CHAPTER 2

RESEARCH METHODOLOGY

2.0 Electrochemical techniques to detect catabolic/anabolic redox molecules/mediator interactions in yeast

Electrochemical techniques have been used in various fields of biotechnology including biosensors, bioelectrochemical synthesis and biofuel cells (Hill, 1987). Electrochemical measurements have been employed for BOD (biochemical oxygen demand) measurements (Pasco et al., 2000; Trosok, Driscoll & Luong, 2001) yeast catabolism (Baronian et al., 2002) and viable cell population determination (Perez et al., 1998). Catabolism involves several redox reactions and specific redox couples that cycle between oxidised and reduced states. Although large numbers of proteins are electrochemically active at the outer cell membrane, they are electrochemically inactive within the cells and are unable to facilitate electron transfer to the external surface owing to cell membrane and other outer cell surface structures (Kim et al., 1999). However, redox mediators can fulfil this requirement by facilitating the transfer of electrons from internal reduced proteins to the external surface.

Mediators are small molecules that undergo redox reactions with external reduced catabolic proteins, which they oxidise. The double mediator system containing 2,3,5,6-tetramethyl 1,4-phenylenediamine (TMPD) or menadione and potassium ferricyanide has been used to probe intracellular redox activity of eukaryotes (Zhao et al., 2005; Heiskanen et al., 2004; Baronian et al., 2002., Rabinowitz et al., 1998). The detection of intracellular redox activity using TMPD or menadione as an electron transfer mediator is based on their diffusion through the cell membrane and reduction by cytosolic and mitochondrial enzymes catalysing electron transfer from NADP(H) to quinone substrates. The electrochemical quantitation of ferricyanide accumulation arising from TMPD/menadione mediated reduction of ferricyanide is an indication of the catabolic rate of the organism.

2.1 Electrochemical equipment and method

2.1.1 Electrochemical cell

Electron transfer reactions or redox reactions involve the transfer of electron from a species with a more electronegative reduction potential to more positive reduction potential, which can be measured by setting up an electrochemical cell.

The most commonly used electrochemical cell in experiments is the three-electrode configuration, comprising a reference electrode, working electrode and an auxiliary electrode (Figure 2.1). The reference electrode enables control of the potential at the surface of the working electrode where the redox reaction detection occurs. The potential can be set to facilitate the wanted redox reaction. For example the detection of cell catabolism can be monitored by monitoring the oxidation of reduced mediator. The current flows between auxiliary electrode and the working electrode.
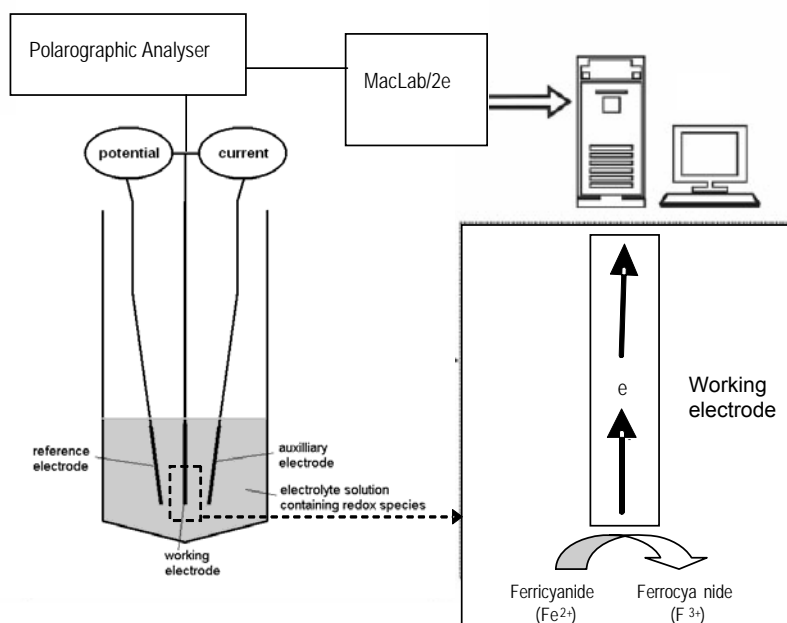
Figure 2.1 A typical three electrode elctrochemcial cell with a Polarographic analyser connected to the Macintosh computer for recording, visualising and archiving voltammogram

Many of the limitations associated with the conventionally sized solid electrodes such as 1 to 5 mm radius disc used in voltammetric techniques can be either been removed or greatly minimised via the use of microelectrodes of the diameter of ranging from a micrometer to about 25 μm (e-mail communication from Dr. Jonathan.O. Howell, Bioanalytical Systems). A platinum micro disc working electrode of 100 μm was used in this study and is categorised as a pseudo microdisc electrode. Although the smaller working microelectrode of 10 μm offers advantage of obtaining steady-state measurements faster, such as providing a faster scan rate, or being able to be held at a fixed potential for shorter time than a 100 μm electrode. In this study the larger diameter Pt pseudo microdisc working electrode was used because it can switch its behaviour depending on the scan rates used. At the very slow scan rate of 5 mV s^{-1} used in this study it behaves like a microrelectrode and at the faster scan rates of 20 -100 mV s^{-1} it behaves like a macroelectrode. In addition the 100 μm Pt pseudo microdisc working electrode has the following advantages:

a. The 100 μm Pt pseudo microdisc working electrode generates currents larger than 10 μm and hence the noise is of a less problem.

b. The 100 μm is more robust i.e. it is easier to retain the working property longer than a 10 μm disc (personal communication - Dr. Alison Downward, Canterbury University, New Zealand).

When electrolysis occurs, a concentration gradient is generated between the electrode surface and the solution. For this reason the current measured at all voltammetric electrodes depends on the modes of mass transport that are operating (Wightman & Wipf, 1996). At microelectrodes the predominant mode is diffusion. Because the flux as a result of diffusion is very large, the effects of convection tend to be less apparent than at electrodes of conventional size and for this reason, the majority of the theoretical treatments of currents at microelectrodes have dealt with diffusion-limited process. A major feature of the microelectrode voltammetry is the ready access to near steady state conditions (radial-diffusion) where the influence of ohmic drop (iR) is considerably reduced relative to that associated with the transient response (linear diffusion). In addition to this under near steady-state conditions, electrochemically derived charging current terms are negligible (Bond, 1994). The geometry of the microelectrode is also an important factor in fabricating microelectrodes and various techniques have been developed to construct microelectrodes geometries of such as hemispheres, discs, cylinders, rings and bands (Wightman & Wipf, 1996). In addition to their spatial

resolution several other features such as improved temporal resolution of electrochemical reactions, much greater fluxes under diffusion controlled conditions and the possibility of the investigation of electrochemical process in high resistance are possible with microelectrodes depending on the experimental conditions (Wightman & Wipf, 1996).

The redox reaction detection occurs at the surface of the Pt pseudo microdisc working electrode (pseudo microdisc electrode), at the appropriate applied potential that results in the mass transport of a new material to the electrode surface and generating the current. The wanted redox reaction at the working electrode can be facilitated by controlling the potential relative to the reference electrode.

Pt wire was used as the auxiliary electrode. Current flows between the working electrode and the auxiliary electrode.

Silver/silver Chloride (Ag/AgCl) was used as the reference electrode ($E^0 = + 0.222$ V, theoretical).

The three methods commonly used to quantify reduced mediator are voltammetry, coulometry and amperometry. Linear steady-state voltammetry has been chosen for this study.

2.2 Voltammetry

Steady-state voltammetry offers a powerful method of studying electrochemical systems with the advantage that the charging currents associated with the voltammetry are absent and the results are typically reproducible (Baronian et al., 2002., Streeter & Compton, 2007). Steady-state voltammetry, either linear sweep or cyclic voltammetry, are commonly used techniques to determine the amounts of oxidised and reduced forms of an electroactive species in solution. In steady-state voltammetry the potential of a working electrode is swept relative to a reference electrode. In voltammetry, the effects of the applied potential and the behaviour of the redox current are described by several well-known laws. In the cases where diffusion plays a controlling part, the current resulting from the redox process (Faradic current) is related to the material flux at the electrode-solution interface and is described by the Fick's law. The applied potential that controls the concentration of the redox species at the electrode surface and the rate of the reactions is described by the Nerst or Butler-Volmer equation. Voltammetry employs measurements of changes over time (T) in the current (I) flowing through the system of electrodes in relation to potential (E) applied to the working electrode. The

registered changes in the current gives a I (T) = f [E (T)] relationship. The plot is called the voltammogram.

2.2.1. Linear-sweep Voltammetry

In linear sweep voltammetry the dependence of the current flowing through the electrochemical measuring cell in which the examined substance is on the linear increase of the potential. The scan-rate of the electrode potential is usually varies between 20 -100 mV s^{-1} (Szczepaniak, 2002), however, in this study 5 mV s^{-1} has been used because with this slow scan rate the 100 µM pseudo microdisc electrode behaves like a microelectrode and gives the desired complete voltammogram showing the oxidised and reduced forms of the electroactive species compared to the faster scan rates ranging form 20 -100 mV s^{-1} which only gives partial data. This slow scan rate used in this study makes it possible to quantify the reduced mediator at the working electrode. The relationship I = f (E) has the shape of the plateau (Figure 2.4). The electrode potential at which the peak (maximum current) is observed is characteristic for a given substance and its height is proportional to the concentration of the substance. The position of the voltammogram obtained through linear sweep voltammetry on the current axis gives the proportions of each oxidation state, and the anodic and cathodic plateau currents allow quantitation of each redox form which is not possible with cyclic voltammetry.

2.2.2. Cyclic voltammetry

Cyclic voltammetry is based on varying the applied potential at a working electrode in both forward and reverse directions at a given scan rate, while monitoring the current. For example, the scan could be in the negative direction to the lowest potential and that point the scan would be reversed and run in the positive direction back to the highest potential. Depending on the analysis, one cycle, partial cycle or series of cycles can be performed. On the basis of the voltammogram, it is possible to determine not only the type and quantity of the substance examined but also to establish if the electrode process is reversible (Cyganski, 2004). This technique is also applied to study the mechanisms of electrode processes (Cyganski, 2004).

The important parameters in a cyclic voltammogram are the peak potentials (E_{pc}, E_{pa}) and peak currents (i_{pc}, i_{pa}) of cathodic and anodic peaks respectively. If the electron transfer process is faster than the other process (such as diffusion) the reaction is said to

be electrochemically reversible. Cyclic voltammetry is conducted in quiescent solutions to ensure diffusion control.

2.3 Cultivation of microorganisms

Microorganisms: *Arxula adeninivorans*, *Rhodotorula rubra*, *Saccharomyces cerevisiae* and *Trichosporon cutaneum* cells were grown in 100 mL yeast extract peptone dextrose (YEPD) medium (see Appendix-I) or in minimal defined medium (see Appendix-II/III) by inoculating well isolated single colony grown on YEPD agar. The flasks are incubated with shaking at 130 rpm for 16 hours at 34ºC for YEPD grown cells and 48 hours for minimal medium grown cells.

2.3.1 Harvesting the yeast cultures

Yeast cells were harvested by centrifugation in an eppendorf centrifuge at 5,000 rpm (equal to 3214 rcf) for 8 min at 10°C. The cells were washed twice in phosphate buffer pH 7 (see Appendix-IV) and re-suspended in phosphate buffered saline pH 7.0 (PBS, see Appendix- IV). The consistency of using fixed number of cells for all the experiments was achieved by maintaining the growth conditions of cells such as incubation time, incubation temperature and shaking speed constant. In addition, used the cells at fixed optical density of 3.0 using a LKB Novaspec II spectrophotometer rather than measuring the growth rate of cells.

2.3.2 Method of adjusting the yeast cell optical density (OD $_{600}$ nm) to 3.0

In order to be within the spectrophotometer's detection range, the yeast cells were diluted four times in PBS (1:4 dilution) and OD $_{600}$ was measured in Acryl-Cuvettes (10 x 4x 45 mm cuvette). The optical density was adjusted to 0.75 to obtain the OD $_{600}$ of 3.0 when multiplied with the dilution factor 4 i.e. 0.75 x 4 = 3.0 (Baronian et al., 2002).

2.3.3 Incubation of yeast cells with mediators and substrate(s)

A total volume of 5 mL of incubation suspension was prepared for each trial. The incubation suspension comprised cell suspension of OD 3.0 (3.675 mL), ferricyanide solution (0.8 mL, final concentration 80 mM, see Appendix- V), menadione (0.025 mL, final concentration of 100 μg/mL, see Appendix- V) TMPD solution (0.125 mL, final concentration 0.5 mM, see Appendix- V) and glucose/PBS (0.375mL, final concentration 7.5 mM).

The cells with substrate(s) were incubated at 34°C with oxygen-free nitrogen sparging for 1 or 8 hours (estrogens) depending on the substrates used in the incubation mixture

(Figure 2.2). At the completion of incubation, cells were pelleted by centrifugation at 8000 rpm at 20°C for 8 minutes. The supernatant was transferred to sterile tubes (Figure 2.2) and used it for electroanalysis. All trials were run in triplicate with the supernatants. Because a basal level of catabolism still operates in the absence of external substrates, all experiments included a negative control to set the base level (endogenous) catabolic activity. This endogenous catabolism is referred to as either 0 mM substrate or control.
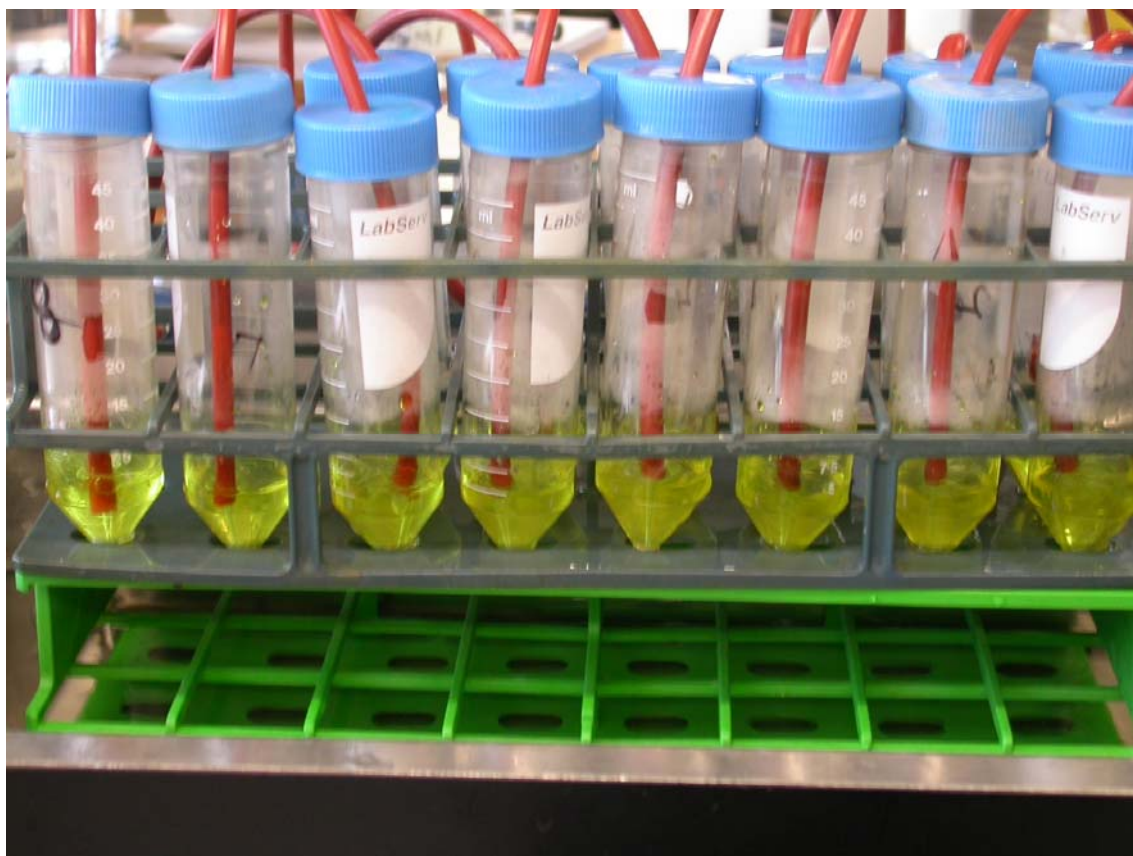
Figure 2.2 The incubation set up: T = 34°C, tubes sparged with nitrogen. Tubes contain yeast cells, mediators and substrates.

2.4 Electroanalysis of the supernatant

In all the experiments the amount of oxidised/reduced mediator in the supernatant was analysed by steady-state voltammetry. Steady-state voltammetry was performed by using a 100 µm diameter Platinum pseudo microdisc working electrode (Bio-Analytical Systems), a Pt wire auxiliary electrode (Bio-Analytical Systems or G-Glass), and a Ag/AgCl reference electrode (Bio-Analytical Systems) controlled by a polarographic analyser (EG&G Princeton Applied Research model 364). An AD Instruments Maclab 2e and an Apple computer were used for signal processing depicted in Figure 2.3.

Figure 2.3 An AD Instruments Maclab 2e and an Apple-computer used for signal processing.

2.4.1 Electrode pre-treatment

Before each trial, the Pt pseudo microdisc electrodes were polished on a Lecloth (Leco) with slurry of 0.3 μm alumina (Leco) at the shape of a figure eight for 40 seconds. It was then washed with distilled water to remove the alumina and polished on Lecloth alone (without alumina) to remove any traces of alumina before each scan.

2.4.2 Steady-state voltammetry

Steady-state voltammograms were obtained after initialising for 20 seconds and scanning from 450 to 100 mV at a scan rate of 5 mV s^{-1} and the steady-state current was measured at 425 mV. 450 mV was used as the starting point to allow for initial fluctuations of the system and allowed the system to stabilise as the anodic current was measured at 425 mV (Figure 2.4).
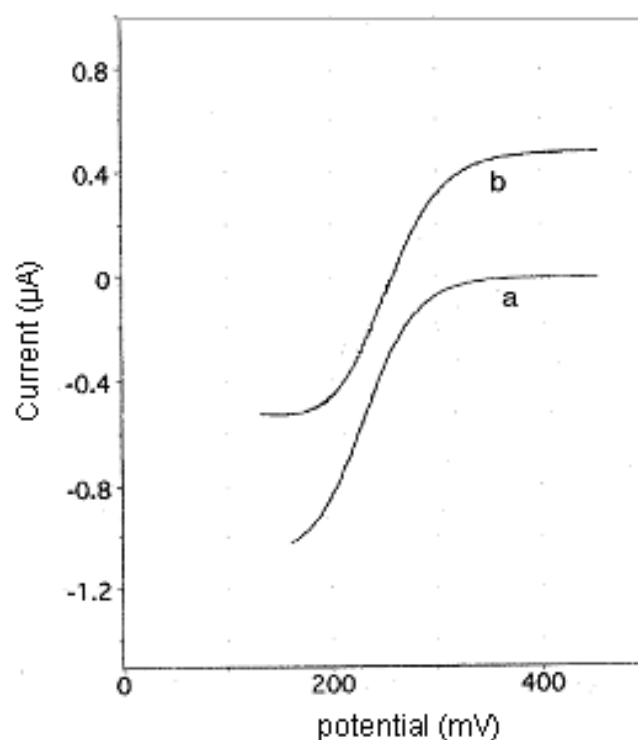
Figure 2.4 Voltammograms of ferricyanide (a) before incubation with *S. cerevisiae* cells and (b) after incubation with *S. cerevisiae* cells with 7.5 mM glucose. The difference in voltammograms represents the catabolic responses of the organism.
(From – Gurazada & Baronian, 2006, Poster presentation in "The Ninth World Congress on Biosensors, Toronto, Canada)

In Figure 2.4 Curve (a) shows that before incubation of cells with ferricyanide, the mediator is completely in the oxidised form, (the voltammogram shows only cathodic current due to reduction of ferricyanide). After incubation of cells for one hour, the voltammogram Curve (b) has shifted up the current axis and there is both anodic current (arising from oxidation of ferricyanide) and cathodic current (arising from reduction of ferricyanide) and represents the catabolic response of the organism to glucose.

The steady-state current at 425 mV was used as the sample signal (response) and the mean current at this potential from three repeat voltammograms was calculated and used in analysis. The measured currents varied by less than 5%. The mean currents from each trial triplicates were used to calculate the mean current and standard deviation for each experiment. The standard deviations are shown as error bars on the plotted data. Total assay time was a 60 min incubation period and an analysis time of less than 5 min per three repeat voltammograms and for estrogen assays a 5-hour incubation period plus an analysis time less than 5 min per three repeat voltammograms.

2.5 Preparation of *Arxula adeninivorans* LS3 genomic DNA

Total genomic DNA was isolated from *A. adeniniovrans LS3* to use as a template to perform polymerase chain reaction to identify the self-splicing intron in 25S rDNA to confirm its authenticity.

Buffers and solutions

1. Sodium phosphate buffer pH 6.5 (12.5 mL)
2. Solution 1 and 2 (see Appendix- VI)
3. Tris Ethidium-bromide (TE) Buffer (see Appendix- VI).
4. TBE buffer (see Appendix- VI)

2.5.1 Genomic DNA isolation from A. adeninivorans

The method of isolating total genomic DNA from *A. adeninivorans* (Appendix X) was followed from the yeast DNA protocols

http://www.le.ac.uk/genetics/teaching/msc/protocols/yeastdna.html

2.6 Polymerase Chain Reaction (PCR)

Oligonucleotide primers (primer I and primer II) were designed to amplify the group I C intron by using the computer programme (http://alces.med.umn.edu/bin/websub). Primers design was based on the published 25S rDNA sequence in NCBI with *Z50840* accession number from *AA25SLS3*. Two primers were designed, one primer designed to amplify the DNA fragment starting from nucleotides 2901 - 3311 base pairs (410 bp) and second primer was designed to amplify the longer fragment (3719 bp) encompassing group IC intron of 25S rDNA starting from nucleotides 121- 3840 bp.

INT - 1 Primers

Forward primer starting from 121+ cgctgaacttaagcatatcaataagc

Reverse primer starting from 3840- acaaggctactctactgcttacaatactc

INT - 2 Primers

Forward primer starting from 2840+ atcgatcctttagtccctcagaa

Reverse primer starting from 3440- ctcacgttccctattagtgggtg

Oligonucleotide primers (INT - I and INT – II) - Purchased from primer direct 'Invitrogen' company as a lyophilised powder form which was reconstituted as per the protocol supplied by the company. Polymerase chain reaction reagents: PCR buffer,

deoxyribonucleotide phosphates (dNTP), Taq DNA polymerase purchased from Invitrogen Company (New Zealand).

2.6.1 Primers reconstitution

Primers are reconstituted by centrifuging the tube for approximately 10 seconds to collect the DNA at the bottom of the tube. The lid was opened carefully and appropriate volumes of TE buffer were added. The lid was then closed for 2 minutes for dehydration, after which the tube was vortexed for 10 sec to prepare 10 μM stock solutions. The reconstituted primers are stored at -20°C. The stability of reconstituted primers is > 6 months.

2.6.2 PCR amplification

Two sets of primers designed based on the sequences of 25S rDNA to amplify the larger fragment of 3719 bp spanning the intron sequence and the other smaller fragment of 600 bp harbouring the 410 bp self-splicing unique intron sequences. The master-mix of each reaction was prepared and is given below

PCR components	Tube-1 (control)	Tube-2 (Control)	Tube-3	Tube-4	Tube-5	Tube-6
Sterile distilled water	19 μL	19 μL	15 μL	11 μL	15 μL	11 μL
PCR buffer with MgCl$_2$ (10X)	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL	2.5 μL
dNTPs 10 mM	0.5 μL	0.5 μL	0.5 μL	0.5 μL	0.5 μL	0.5 μL
DNA Taq.polymerase	1 μL	1 μL	1 μL	1 μL	1 μL	1 μL
DNA	-	-	4 μL	8 μL	4 μL	8 μL
Primer mix Forward & Reverse	2 μL (INT-1)	2 μL (INT-2)	2 μL (INT-1)	2 μL (INT-1)	2 μL (INT-2)	2 μL (INT-2)
Total	25 μL	25 μL	25 μL	25 μL	25 μL	25 μL

The PCR master-mix was prepared as above for two sets of primers with appropriate negative controls (no DNA added and hence no amplification with the two sets of

primers). The following PCR amplification protocol was used for all the samples: the denaturation programme was set for 5 min at 95°C and the 35 PCR cycles were set up in the PCR thermocylcer machine as follows:

1. Denaturation – 94°C for 1 minute.
2. Annealing – 68°C for 1 minute.
3. Extension – 72°C for 3 minutes

After 35 cycles, the tubes were left for extended extension of 7 minutes. At the end of the PCR amplification, tubes were removed from the thermocycler machine and stored at -20 °C, the samples were run in agarose gel electrophoresis to confirm the success of PCR amplification and measurement of the sizes of the DNA fragments amplified.

2.7 Agarose gel

The method for preparing 1% agarose gel and gel electrophoresis is described in Appendix XI.

2.7.1 Materials

Agarose (Sigma), 10 µg/mL ethidium bromide, agarose gel sample-loading buffer, DNA ladder (500bp, DNA marker).

Agarose gel running apparatus

Power pack, leads, Polaroid camera, transilluminator.

2.8 *A. adeninivorans* cultivation and preparation

The YEPD medium (see Appendix- I) was inoculated with isolated single colony of *A. adeninivorans* and incubated overnight at 34°C shaker incubator at 130 rpm. Cells were harvested and cell density was adjusted to an optical density of OD_{600} to 3.0 as described earlier (2.3.2).

2.8.1 Incubation of *A. adeninivorans* cells with mediators and substrate(s)

A total volume of 5 mL of incubation suspension was prepared for each trial. The incubation suspension comprised cell suspension of OD 3.0 (3.7 mL), ferricyanide solution (0.8 mL, final concentration 80 mM, see Appendix V), TMPD solution (0.125 mL, final concentration 0.5 mM, see Appendix V), glucose or PBS (0.375 mL, final concentration 7.5 mM).

The incubation of cells with substrate(s) was performed at 34°C with oxygen-free nitrogen sparging for 1 hour. At the completion of incubation, cells were pelleted by centrifugation and the supernatant was collected and used for electroanalysis.

2.8.2 Electroanalysis of the supernatant

Steady-state voltammetry was performed and steady-state voltammograms were obtained, as described earlier (see 2.4.2).

2.9 Cultivation of *A. adeninivorans* and 'Lentikat' preparation

A. adeninivorans cells were grown by inoculating a single, well isolated colony on the plate into 100 mL YEPD broth in culture flasks and incubated at 34°C with shaking at 130 rpm overnight. The cells were harvested by centrifuging the cells at 8000 rpm for 8 min at 10°C. The pellet was washed with phosphate buffer pH 7.0 (see Appendix IV) and finally resuspended in phosphate buffered saline pH 7.0 (see Appendix IV). The cells were adjusted to OD_{600} = 3.0. Lentikat' solution (supplied by 'Genia Biotechnology') was kept at 40°C. To 70 mL of 'Lentikat 'solution 0.2 mL of cells and 17.3 mL of PBS were added. The cell suspension was mixed thoroughly. The PVA cell suspension was dropped using a 'Lentikat' printer onto pre-weighed (a) plastic Petri dishes to form discs approximately 3 mm in diameter. The Petri dishes + 'Lentikats' were weighed (b). The PVA was cross-linked and stabilised by drying alone. This was achieved by placing them in biohazard cabinet and weighing every 2 hours until the weight of the 'Lentikats' were reduced to 25% of their original weight d. This was achieved by using the following formulae:

c = (b-a) x 0.25

d= a + c

a = weight of plastic Petri dishes.

b = weight of Petri dishes + 'Lentikats'.

c = weight of 'Lentikats'.

d = 25% dry weight of 'Lentikats'

After the Petri dishes attained the target weight, the 'Lentikats' were rehydrated with 'Lentikat' stabilising solution. The 'Lentikat' stabilising solution (supplied by 'Genia Biotechnology') containing the 'Lentikats' was transferred into a 500 mL sterile bottle and stirred with a sterile magnetic stirrer for two hours at room temperature. After two hours of stirring the 'Lentikat' stabilising solution was discarded and the 'Lentikats' were transferred to a sterile 2000 mL conical flask containing 400 mL of sterile YEPD medium. The 'Lentikats' in YEPD medium were incubated overnight at 34°C with shaking. The immobilised cells were finally washed in PBS three times and stored in PBS at 4°C.

2.9.1 'Lentikats' staining and the number of 'Lentikats' used in experiments

'Lentikats' staining was done in 1% Neutral Red dye for half an hour and then washed with excess water. The stained 'Lentikat' was placed on a slide and viewed using bright field microscope at 400X. 'Lentikats' used for glucose experiments were counted manually by using forceps.

2.10 *A. adeninivorans* cultivation and preparation for detecting environmental contaminants

A. adeninivorans was grown in a minimal medium (pH 5.5) (see Appendix II, Atlas, 1996, Second Edition, CRC Press) with either gallic acid, naphthalene or di-butyl phthalate as a sole carbon source. Cells were inoculated into 100 mL of medium in 250 mL culture flasks at 34°C and shaken at 130 rpm to provide good aeration. Gallic acid (0.59M), naphthalene (0.083 mM) or di-butyl phthalate (0.0363 mM) were used as a sole carbon source (1 mg/mL final concentration of all contaminants). Because these compounds are toxic, each was first added at 0.7 mg/mL concentration and incubated for 24 hours, and then a further 0.3 mg/mL was added and incubated for another 24 hours for all the carbon sources except for the naphthalene cultures which were incubated for another 48 hours. The cells were then harvested as described earlier (2.3.1) and finally resuspended in phosphate buffered saline pH 7.0. The optical density was adjusted at $OD_{600} = 1.9$ for all the contaminant molecules except for naphthalene (using naphthalene as a sole carbon source resulted in the low growth and even after three days of incubation, the OD_{600} was 0.920).

2.10.1 Reagents preparation (stock solutions)

1. Gallic acid was dissolved in boiling water to give 0.59 M (100 mg/mL) stock solution.
2. Naphthalene was dissolved in 100% ethanol to give 0.5 M stock solution.
3. Di-butyl phthalate was prepared in 100% ethanol to give 0.5 M stock solution
4. 0.1 M Glucose stock solution was prepared.

All solutions were filter sterilised through a 0.45 µM Millipore filter and stored at 4°C.

2.10.2 Incubation of cells with mediators and substrates

Pre-conditioned cells- A total volume of 5 mL of incubation suspension was prepared for each trial. The incubation suspension comprised 4.060 mL cells, 0.8 mL of ferricyanide (final concentration of 80 mM), 0.125 mL TMPD solution (final

concentration of 0.5 mM) and 0.01 mL of substrate solution or 0.01 mL ethanol. For glucose experiments, 0.005 mL glucose or 0.005 mL PBS was added.

The cells with mediators and substrate were incubated at 34°C for one and half hours with oxygen sparging for naphthalene. Oxygen is required with the mediators in the incubation mix to break open the naphthalene ring structure and to enable its utilisation by *A. adenininovrans* cells (standardised the experimental conditions after several experimental trials). For gallic acid and di-butyl phthalate one-hour incubation at 34°C with oxygen-free nitrogen sparging was performed. There is no oxygen requirement in the incubation mix for the utilisation of di-bytyl phthalate and gallic acid and the presence of oxygen will compete with the mediators for electrons that results in smaller response, hence oxygen free nitrogen sparging was used. At the completion of incubation, cells were pelleted by centrifugation at 8000 rpm for 8 min at 20°C. The supernatant was transferred to fresh tube and electroanalysis was performed. All the trials were run in triplicate.

2.10.3 Electroanalysis of the supernatant
Steady-state voltammetry was performed and steady-state voltammograms were obtained as described earlier in section 2.4.2.

2.11 Minimal medium pH (7.1)
Minimal medium (pH 7.1) was prepared using the Routledge and Sumpter (1996) protocol (Appendix- III). Growth medium was prepared by adding 5 mL of 20% glucose solution, 1.25 mL of 4 mg/mL L-asparitic acid solution, 125 µL of 20 mM copper (II) sulphate solution and 0.5 mL vitamin solution to 45 mL single strength minimal medium in a sterile conical flask.

2.11.1 Preparation of substrate stock solutions
Glucose and lactose (BDH AnalaR), 0.1 M stocks, 180 mg L^{-1} peptone (GibcoBRL Peptone 140, casein hydrolysate) were prepared in distilled water. 17β-estradiol (sigma) stock solutions were prepared in 96% ethanol. Initially 17β-estradiol 3 mg L^{-1} stock was prepared and did serial dilutions from that stock (1:10) to prepare 300 µg L^{-1}, 30 µg L^{-1}, 3 µg L^{-1}, 0.3 µg L^{-1}, 0.03 µg L^{-1} and 0.003 µg L^{-1} stock solutions. 17α-estradiol, 17α-ethynylestradiol and estrone (sigma) were dissolved in 96% ethanol to give 3 mg L^{-1} stock and did serial dilutions (1:10) to prepare 300 µg L^{-1}, 30 µg L^{-1}, 3 µg L^{-1}. All solutions were filter-sterilised (0.45 µM Millipore filters) and stored at 4°C. All glass

and plasticware with the exception of pipette tips were washed in absolute ethanol to remove traces of estrogenic substances.

2.11.2 hER S. *cerevisiae* cells cultivation and preparation

hER *S. cerevisiae* was made available by Environmental Science and Research (ESR) Christchurch, New Zealand. The strain was developed by Genetics Department at Glaxo Wellcome, Stevenage, Hertfordshire, U.K. hER *S. cerevisiae* was constructed by integration of the human estrogen receptor gene (hERα) into the *S. cerevisiae* genome and an expression plasmid carrying estrogen responsive elements (ERE) coupled to a *lac Z* reporter gene. hER *S. cerevisiae* was used in YES screen to identify estrogenic compounds. (Rutledge and Sumpter, 1996).

A well isolated single colony of hER *S. cerevisiae* from a plate was inoculated in 100 mL minimal medium (growth medium) and incubated at 30°C shaker incubator at 130 rpm for two days. Cells were harvested by centrifugation at 5000 rpm for 8 min at 10°C. The cells were washed twice in phosphate buffer (pH 7.0) and re-suspended in phosphate buffered saline (pH 7.0). Cell density was adjusted to an optical density at 600nm (OD_{600}) = 3.0 using LKB Novaspec II spectrophotometer as described above. Cells were immediately incubated with appropriate substrates at 34°C with oxygen-free nitrogen sparging for 8 hours.

2.11.3 Incubation of cells with mediators and estrogen substrate(s)

A total volume of 5mL of incubation suspension was prepared for each trial. The incubation suspension comprised a cell suspension of OD = 3.0 (3.524 mL), ferricyanide (0.8 mL, final concentration 80 mM), TMPD (0.125 mL, final concentration 0.5 mM), lactose (0.375 mL, final concentration 7.5 mM) and 0.166 mL or less of estrogen substrate (final concentration of 3.7×10^{-6} nM, 3.7×10^{-5} nM, 3.7×10^{-4} nM, 3.7×10^{-3} nM, 3.7×10^{-2} nM, 3.67 nM , 11 nM, 22 nM, 55 nM and 110 nM) and 96% ethanol (0.166 mL in endogenous control groups where required), 10 µL made up of 5 µL 0.1 mM glucose and/or fructose (final concentration 0.1 mM) and/or 5 µL peptone final concentration 0.018% with or without 5 µL PBS where required.

The incubation of cells with substrate(s) was done at 34°C with oxygen-free nitrogen sparging for 8 hours. On completion, the cells were pelleted by centrifugation (8000 rpm at 20°C for 8 min) and the supernatant was collected for electroanalysis. All trials were run in triplicate. Because a basal level of catabolism still operates in the absence of

external substrates, all experiments included a negative control to set the base level (endogenous) catabolic activity. This endogenous catabolism is referred to either 0 mM substrate or control.

2.11.4 Electroanalysis of the supernatant

Steady-state voltammetry was performed and steady-state voltammograms were obtained as described earlier.

2.12 Wild -type *Saccharomyces cerevisiae* NCTC 10716 and *Candida albicans* CDC S-24

2.12.1 Cultivation of cells

S. cerevisiae and *C. albicans* were cultivated in minimal medium (Routledge & Sumpter, 1996) containing glucose as a sole carbon (energy) source. In one of the experiments with *S. cerevisiae* used YEPD medium. Cells, which were grown in the minimal defined medium, were incubated for 48 hours, and cells that were grown in YEPD were incubated for 16 hours. Both groups were shaken at 130 rpm in indented flasks at 30ºC, and harvested by centrifugation at 5000 rpm for 10 min at 10ºC. The cell pellet was washed twice with phosphate buffer (pH 7) and resuspended in phosphate buffered saline (pH 7) to obtain an optical density of 3.0 at OD_{600}. Incubation of cells with mediators and substrates and incubation as described for hER *S. cerevisiae* (2.11.3) except 0.375 µL lactose was replaced with 0.375 µL PBS.

2.13 Environmental sample collection and preparation

The samples were collected in sterile bottles. The samples and collection sites are given below

1. Trickling Filters influent and effluent – The staff of Wastewater Treatment Plant (WTP), Christchurch, supplied the Trickling Filters influent and effluent samples.
2. Avon River water – Sample was collected from Avon River at the banks of Antigua Boatsheds site, Cambridge Terrace, Christchurch.
3. Halswell River water – Halswell River water was collected from the banks of Halswell River, Leadleys road, Halswell, Christchurch.
4. Tai Tapu River – Tai Tapu River water was collected form the banks of Tai Tapu River, Lincoln Tai Tapu Road, Canterbury.

The samples were centrifuged for 10 min, 8000 rpm at 10ºC to remove solid particles. The supernatant was transferred into a sterile bottle and then filter sterilised to remove microbes and stored in a sterile bottle at -20ºC.

2.13.1 Mediators and substrate
As described for hER *S. cerevisiae* (2.11.3).

2.13.2 Environmental samples preparation for the quantitation of total estrogenic compounds
Environmental samples were analysed for total estrogenic compounds using different dilutions such as 1:700, 1:50, 1:70 and 1:7 to be within the systems limitations. Environmental samples were prepared in a total 7 mL volume with appropriate control (no sample or estradiol, 1mL buffer and 0.024 mL ethanol) and l mL of sample of each dilution. 0.024 µL of estradiol or ethanol to give estrogen (0.367 nM and 0.037 nM) standards. Potassium ferricyanide 1.12 mL to give 80 mM final concentration and 0.175 mL of TMPD to give 0.5 mM final concentration and 0.007 mL of glucose to give 0.1 mM final concentration and *S. cerevisiae* cells 4.674 mL was used to give total volume of 7 mL.

The incubation of cells with substrate(s) was performed at 34°C with oxygen-free nitrogen sparging for 5 hours. At the completion of incubation, cells were pelleted by centrifugation (8000 rpm at 20°C for 8 min) and the supernatant was removed for analysis. All trials were run in triplicate. Because a basal level of catabolism still operates in the absence of external substrates, all experiments included a negative control to set the base level (endogenous) catabolic activity. This endogenous catabolism is referred to either 0 mM substrate or control.

2.13.3 Incubation of cells with mediators and substrate(s)
As described for hER *S. cerevisiae* except the incubations was performed for 5 hours.

2.13.4 Quantitation of glucose in the environmental samples
Glucose was quantified in the environmental samples prior to quantification of total estrogens. The enzyme glucose oxidase enzyme (1000 U) was used for each sample (1 mL of sample in 4 ml of phosphate buffer saline, pH 7.0 and potassium ferricyanide). The tubes containing the sample, enzyme and mediator were incubated for half-an hour

at 34°C. The glucose in the environmental sample was oxidised by glucose oxidase enzyme to give gluconic acid and hydrogen peroxide and the potassium ferricyanide was reduced. The reduced ferricyanide was quantified which gave a concentration of glucose in the environmental samples (Pan et al., 2005).

2.13.5 Electroanalysis of the supernatant

Steady-state voltammetry was performed and steady-state voltammograms were obtained as described earlier. The steady-state current at 425 mV was measured and was used as a relative measure of the amount of ferricyanide produced, hence the number of cell redox molecules that are oxidised by TMPD (either by yeast catabolism and/or estrogen mediated interaction modifying the estrogen binding protein's (EBP) NADPH oxidoreductase enzyme activity depicted in Figure 4.6).

2.14 Graphical data analysis

R^2, Regression line, equation, standard deviations, error bars, statistical analysis and other graphical analysis were performed using Microsoft Excel. Error bars are 1 standard deviation. EC_{50} values were calculated from the equation obtained by performing regression analysis of dose responsive curves data.

2.14.1 Limit of detection

It is important for any analytical measurements to calculate the limits of detection and determination. The limit of detection (y_L) was calculated by the blank-value procedure from the blank sample B, the Standard deviation (variation) of the blank sample s_B.

The limit of detection is given by:

$y_L = B + 4.65\ s_B$., (Riedel et al., 1988)

There is 5% chance of misclassification when the means of the distributions of the blank and limit of detection are separated by 4.65 SD units compared to 3.29 SD units for the well-known blank model (Kichmer, C.J, 1988).

2.14.2 Limit of determination

The limit of determination (y_D)/quantitation is the value calculated from the blank value. The limit of determination is given by

$y_D = B + 14.1\ s_B$., (Riedel et al., 1988)

2.14.3 Statistical Analysis

ANOVA Analysis – This is also known as analysis of variance. If there are only two groups, a t-test can be used, however one could use ANOVA and in this study, ANOVA has been used. It is used to test the null hypothesis Mean 1 = Mean 2 = Mean 3
against the alternative hypothesis that not all means are equal

The aim of performing ANOVA is to test the null hypothesis at a certain confidence level. The p-value or calculated probability is the estimated probability that the data is statistically similar or different. If the p - values obtained in the ANOVA analysis are less than 0.05 (alpha) then they indicate that not all means are equal at the 95% confidence level and the null hypothesis is rejected, if p-values are more than 0.05 then they are not significantly different (similar) and the null hypothesis is accepted.

The assumptions of ANOVA

There are several important assumptions of ANOVA, which should be adhered to rather closely. They are:

1. Each of the groups is a random sample from the population of interest.

2. The measured variable is continuous or if discrete, it may assume a large range of values.

3. Measurement is on a ratio or intervals scales.

4. The error variances of the different groups are equal.

5. The residuals are approximately normally distributed.

The assumptions are not quiet as simple and may be relaxed under certain conditions (Hampton and Hovel 2nd Ed, 2006)

Linear Regression analysis and assumptions

Regression analysis is used to estimate an equation that describes the linear relationship between the two variables in question. This is called the regression equation or the regression function. From this equation it is possible to construct a line through the points of a scatter plot, which is called the least squares regression line. The regression equation may be used to predict the mean value of the dependent variable (y) at various values of the independent variable (x) and can also calculate a confidence interval for the prediction to give a measure of the error or likely range of possible responses. Regression analysis is based on several assumptions that are given below:

1. The independent variable is fixed. This means, in effect, that values of the independent variable are chosen by the investigator and do not represent a random variable in the population. There is thus no variance associated with the independent variables.

2. For any value of the independent variable (x) there exists a normally distributed population of values of the dependent variable, y

3. The variances of residuals for all values of x are equal.

4. Observations are independent.

5. The functional relationship is linear which can be checked by plotting residuals.

2.15 The use of logarithmic transformation of the data

The most common use of transformation in biology is to help the data meet the distributional and variance assumptions required for linear models.

Logarithmic transformation of dependent variable - Emerson (1991), Sokal and Rohlf (1995), Tabachnick, and Fidel (1996) provide excellent descriptions and justifications of data transformation. The most common transformation is logarithmic transformation, where the transformed data is simply the logs (to any base) of the original data and there are choices of which variables to transform. The significance of "the logarithmic transformation" phenomenon in terms of the dynamics of a biological system was specified (Bartlett, 1947). In the case of a variance greater than the mean, the literature advises considering a logarithmic transformation of the data (Bartlett, 1947; Quenouille, 1950). Quenouille (1950) stated that the logarithmic transformation tends to restore normality in the distribution and equalise the variances simultaneously whereas Hoyle (1973) cites a number of studies empirically showing the logarithmic transformation as a way of making the data conform to the three linear-model assumptions of additivity, constant variance, and normality. Aitchison and Brown (1957) defined the lognormal distribution as "the distribution of a variate whose logarithm obeys the normal law of probability". According to them, many of the properties of the lognormal may be immediately derived from those of the normal distribution. Keynes (1921) regarded the main advantage of the lognormal distribution the possibility it offered of adapting without much trouble to asymmetrical phenomena numerous expressions that had already been calculated for the normal law of error. In contrast to the normal distribution, which is centred on the arithmetic mean, the lognormal distribution is centred on the geometric mean, which can be calculated by first calculating the arithmetic mean of the logarithmically transformed data and then taking this mean's antilogarithm. Thus, we can see that the purpose of the logarithmic

transformation is to create a model that conforms to the requirements of the normal law of error for inferential purposes. It does this by artificially reducing the amount of variance to that of the normal distribution. In summary, the logarithmic transformation of data enables the analyst to switch the law of error for tests of significance in linear models from the normal distribution to the lognormal distribution. It is important that confidence intervals for predictions based on transformation of data will be meaningful as the probability assumptions used in making these assumptions are valid if the residuals are Normal. Log transformation if Y also linearise exponential function.

There are several aims of data transformation for statistical analysis, especially for linear models (Quinn & Keough, 2002):

- To make the data and the model error terms closer to a normal distribution.
- To improve homogeneity of variances, often because of improving normality.
- To reduce the influence of outliers especially when they are at the one end of distribution.
- To reduce the size of interaction factors.

Logarithmic transformation of independent variable - Plotting doses or concentrations on a logarithmic scale is a standard procedure for the analysis of pharmacological and toxicological experiments, because the dose steps used are often spaced widely and in logarithmic steps (Lutz et al., 2005). If there is no evidence of variance heterogeneity, then it is best to transform X to try to linearise the relationship (Neter et al., 1996). Sampling variability can produce nonlinear shapes even if the true response is linear. This is particularly important for bioassays for carcinogenicity, because this test is usually not repeated (Lutz et al., 2005). In a comprehensive review on dose-response relationships for carcinogens (Zeise, Wilson & Crouch, 1987), the authors reported that nonlinearity is common in rodents, even for high dose data. By log transformation of the substrate concentration, an adequate model was obtained from which reliable kinetic constants and pH profiles were determined for the activities of horse liver alcohol dehydrogenase catalysed reduction of cyclohexanone (Anderson & Adlercreutz, 1999). Since there is sufficient evidence available in the literature for the data transformation for bioassays and microbial reactions, in this study the data has been plotted on log scale wherever applicable.

CHAPTER 3

RESULTS

Objectives

1. To confirm the authenticity of *A. adeninivorans* to use it as a biocomponent in biosensors.
2. To investigate the effect of nitrogen sparging against nitrogen overlay on the efficiency of the mediators' oxidation.
3. To investigate the generality of single and double mediator interactions in relation to glucose catabolism in four yeast species.

3.0 Molecular characterisation of *A. adeninivorans*

The authenticity of *A. adeninivorans* before using it as a biocomponent. This was tested by designing appropriate primers that would amplify the unique self-splicing intron that is located within the 25S rDNA and performed polymerase chain reaction (PCR) to amplify the desired fragments using appropriate controls. The protocol for PCR reactions and PCR conditions were given earlier (2.6.2). The amplified DNA fragment sizes were determined by running 1% agarose gel electrophoresis shown in next page (Figure 3.1).
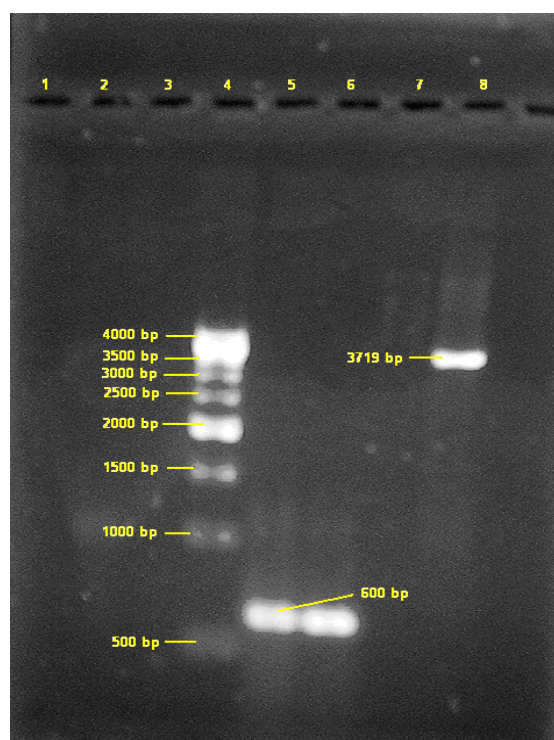
Figure 3.1 PCR fragments amplified from *A. adeninivorans* DNA template using INT - 1 (3719 bp) and INT - 2 (600 bp) primers. The order of loading samples in the gel is given as well numbers: controls with no DNA sample (1 & 2), DNA ladder (4), INT - II PCR samples (5 & 6) and INT - I PCR samples (7 & 8)

The results in Figure 3.1 show that the desired fragments of 600 bp from INT II and 3719 bp DNA fragments from INT I (sizes determined from the gel by comparing with the DNA ladder) have been amplified confirming the presence of the self-splicing intron within the 25 S rDNA of *A. adeninivorans* confirming its authenticity.

3.1. Optimisation of the lipophilic mediator's concentration

The existing double mediator system uses potassium ferricyanide (80 mM) and menadione at 100 µM (Baronian et al., 2002). Optimisation of TMPD concentration was required before experiments using yeast as a sensor biocomponent could begin (Figure 3.2).
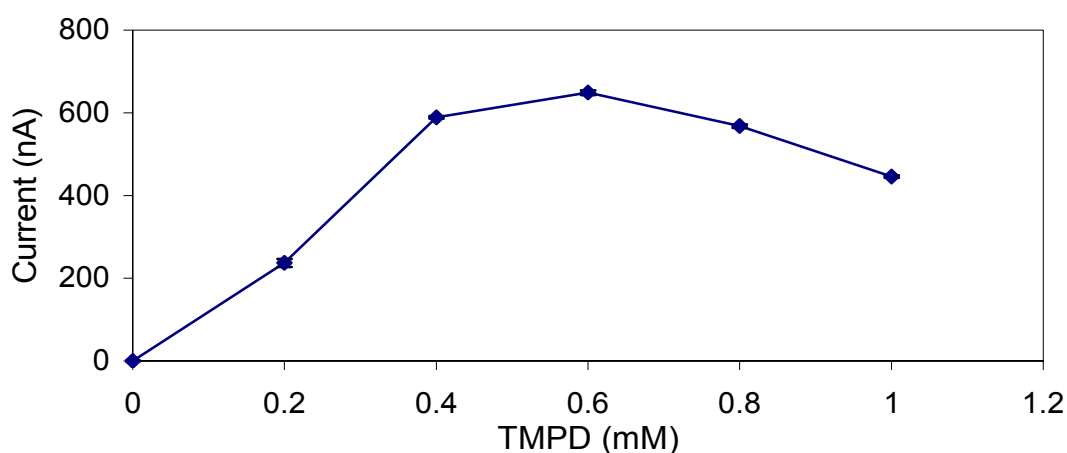
Figure 3.2 *A. adeninivorans* response to ferricyanide, 7.5 mM glucose and increasing TMPD concentration.

The results in Figure 3.2 show that glucose mediated catabolic responses of *A. adeninivorans* with ferricyanide and increasing TMPD concentration gave optimum response between 0.5 - 0.6 mM TMPD. This is consistent with the reported concentration range of 0.5 - 0.6 mM for TMPD in *S. cerevisiae* (Baronian et al., 2002). Hence, it was decided to use 0.5 mM TMPD and the yeast cell density of OD – 3.0 for all the subsequent experiments.

The results in Figure 3.2 show that above 0.6 mM the signal dropped, suggesting some kind of toxicity to the cells at the higher concentration. While there is no direct evidence that TMPD is specifically toxic or depress a known metabolic pathway, the significant depression of metabolic activity at higher concentrations is clearly some form of toxicity. The mechanism of this depression could be fruitful future investigation.

3.2. Effect of nitrogen sparging and incubation time on the efficiency of mediator oxidation of cellular redox molecules of *A. adeninivorans*

The effect of nitrogen sparging against nitrogen overlay on oxidation of mediator efficiency was investigated (Figure 3.3).
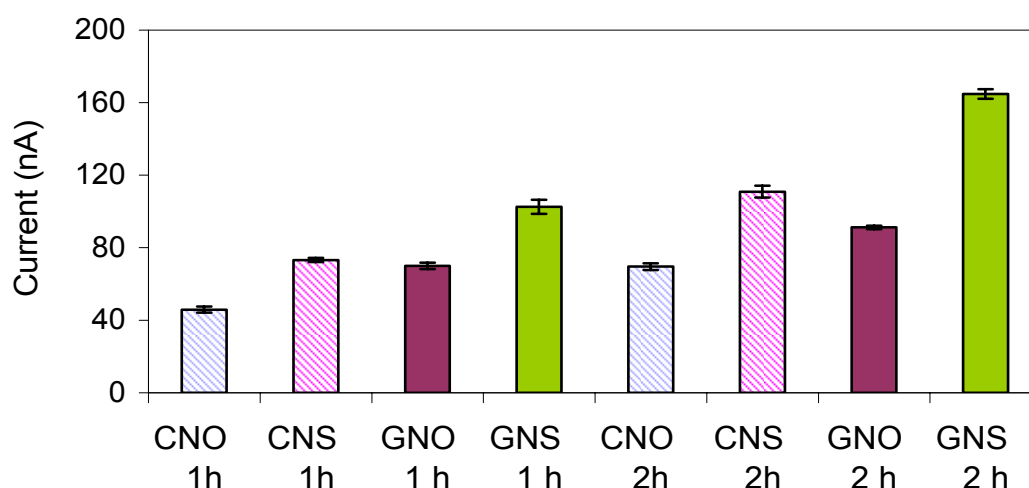
Figure 3.3 Effect of nitrogen sparging and incubation time on the efficiency of mediator oxidation of cellular redox molecules of *A. adeninivorans*. (CNO - control nitrogen overlay; GNO - glucose nitrogen overlay; CNS - control nitrogen sparging; GNS - glucose nitrogen sparging).

The effect of incubation time and nitrogen sparging vs. nitrogen overlay on the efficiency of mediator oxidation efficiency was compared and expressed as a percentage of the control response (Table 3.1).

Table 3.1 Effect of nitrogen sparging and incubation time on the efficiency of mediator oxidation of cellular redox molecules of *A. adeninivorans*

Incubation conditions	1 hour	Difference 1h	2 hours	Difference 2h
Control with nitrogen overlay (CNO)	46 nA (100%)	0 %	70 nA (100%)	0%
Control with nitrogen sparging (CNS)	73 nA (159%)	59%	111 nA (159%)	59%
Glucose with nitrogen overlay (GNO)	70 nA (100%)	0%	91 nA (100%)	0%
Glucose with nitrogen sparging (GNS)	103 nA (147%)	47%	165 nA (181%)	81%

The results in Figure 3.3 and Table 3.1 show that nitrogen sparging enhanced the signals in *A. adeninivorans* cells compared to nitrogen overlay. Nitrogen sparging efficiently removes the oxygen that competes with the mediators and increases the mediator's oxidation efficiency. The incubation time obviously enhances the signal, but the percentage of enhanced signal is the same except for glucose with nitrogen sparging

at 2 hours instead of 47% it is higher (81%). However, these results confirm that nitrogen sparging is necessary to remove oxygen and one-hour incubation is sufficient to give the dynamic range, hence it was decided that nitrogen sparging and one hour incubation is to be used unless mentioned specifically, for example, the naphthalene experiments (no nitrogen sparging) and the estrogen experiments (incubations of 4 to 8 hours).

3.3 Investigation of glucose dependent catabolic responses in four yeast species using single and double mediator systems

It is important to investigate the generality of single and double mediator interactions in relation to glucose catabolism. This investigation involved four species of yeast that belong to Basidiomycota (*A. adeninivorans* and *R. rubra*) and Ascomycota (*S. cerevisiae* and *T. cutaneum*) to check for inter-generic and inter-phyla variations. Single hydrophilic mediator ferricyanide (80 mM) and double mediator systems of ferricyanide coupled with a lipophilic mediator menadione (0.1 mM) or TMPD 0.5 mM were used to investigate the differences in responses between the two mediator systems and among yeast species (Figures 3.4, 3.5, 3.6 & 3.7).
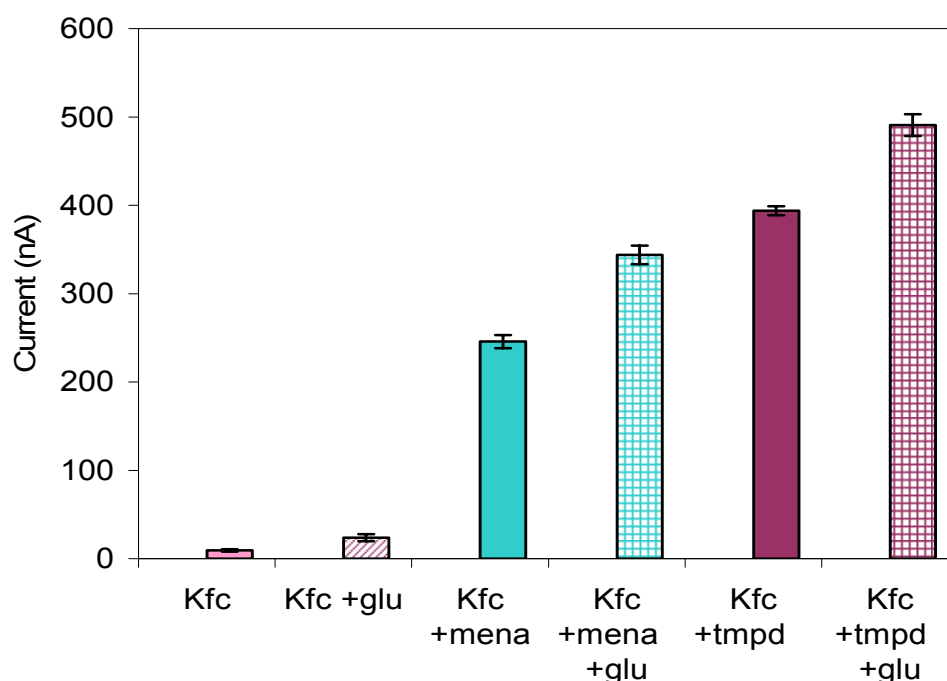
Figure 3.4 Glucose dependent catabolic responses of *A. adeninivorans* with single and double mediator systems (Kfc – potassium ferricyanide; glu – glucose; mena – menadione and tmpd-TMPD).
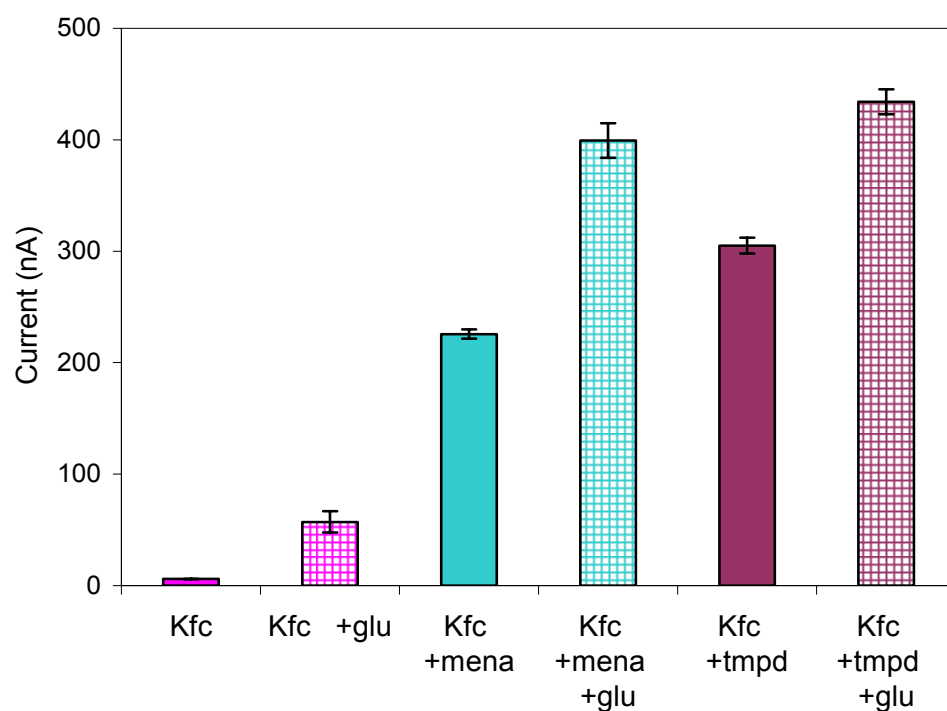
Figure 3.5 Glucose dependent catabolic responses of *S. cerevisiae* with single and double mediator systems.
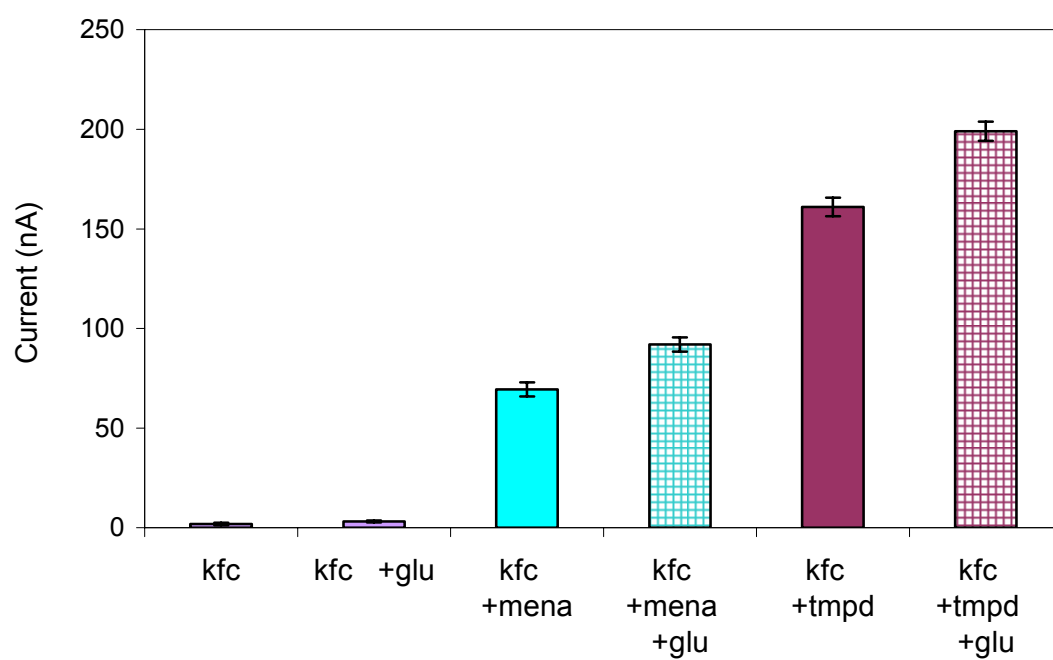
Figure 3.6 Glucose dependent catabolic responses of *T. cutaneum* with single and double mediator systems.
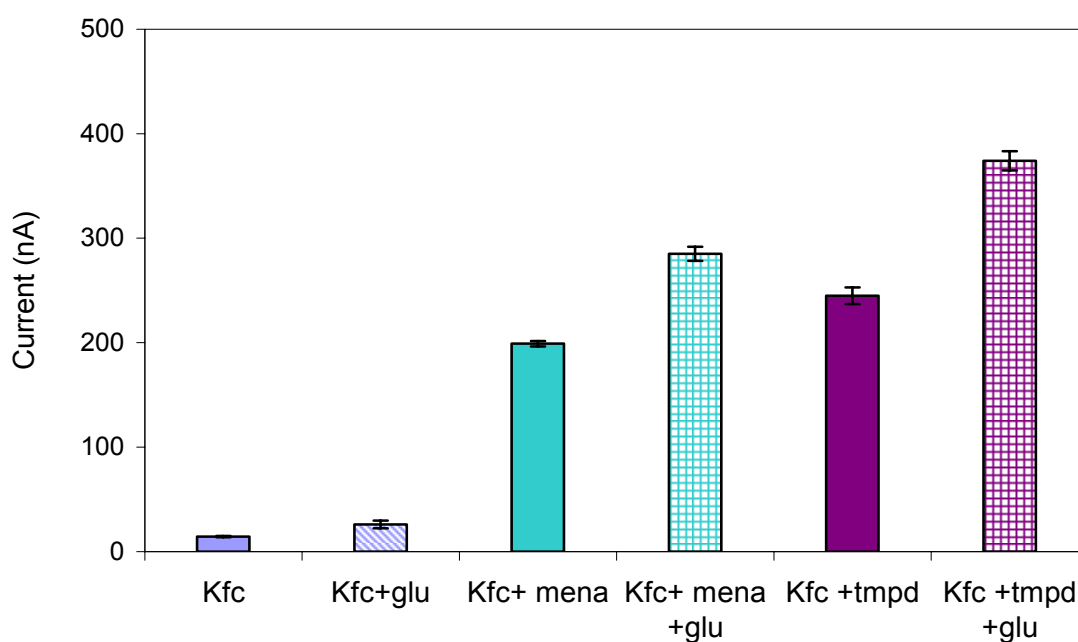
Figure 3.7 Glucose dependent catabolic responses of *R. rubra* with single and double mediator systems.

The responses of yeast cells to single and double mediators presented in Figures 3.4 to 3.7 are tabulated in Table 3.2 for the sake of convenience in interpreting and comparing.

Table 3.2 Summary showing the responses (means) of all four yeast species responses to single and double mediator systems (mena – menadione, glu – glucose)

Mediators + substrates	*A. adeninivorans*	*S.cerevisiae*	*T. cutaneum*	*R. rubra*
Potassium ferricyanide	9 nA	6 nA	2 nA	15 nA
Potassium ferricyanide + glu	24 nA	57 nA	3 nA	26 nA
Potassium ferricyanide + mena	246 nA	226 nA	69 nA	199 nA
Potassium ferricyanide + mena + glu	344 nA	399 nA	92 nA	285 nA
Potassium ferricyanide + TMPD	393 nA	305 nA	162 nA	245 nA
Potassium ferricyanide + TMPD+glu	491 nA	434.1 nA	199 nA	374 nA

The two double mediator responses in yeast cells were compared to the single hydrophilic mediator (potassium ferricyanide) response and expressed as a percentage (Table 3.3).

Table 3.3 Summary responses of all four yeast species responses to double mediator systems expressed as a percentage of the potassium ferricyanide response.

Mediators	*A. adeninivorans*	*S. cerevisiae*	*T. cutaneum*	*R. rubra*
	% ferricyanide response.	% ferricyanide response.	% ferricyanide response.	% ferricyanide response.
Potassium ferricyanide + menadione	2608	3831	3637	1327
Potassium ferricyanide + TMPD	4159	5170	8481	1620

The purpose of this comparison is to compare the single mediator responses with double mediator responses in different yeast to select the double mediator system that would result in better noise: response ratios. The purpose of expressing the double mediator responses as percentage of single mediator response is to give a clear **qualitative** comparison of the oxidation efficiency of the double mediator systems vs. single mediator systems. In *T. cutaneum* (Figure 3.5), the responses were smaller than other yeast species tested (Table 3.2) which could be due to its low growth rate compared to other yeast.

Objectives

1. Investigate the catabolic responses of *A. adeninivorans* free and immobilised cells in 'Lentikats' to glucose.
2. Compare and contrast the activity of free and immobilised *A. adeninivorans* cells.
3. Determination of 'Limits of detection and determination' of glucose.

3.3.1 Investigating the glucose response range using the double mediated electrochemical responses of free *A. adeninivorans*

Normally potentiometric sensors respond linearly when the concentration of the substrate is plotted logarithmically (the Nernst equation) but amperometric sensors generally do not. In this and in subsequent experiments however, it was found that these responses are also related to the logarithm of the concentration of substrate. This may be because this amperometric sensor is based on enzyme reactions within the cell and these reactions may not be as simple as inorganic chemical reactions where this rule generally applies.

The catabolic responses of *A. adeninivorans* to glucose were investigated to identify the useful response range i.e. the range in which it was possible to get a linear response (Figure 3.8).
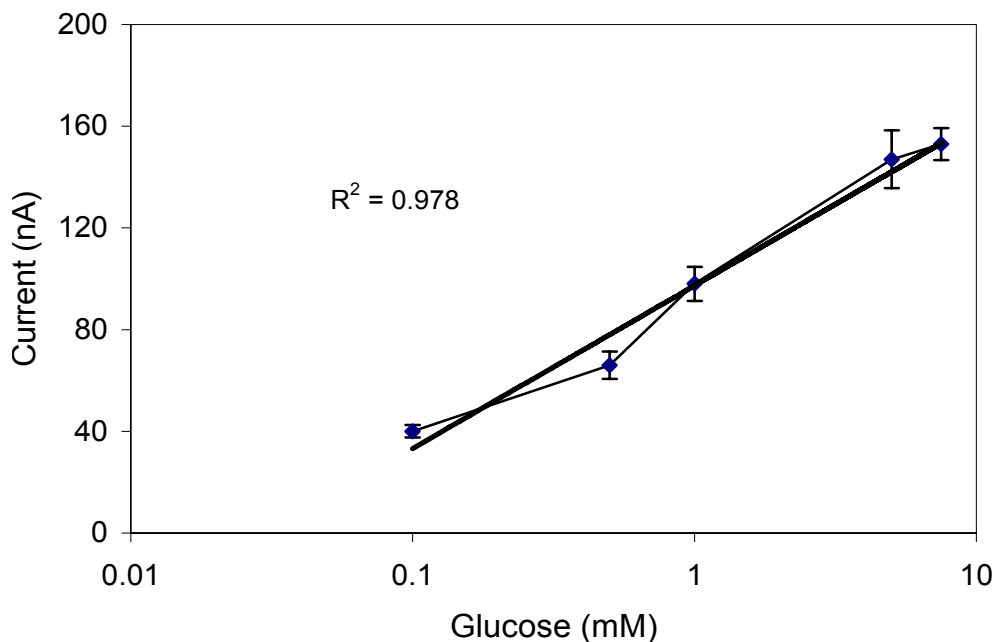
Figure 3.8 Logarithmically related *A. adeninivorans* (free cells) response to 0.1 – 7.5 mM glucose. The endogenous response (0 mM) was subtracted from these responses.

Results in Figure3.8 show *A. adeninivorans* responses were linear to the log of glucose from 0.1 – 7.5 mM.

The ageing effect on glucose mediated catabolic responses of *A. adeninivorans* was investigated using cells of different ages exposed to 7.5 mM glucose (Figure 3.9).
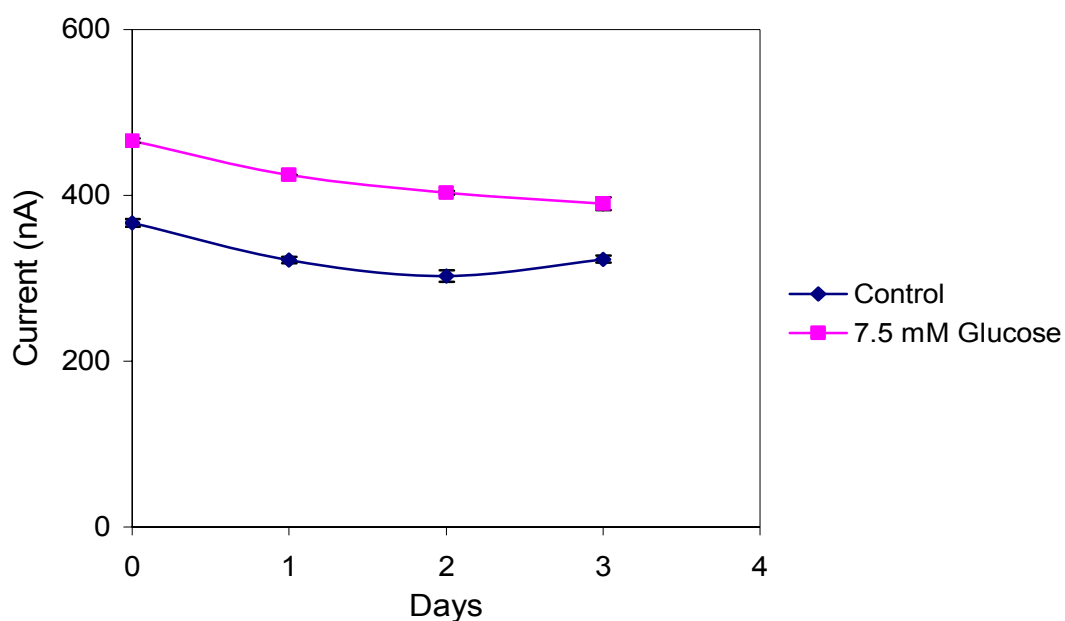
Figure 3.9 *A. adeninivorans* (free cells) responses to 7.5 mM glucose: ageing effect.

The results of in Figure 3.9 show that the free *A. adeninivorans* responses dropped more in the ageing cells compared to the fresh cells.

3.3.2 Immobilised *A. adeninivorans*

A. adeninivorans immobilised cells in 'Lentikats' were stained and viewed under a bright field microscope (Figure 3.10).
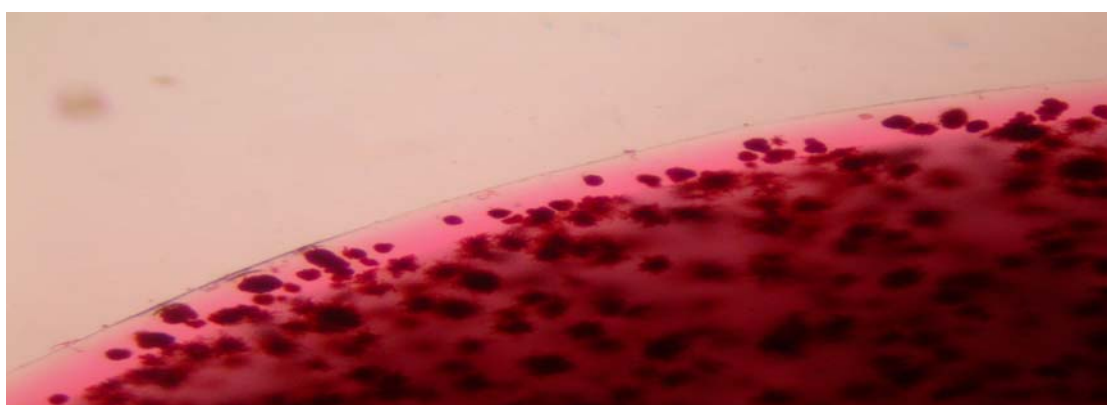
Figure 3.10 Microscopic picture of stained immobilised *A. adeninivorans* cells in 'Lentikat' (400X).

The microscopic picture of stained 'Lentikat' in Figure 3.10 showing yeast colonies immobilised on one 'Lentikat'.

3.3.3 Experiments to standardise the number of 'Lentikats' and to get optimum glucose dependent catabolic responses of immobilised. *A. adeninivorans*

The catabolic responses of immobilised *A. adeninivorans* cells to glucose were investigated. The longevity of immobilised cells was also compared with that of free cells. It is important to identify the number of 'Lentikats' that are required to give a useful response range. In order to get the ideal 'Lentikats' number, giving a useful response range 20 and 100 'Lentikats' and different glucose concentrations were used. The practicality of counting numbers has been taken into account for this experimental design. Initially glucose range (1.5 mM – 7.5 mM glucose) was tested (Figure 3.11).
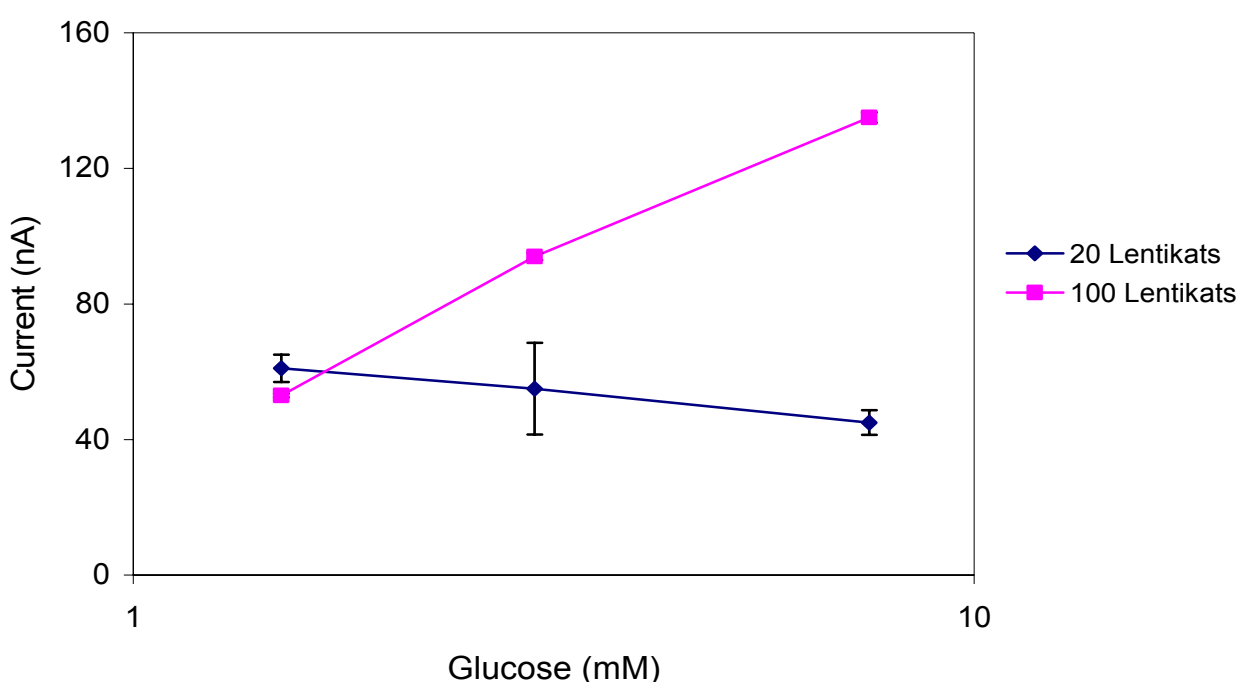
Figure 3.11 Logarithmically related immobilised *A. adeninivorans* response to 1.5 - 7.5 mM glucose (20 and 100 'Lentikats'). The endogenous response (0 mM) was subtracted from these responses.

The results with two different 'Lentikats' numbers and different ranges of glucose concentration in Figure 3.11 show that 100 'Lentikats' number gives a useful response; thus it was decided to use 100 'Lentikats' for all the subsequent experiments investigating the glucose linear response range. Owing to practical difficulty of counting more than 100 'Lentikats' in each tube, experiments with more than 100 'Lentikats' were not performed. It is probable that a higher number could have resulted in a larger response.

The ageing effect on the activity of 'Lentikats' was investigated using fresh and aged 'Lentikats' to compare and contrast the activity loss with ageing with free *A. adeninivorans* cells (Figure 3.12).
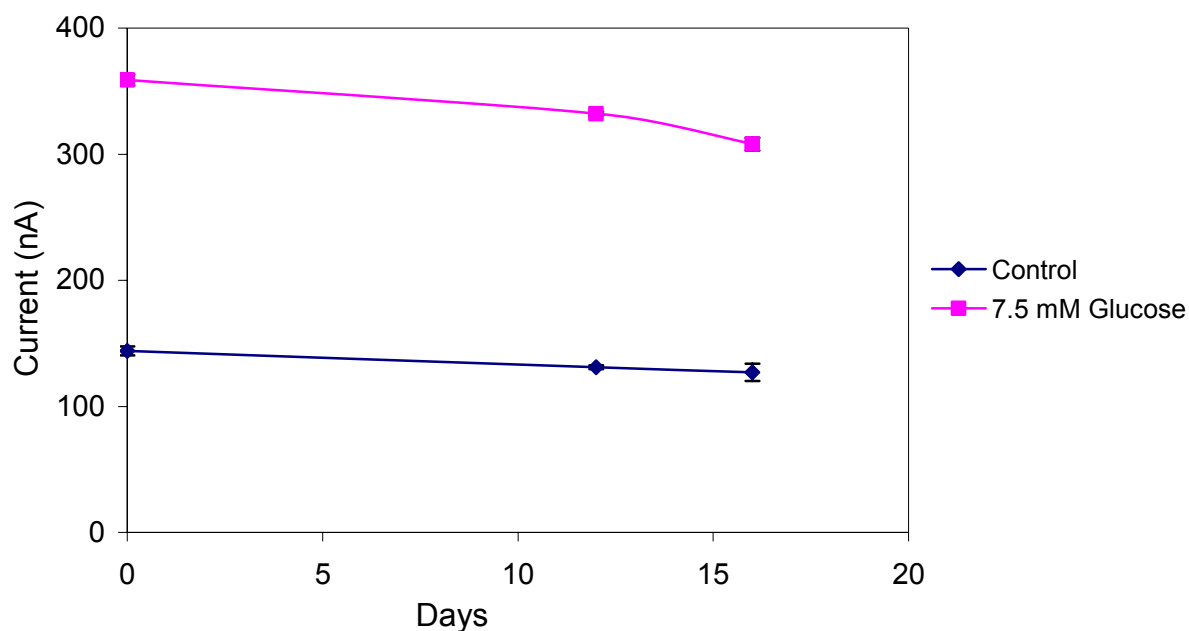
Figure 3.12 Immobilised *A. adeninivorans* (100 'Lentikats'), responses to glucose: ageing effect.

The results in Figure 3.12 show that the cells immobilised in the 'Lentikats' did show some loss in activity (responses), but compared to free cells at the same glucose concentration (7.5 mM), the loss of activity in 16 days was less than the loss seen in free cells in 3 days (see Figure 3.9).

Glucose responses were investigated by performing experiments using a lower glucose concentration range between 0.001 - 0.75 mM (Figure 3. 13).
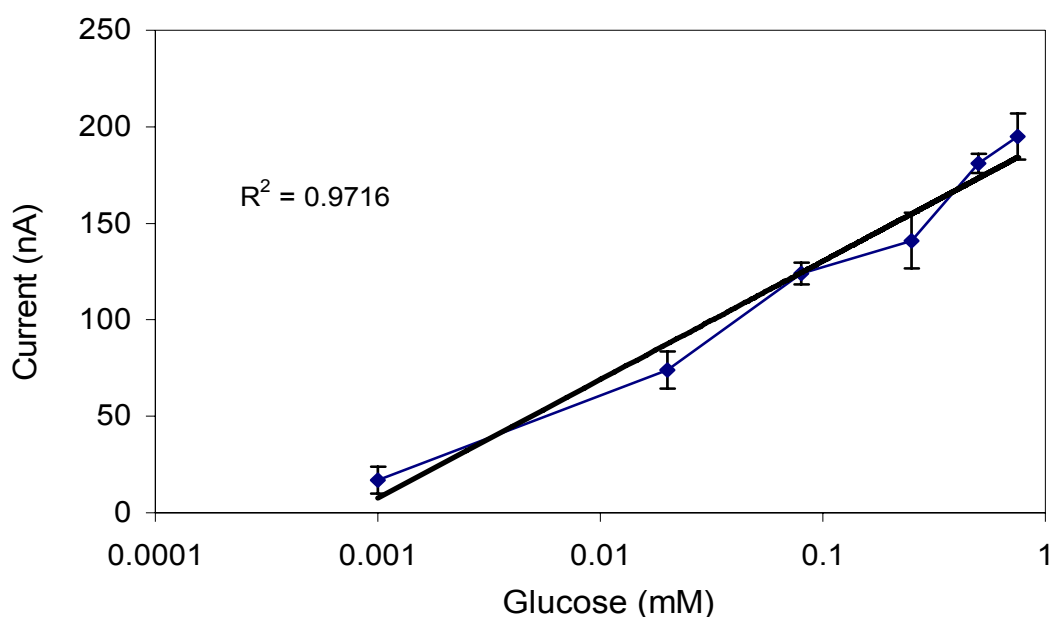
Figure 3.13 Logarithmically related responses of immobilised *A. adeninivorans* (100 'Lentikats') to 0.001 - 0.75 mM glucose. The endogenous response (0 mM) was subtracted from these responses.

The results in Figure 3.13 show *A. adeninivorans* responses were log linear from 0.001 mM - 0.75 mM. The results of separate experiments were combined to reveal the trend of *A. adeninivorans* responses over the entire tested glucose concentration range (Figure 3.14).
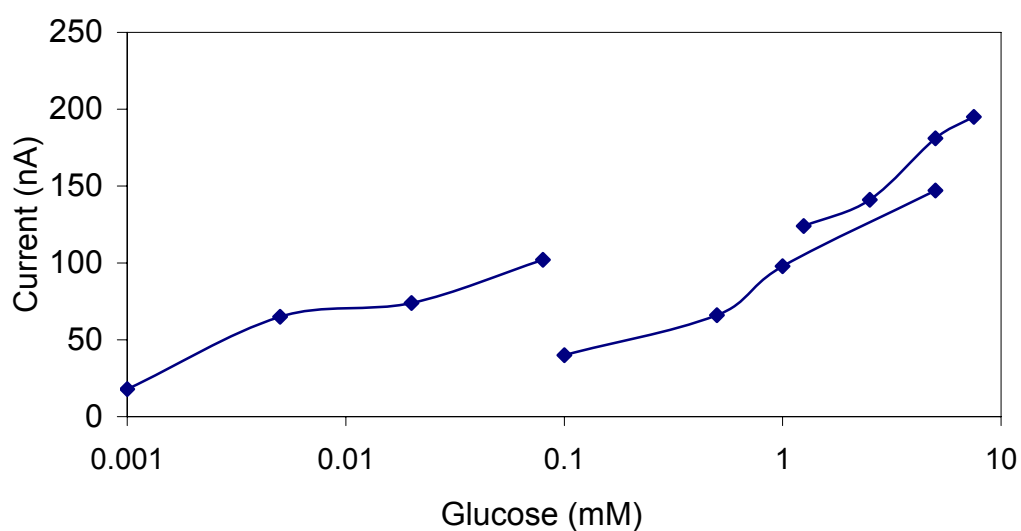
Figure 3.14 Logarithmically related responses of immobilised *A. adeninivorans* (100 'Lentikats') to glucose (summary of *A. adeninivorans* responses to a wide range of glucose). The endogenous response (0 mM) was subtracted from these responses.

The summary responses in Figure 3.14 shows the trend of logarithmically related glucose dependent responses of different batches of *A. adeninivorans* cells to the wide range of glucose concentrations tested. Responses of *A. adeninivorans* at the lower glucose ranges up to 0.08 mM were relatively larger than those at higher glucose concentrations (1 mM to 7.5 mM). The different currents (responses) seen in the three slopes due to either batch-to-batch variations of cells, or because the yeast cells respond differentially to different glucose concentrations. More likely the former explanation may be correct as the above results are, with the exception of 7.5 mM, from concentrations below 'very low' (5mM) glucose concentration categories given by Yin et al. (2000). The detection limit of glucose using the immobilised *A. adeninivorans* 'Lentikats' responses to very low glucose (μM) was investigated (Figure 3.15).

$$y = 11.1Ln(x) + 30.149$$
$$R^2 = 0.9665$$
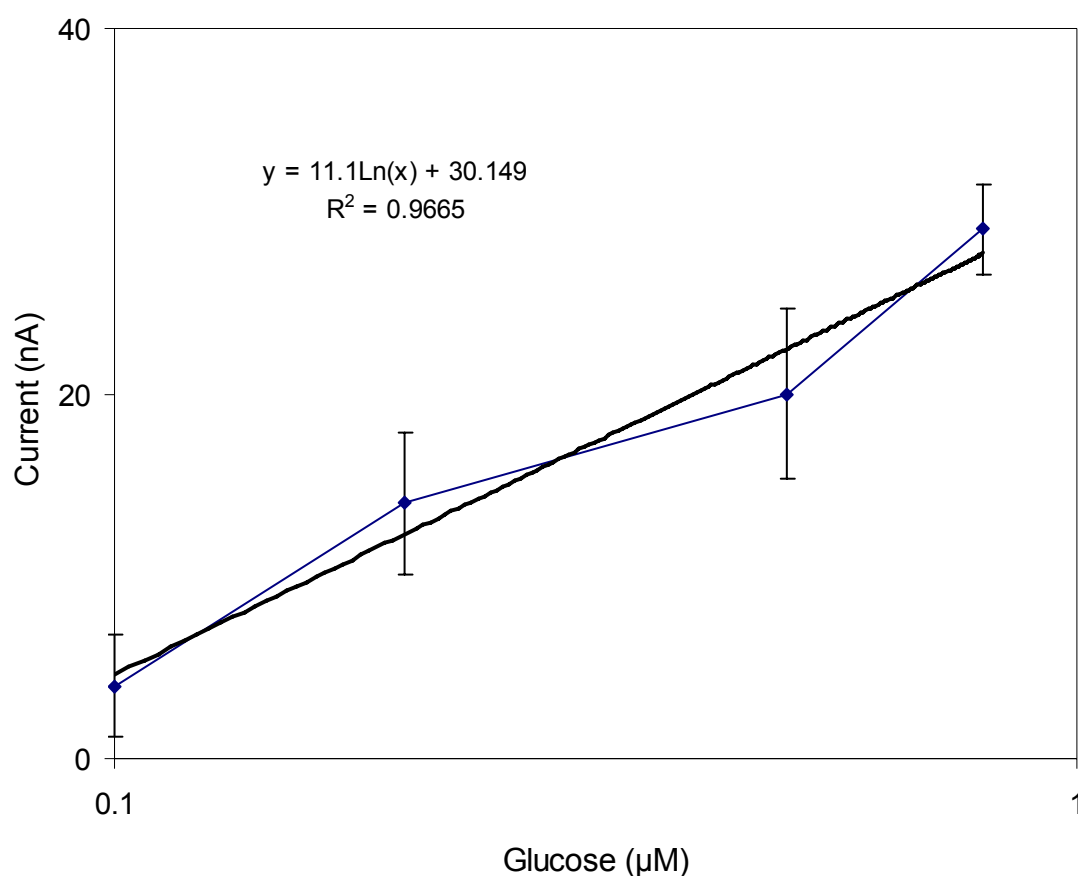
Figure 3.15 Logarithmically related responses of immobilised (100 'Lentikats') *A. adeninivorans* to very low glucose (0.1 – 0.8 μM). The endogenous response (0 μM) was subtracted from these responses. R^2, regression line and the equation were obtained using Microsoft Excel.

The results in Figure 3.15 show *A. adeninivorans* responses were linear to the log of glucose from 0.1 to 0.8 µM.

3.3.4 Glucose limit of detection and determination

The formulas used to determine the limit of detection and limit of determination were obtained from Riedel et al. (1998)

a. The formula used to calculate the limit of detection is given by:

$y_L = B + 4.65s_B$

(y_L = limit of detection, B = blank sample, s_B = standard deviation of the blank)

Example:

$y_L = 0 + 4.65 \times 3.033 = 14.104$

$y = 11.1 Ln(x) + 30.149$ (equation obtained from data presented in Figure 3.15)

$Ln(x) = 14.104 - 30.149 / 11.1 = -1.4459$

$x = 0.236$ µM (2.36×10^{-7} M)

b. The formula used to calculate the limit of determination is given by:

$y_D = B + 14.1s_B$

(y_D = Limit of determination, B = Blank sample, s_B = standard deviation of the blank)

Example:

$y_D = 0 + 14.1 \times 3.033 = 44. 312$

$y = 11.1 Ln(x) + 30.149$

$44.312 = 11.1 Ln(x) + 30.149$

$Ln(x) = 44.312 - 30.149 / 11.1$

$x = 3.58$ µM (3.58×10^{-6} M)

The limits of detection and determination achieved by the double mediated electrochemical detection system are 0.236 µM and 3.58 µM respectively.

Objective

 To test the validity of the "Isogenic Differential Enzyme Analysis (IDEA) "concept in the development of the biosensors to detect environmental contaminants: Gallic acid, naphthalene and di-butyl phthalate

3.4 Development of biosensors based on the "IDEA" concept to detect priority environmental contaminants

The ability of *A. adeninivorans* to use gallic acid as a sole carbon energy source and its responses to gallic acid grown (pre-conditioned) and glucose grown (unconditioned) growth medium (IDEA) were investigated (Figure 3.16).
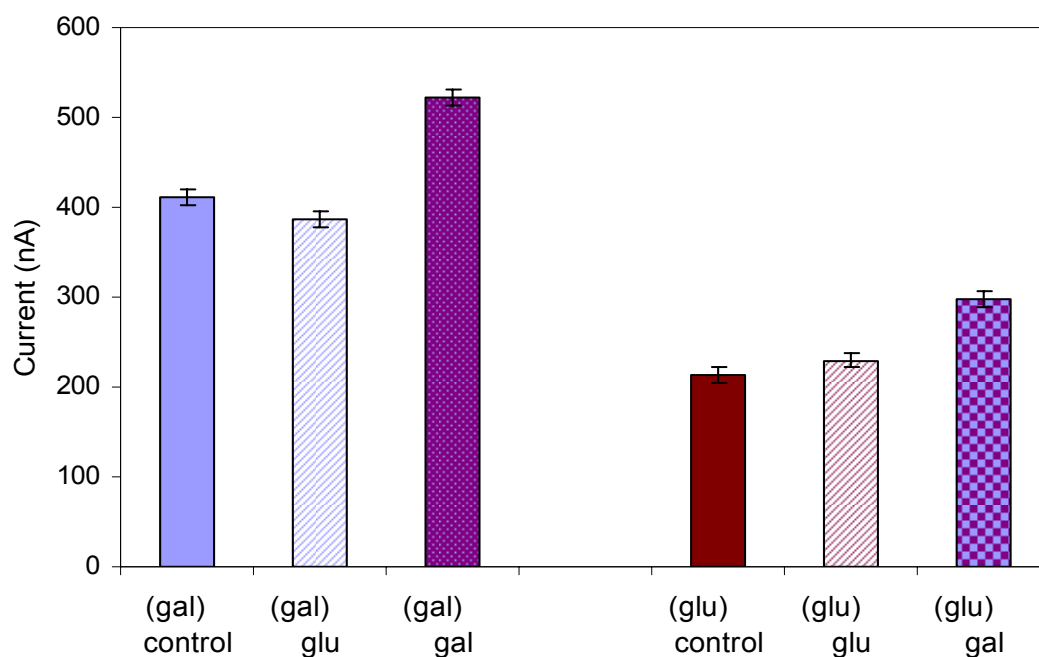
Figure 3.16 Comparison of *A. adeninivorans* catabolic response to 0.1 mM gallic acid (gal) grown cells and 0.1 mM glucose (glu) grown cells. Control is no substrate.
[Parenthesis represents the pre-conditioning of the cells with glucose (glu) or gallic acid (gal)].

The results in Figure 3.16 show that *A. adeninivorans* cells did not respond differentially to gallic acid. Interestingly the gallic acid as a substrate in gallic acid grown and glucose grown cells gave larger responses than with glucose and the addition of glucose in gallic acid grown cells, gave a response that was less than the endogenous response.

3.4.1 Investigation of *A. adeninivorans* ability to utilize naphthalene as sole carbon energy source and the ability of pre-conditioned and un-conditioned cells to respond differentially to naphthalene (IDEA)

The ability of *A. adeninivorans* cells to use naphthalene as a sole carbon energy source in minimal defined medium was tested. *A. adeninivorans* responses to naphthalene from 0.1 mM - 5 mM range was investigated (Figure 3.17).
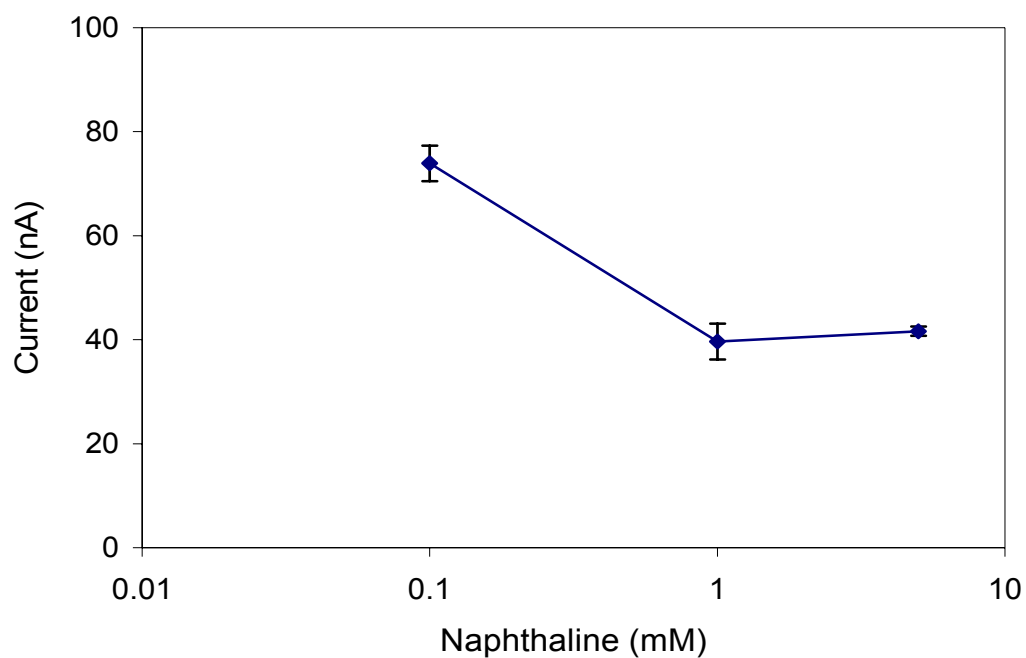
Figure 3.17 Logarithmically related *A. adeninivorans* responses to 0.1 - 5 mM naphthalene. The endogenous response (0 mM) was subtracted from these responses.

The results in Figure 3.17 show *A. adeninivorans* cells response was significant at 0.1 mM and from 0.1 mM and higher concentrations, the response dropped dramatically, suggesting that naphthalene at higher concentrations than 0.1 mM could be toxic to cells.

The naphthalene range between 0.09 mM - 0.5 mM was then investigated to identify the detection limit using this system (Figure 3.18).

$y = 21.777 Ln(x) + 77.937$
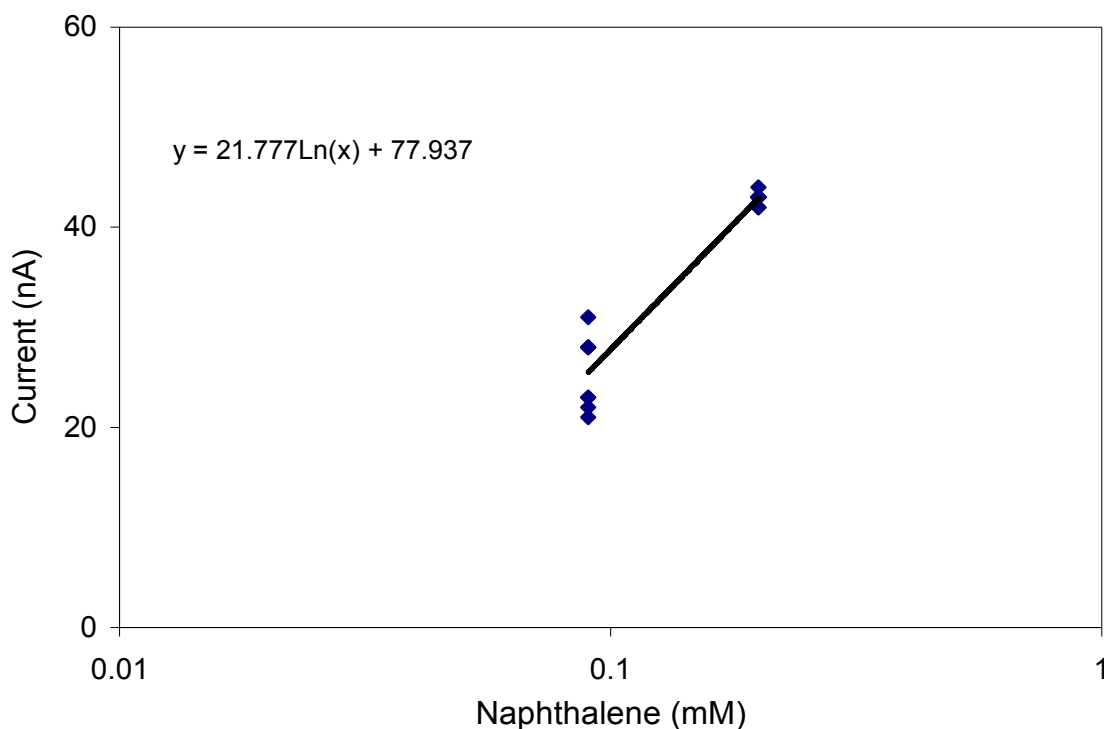
Figure 3.18 Logarithmically related *A. adeninivorans* responses to 0.09 – 0.2 mM naphthalene. The endogenous response (0 mM) was subtracted from these responses. The equation and regression line were obtained using Microsoft Excel.

Limits of detection and determination

The results in Figure 3.18 show increase in the responses of *A. adeninivorans* cells to the log of naphthalene up to 0.2 mM however, above 0.2 mM the responses dropped dramatically (data not shown). The limits of detection and determination were calculated using the equation presented in Figure 3.18 and using the same method described earlier (3.3.4). Although the data has some robustness, using only two data points is not ideal and the results are only applicable within the range of data, i.e. from 0.09 – 0.2 mM, therefore, is as optimal as possible under practical constraints. The limits of detection and determination were calculated based on that data. The limits of detection and determination of naphthalene achieved by this method are 0.060 mM (6x 10^{-5} M) and 0.317 mM (3.17 x 10^{-4} M) respectively well above the limits of environmentally significant concentrations 4 – 40 nM (4 x 10^{-9} M to 4 x10^{-8} M) and are not useful for environmental applications. Because the "IDEA" concept was not viable, the gas phase analysis of naphthalene was not pursued in this study

The differential response of *A. adeninivorans* cells that were glucose grown (unconditioned) and naphthalene grown (pre-conditioned) was investigated to verify the viability of the "IDEA" concept in the development of a biosensor, to detect the target contaminant molecule naphthalene in environmental applications.
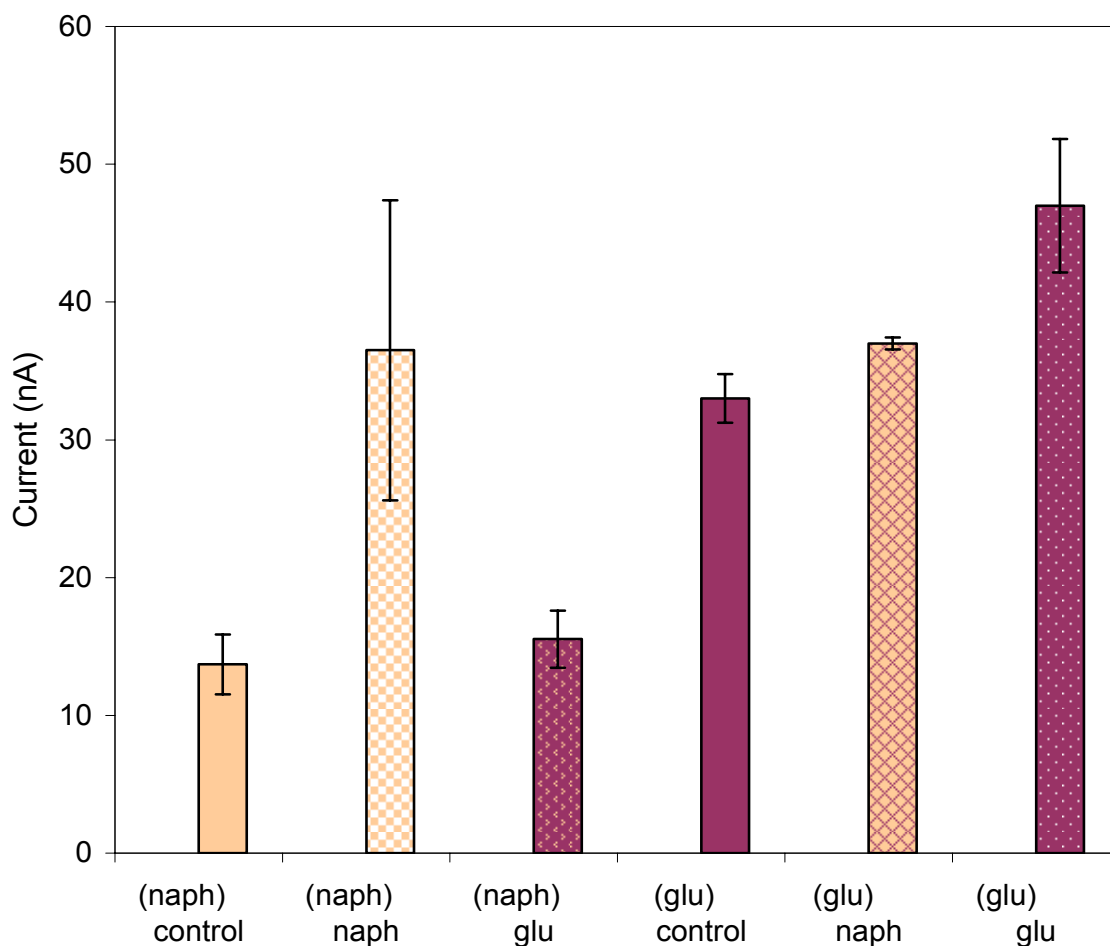
Figure 3.19 Catabolic responses of *A. adeninivorans* grown in naphthalene (naph) or glucose (glu). No substrate (control), 0.1 mM naphthalene (naph), and 0.1 mM glucose (glu).

ANOVA Single Factor Analysis was performed using the raw data obtained from naphthalene grown cells and glucose grown cells using Microsoft Excel. The method used for the ANOVA analysis is given below. The same method was followed for the rest of the experiments where the ANOVA analysis was performed.

ANOVA Single Factor Analysis of naphthalene grown *A. adeninivorans* cells.

Control	Glucose
17	13
16	12
16	12
12	17
12	17
12	18
11	15
12	15
12	16

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	9	123.42	13.71	4.67
Column 2	9	139.56	15.53	4.28

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	14.40	1	14.40	3.21	0.0919	4.49
Within Groups	71.70	16	4.48			
Total	86.10	17				

P-values were obtained by ANOVA Single Factor Analysis using naphthalene grown cells and glucose grown cells, these values were given below (Table 3.4).

Table 3.4 ANOVA Single Factor analysis of naphthalene grown (naph) cells and glucose grown (glu) cells

Substrate groups	P-value
(naph) Control - (naph) glucose	9.19×10^{-2}
(naph) Control - (naph) naphthalene	5.38×10^{-15}
(naph) Glucose - (naph) naphthalene	1.14×10^{-14}
(glu) Control - (glu) naphthalene	1.01×10^{-6}
(glu) Naphthalene- (glu) glucose	1.06×10^{-5}
(glu) Control - (glu) glucose	5.71×10^{-7}

The results in Figure 3.19 and Table 3.4 show that the *A. adeninivorans* cells differential response was partial, because of the naphthalene grown cells responses to glucose were statistically not significantly different to control ($p > 0.05$) as expected. However, the glucose grown cells have shown a response to naphthalene ($p < 0.05$). This suggests that the cells that were grown in glucose already have some naphthalene metabolic enzymes present and thus do not fulfil the requirement of the "IDEA" concept.

Cells were examined microscopically in an attempt to find the morphological differences between cells grown on glucose (Figure 3.20) and naphthalene (Figure 3.21) as their sole carbon energy source.
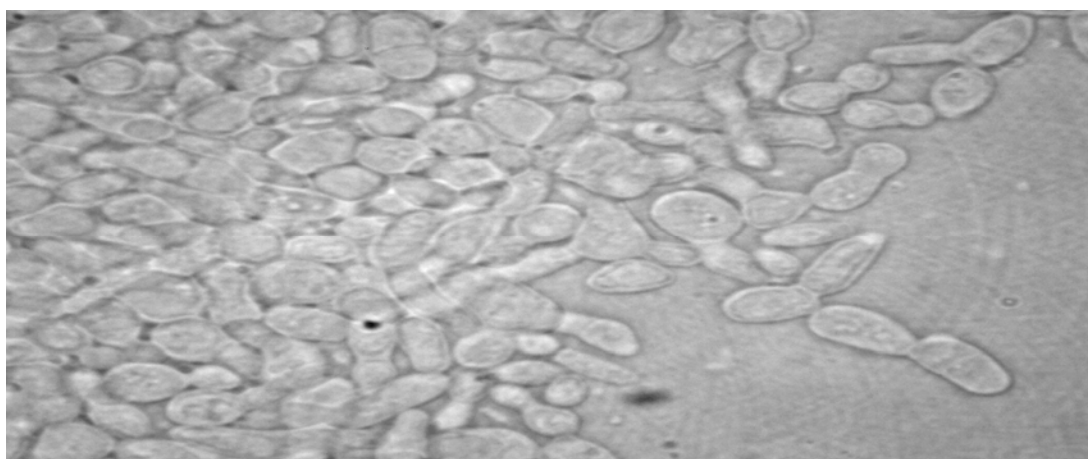
Figure 3.20 *A. adeninivorans* cells grown in minimal medium with glucose as a sole carbon source. (400 x)
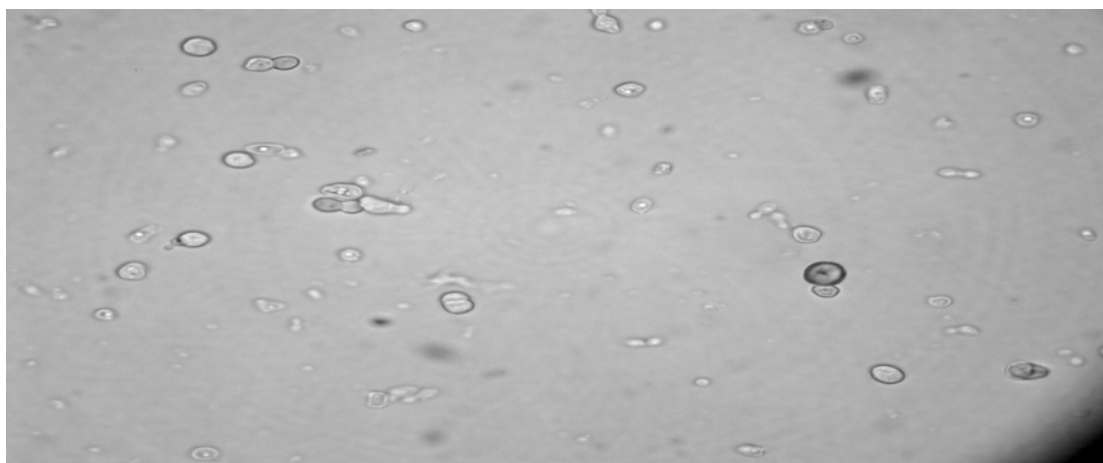
Figure 3.21 *A. adeninivorans* cells grown in minimal medium with naphthalene as a sole carbon source. (400 x)

The microscopic pictures of *A. adeninivorans* grown in minimal medium with glucose (Figure 3.20) are more elongated compared to the oval shaped cells grown in a minimal medium with naphthalene (Figure 3.21) as a sole carbon source. *A. adeninivorans* grew poorly when naphthalene was used as a sole carbon source compared to glucose as a sole carbon source (3 days to grow to the OD_{600} of 0.9 in naphthalene as a sole carbon source, but cells are viable as they responded to naphthalene compared to control with no substrate).

3.4.2 Investigation of *A. adeninivorans* ability to utilize di-butyl phthalate (DBP) as a sole carbon energy source and its ability to respond differentially to DBP pre-conditioned and unconditioned cells

The response of *A. adeninivorans* cells to di-butyl phthalate was investigated using 0.025 – 0.075 mM DBP (Figure 3.22).
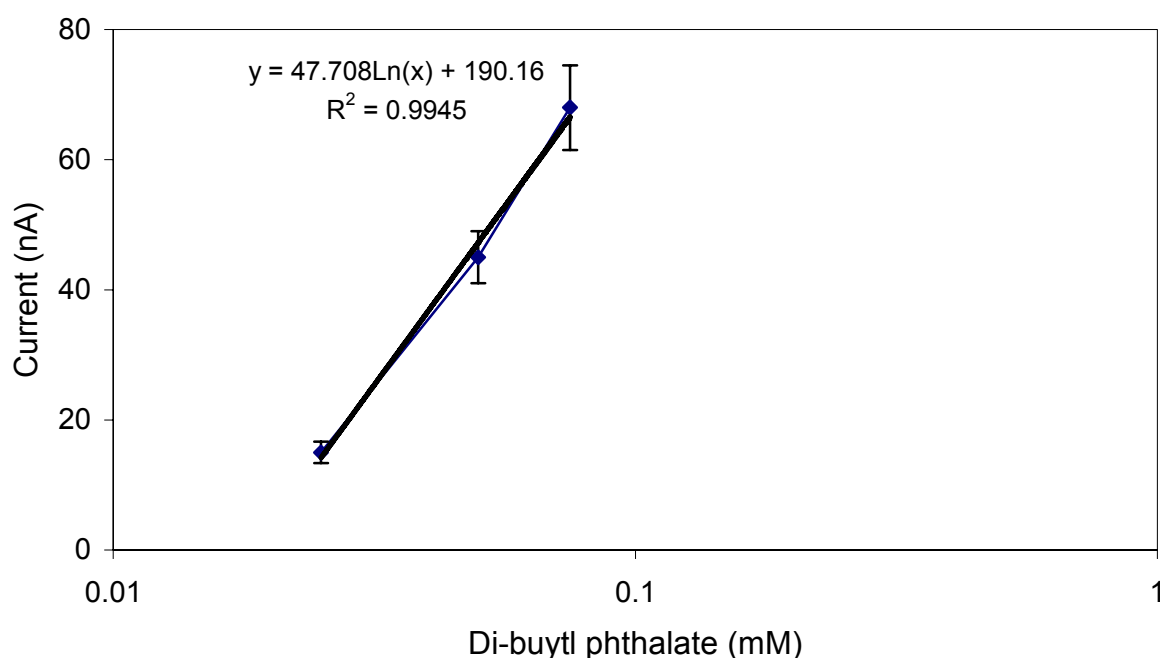
Figure 3.22 Logarithmically related *A. adeninivorans* responses to 0.025 - 0.075 mM di-butyl phthalate. The endogenous response (0 mM) was subtracted from these responses. Equation, regression line and R^2 calculated using Microsoft Excel.

Results in Figure 3.22 show good linear responses to the log of di-butyl phthalate from 0.025 – 0.075 mM.

Limits of Detection and Determination

The limits of detection and determination of di-butyl phthalate achieved by this method are 0.025 mM (2.5×10^{-5} M) and 0.050 mM (5×10^{-5} M) respectively. These were calculated using the data and equation presented in Figure 3.22 by using the same method described earlier.

The differential catabolic responses of *A. adeninivorans* cells that were glucose grown (unconditioned) and di-butyl phthalate grown (pre-conditioned) was investigated to verify the viability of the "IDEA" concept for the development of di-butyl phthalate biosensor for environmental applications (Figure 3.23).
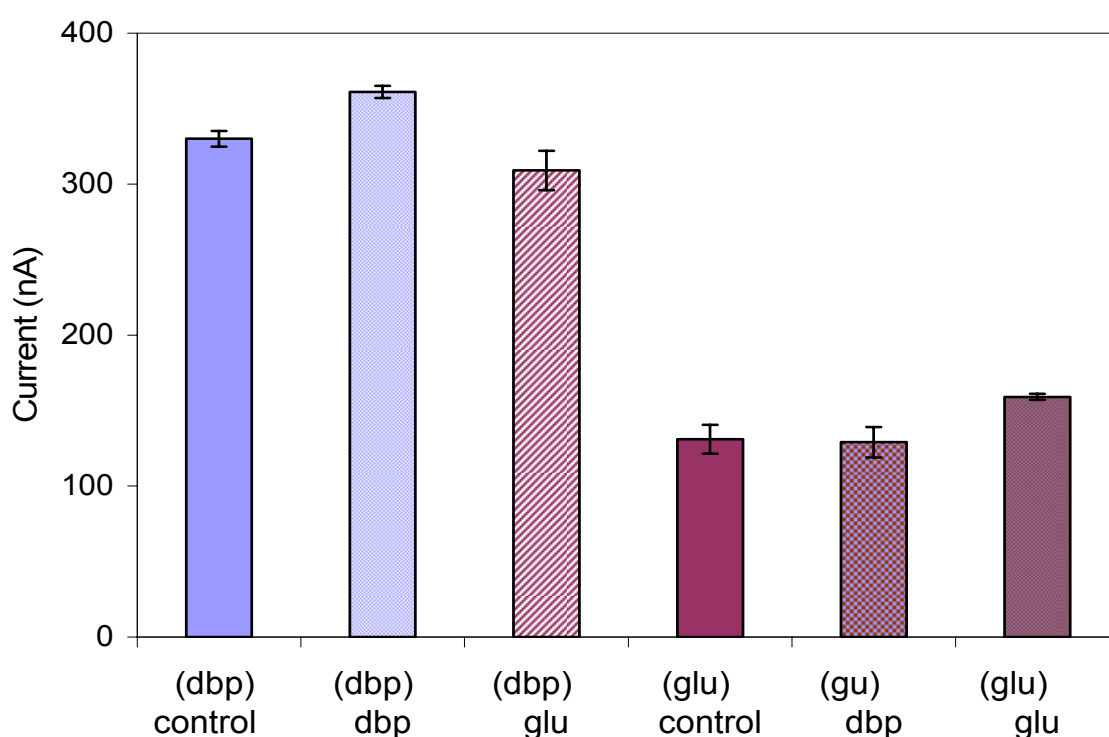
Figure 3.23 Catabolic responses of *A. adeninivorans* to di-butyl phthalate grown (dbp) and glucose (glu) grown to 0.1 mM di-butyl phthalate, 0.1 mM glucose and no substrate (control).

P-values were obtained by performing ANOVA analysis using raw data (Table 3.5).

Table 3.5 ANOVA Single Factor analysis of di-butyl phthalate grown (dbp) cells and glucose grown (glu) cells

Substrate groups	P-value
(dbp) Control - (dbp) glucose	9.91×10^{-5}
(dbp) Control – (dbp) di-butyl phthalate	4.44×10^{-9}
(dbp) Glucose – (dbp) di-butyl phthalate	4.84×10^{-11}
(glu) Control – (glu) di-butyl phthalate	6.21×10^{-2}
(glu) Control - (glu) glucose	5.64×10^{-9}
(glu) di-butyl phthalate - (glu) glucose	3.07×10^{-9}

The results in Figure 3.23 show that *A. adeninivorans* exhibited a differential response to di-butyl phthalate grown cells and glucose grown cells supporting the "IDEA" concept that cells exposed to pre-conditioning induce the degradative enzymes specific to the target contaminant molecule di-butyl phthalate. P-value < 0.05 for (dbp) control – (dbp) di-butyl phthalate is statistically significantly different compared to un-conditioned cells (p > 0.05), which confirms *A. adeninivorans* differential response to pre-conditioned and un-conditioned cells (Baronian, Gurazada & Thomas, 2005). Because the presence of glucose depressed the di-butyl phthalate response and the endogenous response significantly p-value > 0.05 in pre-conditioned cells, the mechanism of glucose depression of di-butyl phthalate response was investigated.

3.4.3 Investigation of glucose depression of di-butyl phthalate response of *A. adeninivorans*
The glucose depression of di-butyl phthalate response in *A. adeninivorans* was investigated (Figure 3.24).
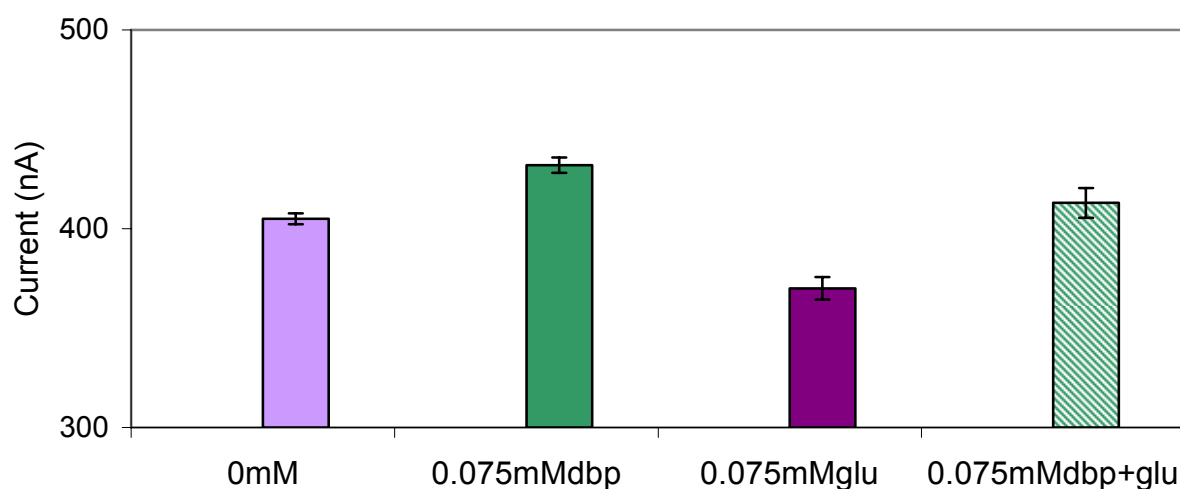
Figure 3.24 Glucose (glu) depression of *A. adeninivorans* endogenous response and di-butyl phthalate (dbp) response.

P-values were obtained by performing ANOVA analysis using raw data (Table 3.6).

Table 3.6 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression

Substrate groups	P-value
0 mM - 0.075 mM dbp	1.36×10^{-11}
0 mM - 0.075 mM dbp + glucose	5.83×10^{-3}
0.075 mM dbp - 0.075 mM dbp + glucose	8.57×10^{-6}

The results in Figure 3.24 show that the presence of glucose not only depressed the di-butyl phthalate catabolic responses, but also the endogenous response. P-values obtained by performing ANOVA analysis (Table 3.6) confirmed the existence of glucose depression phenomenon. The effect of glucose concentration on this depression mechanism was investigated (Figure 3.25).
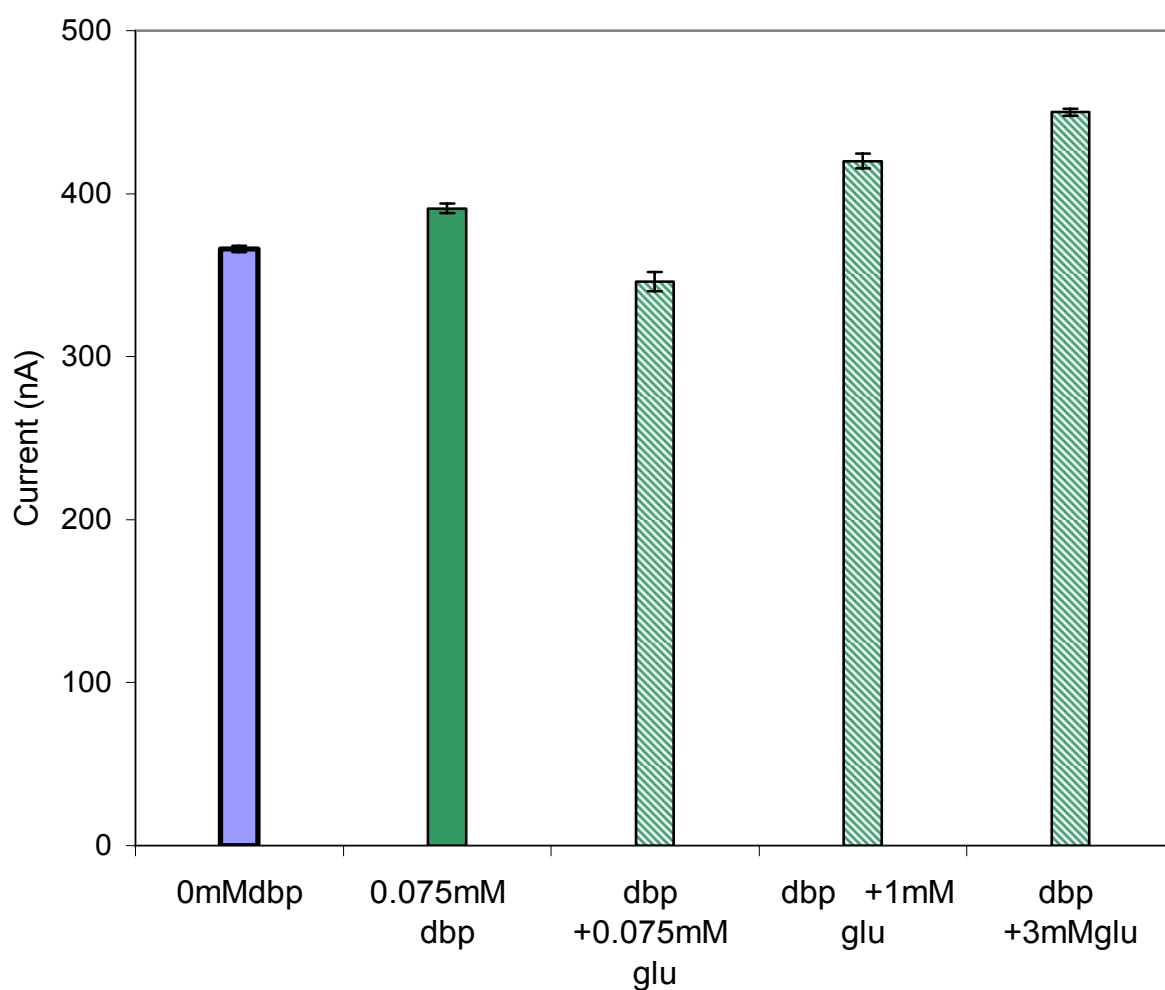
Figure 3.25 A. *adeninivorans* response to combined 0.075 mM di-butyl phthalate (dbp) and increasing glucose (glu) concentrations.

P-values were obtained by performing ANOVA analysis using raw data is presented in (Table 3.7).

Table 3.7 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression (1 – 3 mM glucose)

Substrate groups	P-value
0 mM - 0.075 mM dbp	1.07×10^{-12}
0 mM - dbp + 0.075 mM glucose	8.86×10^{-3}
0 mM - dbp + 1 mM glucose	5.35×10^{-12}
0 mM - dbp + 3 mM glucose	7.92×10^{-17}

The results in Figure 3.25 show that glucose depression was observed at dbp + 0.075 mM glucose, which was less than endogenous response and not observed in the previous experiment (see Figure 3.24) and will be discussed shortly. Results in Figure 3.25 and P-values in Table 3.7 show that the glucose depression was concentration dependent and the depression was relieved from 1 mM glucose. These results led to an investigation of the lower ranges of glucose to identify the concentration at which this depression mechanism begins and the concentration range of 0.035 - 0.1 mM glucose was used (Figure 3.26).
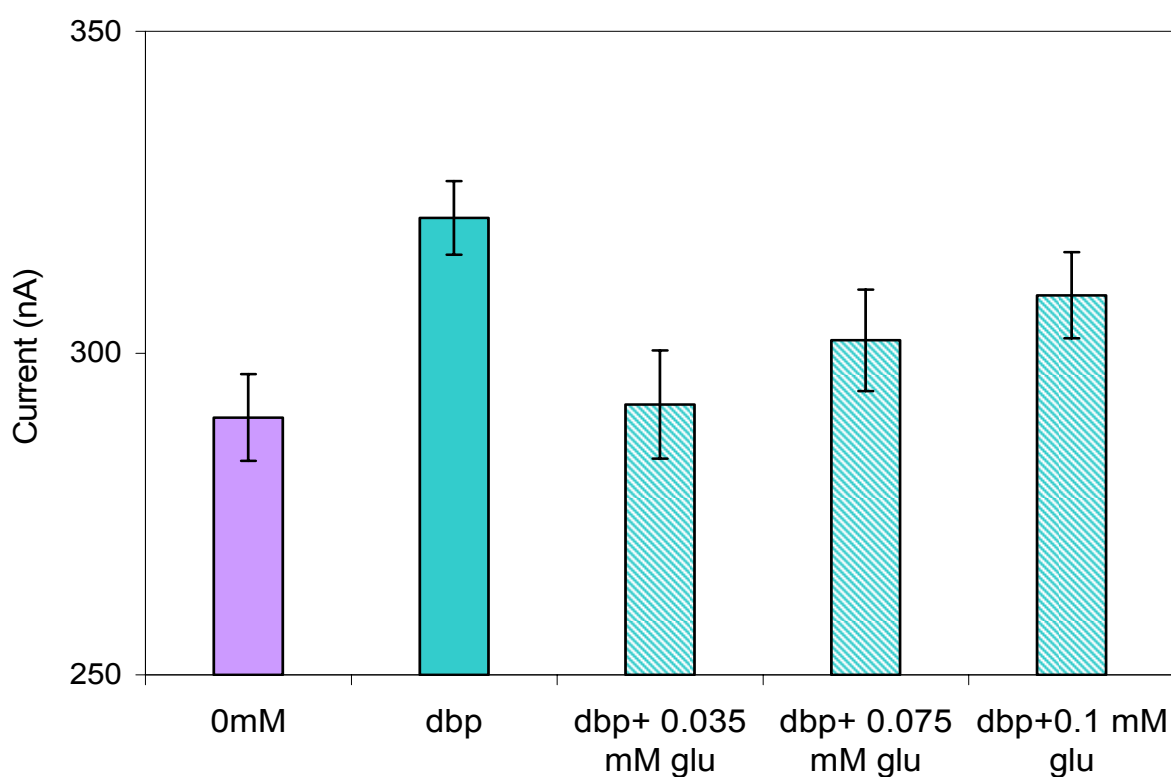
Figure 3.26 *A. adeninivorans* response to 0.075 mM di-butyl phthalate (dbp) and 0.075 mM di-butyl phthalate (dbp) + increasing glucose concentrations.

P-values were obtained by performing ANOVA analysis using raw data (Table 3.8).

Table 3.8 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression (0.035 mM – 0.1 mM glucose)

Substrate groups	P-value
0 mM - 0.075 mM dbp	1.66×10^{-9}
0 mM - dbp + 0.035 mM glucose	6.84×10^{-2}
dbp - dbp + 0.035 mM glucose	1.62×10^{-7}
dbp - dbp + 0.075 mM glucose	7.25×10^{-5}
dbp - dbp + 0.1 mM glucose	4.21×10^{-4}

The results in Figure 3.26 and Table 3.8 show that glucose at all concentrations depressed the di-butyl phthalate response but not less than endogenous response when it was present along with di-butyl phthalate (in only one instance did glucose, in presence of di-butyl phthalate, depress the response to less than endogenous response and this could be an artefact).

3.4.4 Investigation of time-dependent glucose depression pattern: glucose gene induction

It is speculated that the glucose depression is related to the glucose transporters gene induction/expression, which is a time dependent process. To identify the time that is required to trigger this mechanism experiments with different incubation times were performed. Since the glucose depression of di-butyl phthalate response was observed after a one-hour incubation time, 2 and 3 hours incubations were undertaken to observe the duration of glucose depression (Figure 3.27).
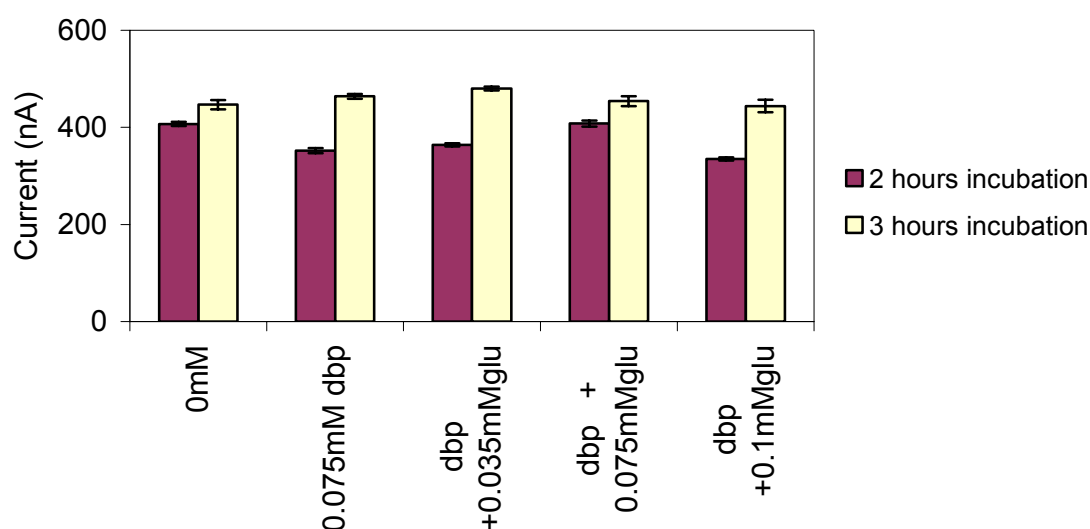
Figure 3.27 *A. adeninivorans* time- dependent response (2 and 3 hours) to di-butyl phthalate (dbp) and dbp + glucose (glu).

The results in Figure 3.27 indicated that the di-butyl phthalate response itself was not observed at two and three hours incubation suggesting that this phenomenon of glucose depression of di-butyl phthalate is time dependent and could be within an hour of the disappearance of the di-butyl phthalate response itself. The mechanism could be more prominent within one hour and could be related to glucose transport gene induction. This hypothesis was tested by performing experiments using 15, 30 and 45 minutes incubation periods. The same batch of fresh cells of OD_{600} - 3.0 was used for all three experiments (Figures 3.28, 3.29 and 3.30).
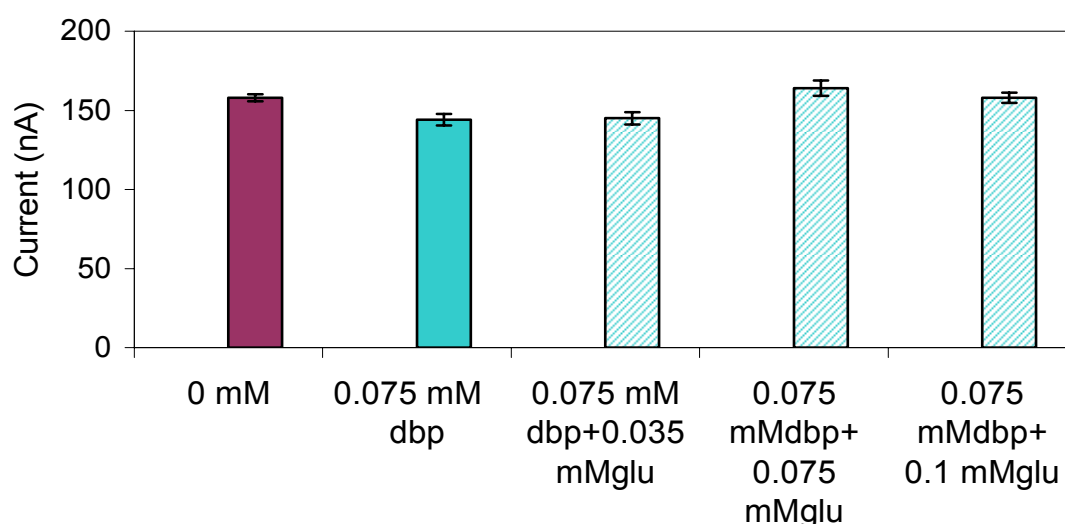
Figure 3.28 Glucose depression of *A. adeninivorans* response to di-butyl phthalate at 15 minutes incubation.
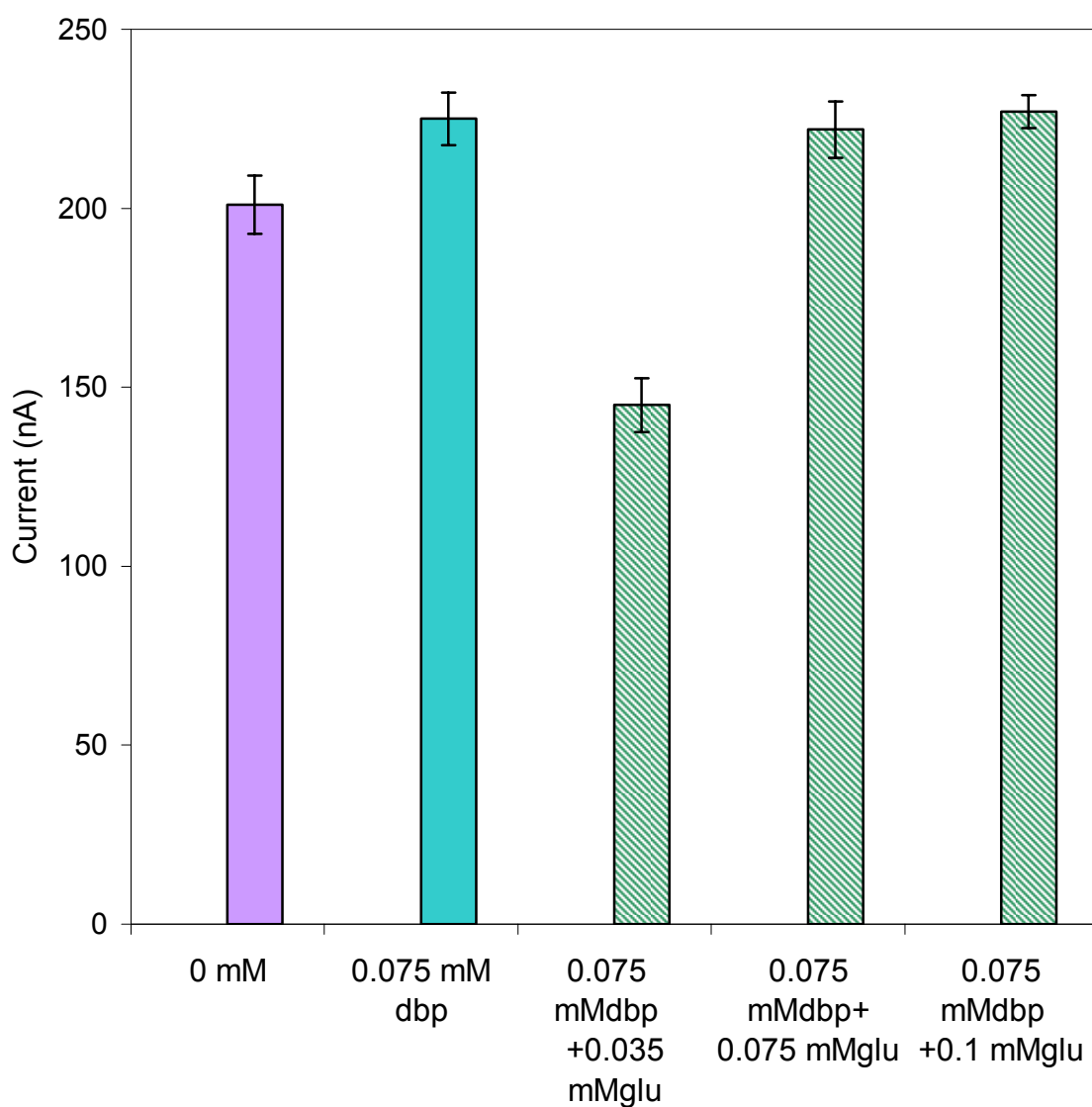
Figure 3.29 Glucose depression of *A. adeninivorans* response to di-butyl phthalate at 30 minutes incubation.

P-values were obtained by performing ANOVA analysis using raw data (Table 3.9).

Table 3.9 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression (30 minutes incubation)

Substrate groups	P-value
0 mM - 0.075 mM dbp	5.98×10^{-6}
0 mM - dbp + 0.035 mM glucose	1.28×10^{-12}
dbp - dbp + 0.035 mM glucose	4.33×10^{-7}
dbp - dbp + 0.075 mM glucose	1.01×10^{-4}
dbp - dbp + 0.1 mM glucose	1.46×10^{-2}
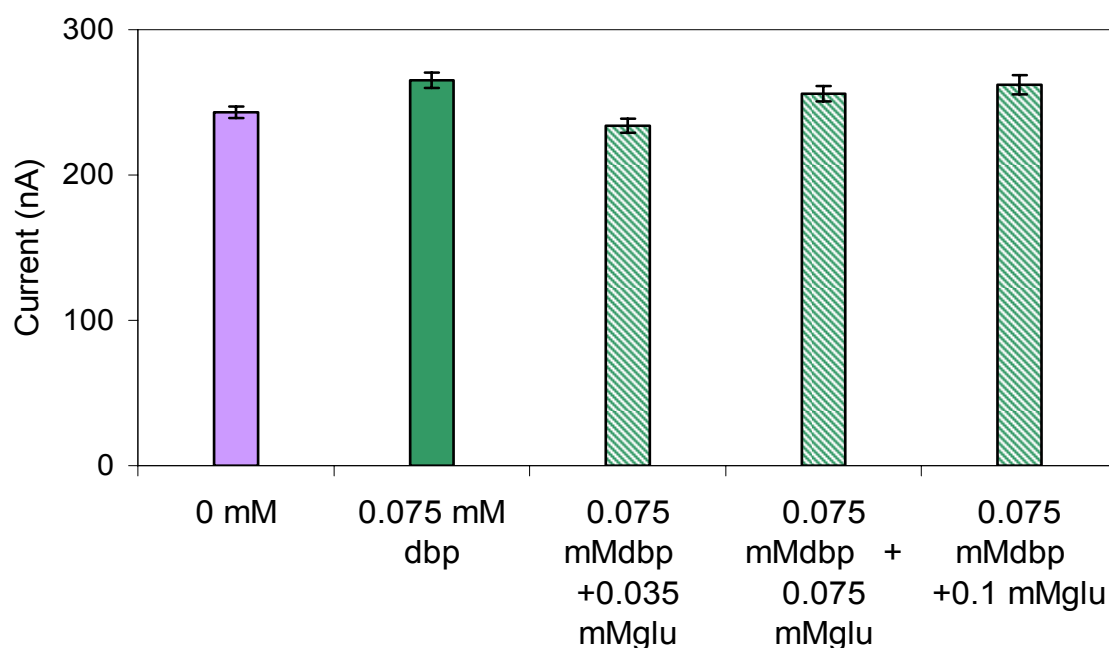
Figure 3.30 Glucose depression of *A. adeninivorans* response to di-butyl phthalate at 45 minutes incubation.

P-values were obtained by performing ANOVA analysis using raw data (Table 3.10).

Table 3.10 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression (45 minutes incubation**)**

Substrate groups	P-value
0 mM - 0.075 mM dbp	2.31×10^{-8}
0 mM - dbp + 0.035 mM glucose	5.33×10^{-4}
dbp - dbp + 0.035 mM glucose	6.37×10^{-10}
dbp - dbp + 0.075 mM glucose	7.6×10^{-5}
dbp - dbp + 0.1 mM glucose	1.8×10^{-4}

The gene induction experiments in Figures 3.28, 3.29 & 3.30 and Tables 3.9 and 3.10 show that at 15 min, even the di-butyl phthalate response was not observed, but at 30 min and 45 min the DBP response was observed and the glucose at 0.035 mM and 0.075 mM depressed the DBP signal significantly, especially glucose which at 0.035 mM not only depressed the di-butyl phthalate mediated response but also the endogenous response. These results show that the glucose was depressing not only the di-butyl phthalate signal but also the endogenous response suggesting that the glucose

transporter genes could be transiently expressed during 30 - 45 min and might be interfering with not only the di-butyl phthalate catabolic responses but also the basal catabolic responses. This glucose based depression is time and concentration dependent and disappears after one hour. In summary glucose depression was seen as a transient phenomenon, might be related to glucose transporter gene induction and was relieved after one hour.

3.4.5 Investigation of the location of the metabolic pathway that is involved in the glucose depression of di-butyl phthalate response using single hydrophilic mediator potassium ferricyanide

The origin of the metabolic pathway involved in glucose depression of di-butyl phthalate was investigated using single hydrophilic mediator (Figure 3.31).
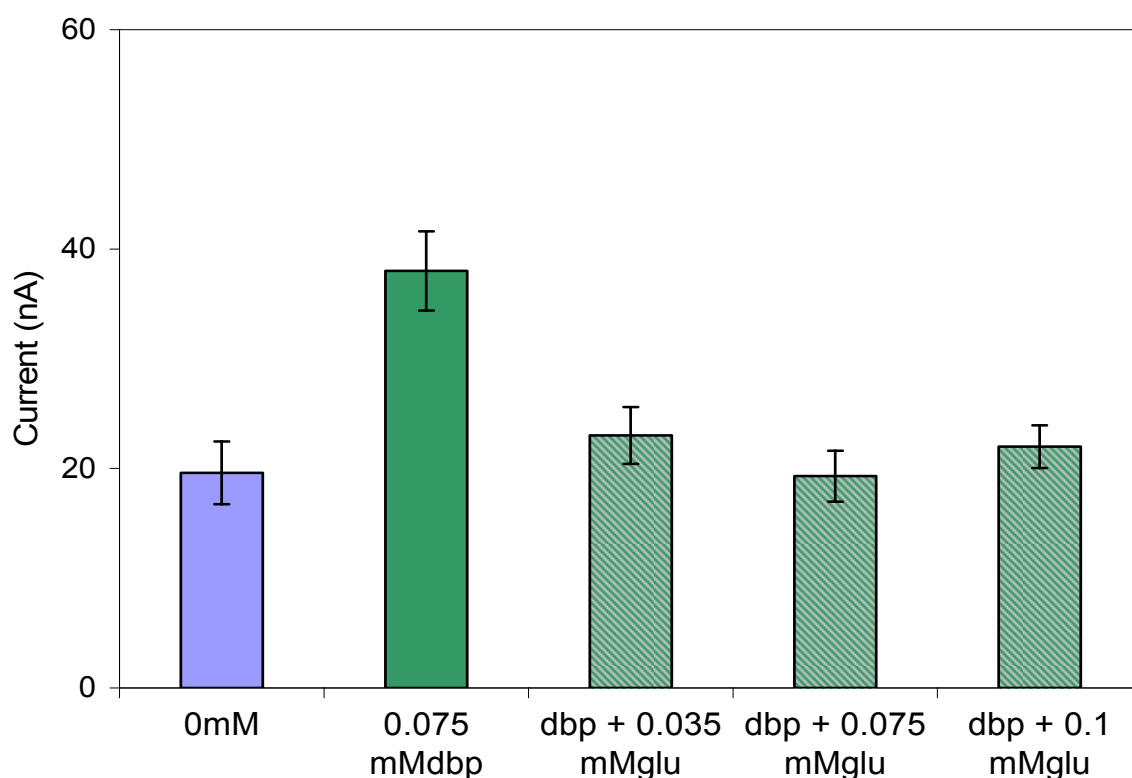
Figure 3.31 Investigation of the location of the metabolic pathway that is involved in glucose depression of *A. adeninivorans* response to di-butyl phthalate to glycolytic pathway at 45 minutes incubation: Single mediator - potassium ferricyanide.

P-values were obtained by performing ANOVA analysis using raw data has shown in the next page (Table 3.11).

Table 3.11 ANOVA Single Factor analysis of di-butyl phthalate grown cells exhibiting glucose depression (single mediator, kfc)

Substrate groups	P-value
0 mM - 0.075 mM dbp	7.42×10^{-8}
0 mM - dbp + 0.035 mM glucose	1.30×10^{-2}
dbp - dbp + 0.035 mM glucose	5.73×10^{-7}
dbp - dbp + 0.075 mM glucose	9.17×10^{-7}
dbp - dbp + 0.1 mM glucose	1.34×10^{-6}

Results in Figure 3.31 and Table 3.11 show the glucose depression of DBP for the three glucose concentrations used. This depression pattern is similar to that seen in the double mediator system. The hydrophilic mediator potassium ferricyanide can only access the electrons that are available from the redox molecules on the surface of the cytoplasmic membrane to reduce, resulting in smaller signal sizes than double mediator system. The depression pattern that was clearly demonstrated with single hydrophilic mediator suggests that the depression is occurring in the cytoplasm where glycolysis occurs because the ferricyanide can only access the trans-plasma membrane electron transport (tPMET) molecules in the cytoplasmic membrane and tPMET use NADH to reduce ferricyanide. The NADH cannot cross the cell membrane (not from mitochondrial source) and the only source of NADH that reduces ferricyanide in this single mediator system is from glycolysis.

Objectives

1. The application of double mediated electrochemical detection system to detect estrogens using genetically modified hER *S. cerevisiae* to reduce the incubation period of 72 hours of current optical detection method and improve the sensitivity.

2. To exploit glucose catabolite repression property to block the catabolism of all other catabolisable molecules and achieve specificity to estrogens

3.5 Application of the double mediated electrochemical detection system to detect estrogens using hER *S. cerevisiae*

The current optical screen using hER *S. cerevisiae* takes 72 hours to detect estrogens. In this study the attempt was made to reduce the incubation time in detecting estrogens by the double mediated electrochemical detection system The chromogenic substrate CPRG of the optical detection system was replaced with lactose in the mediated detection system. Previous work in our laboratory show that 7 hours was needed to get an acceptable estrogen response in hER *S. cerevisiae* (Baronian, Gurazada & Thomas, 2005), thus 7 hours of incubation was used in the following experiment (Figure 3.32).
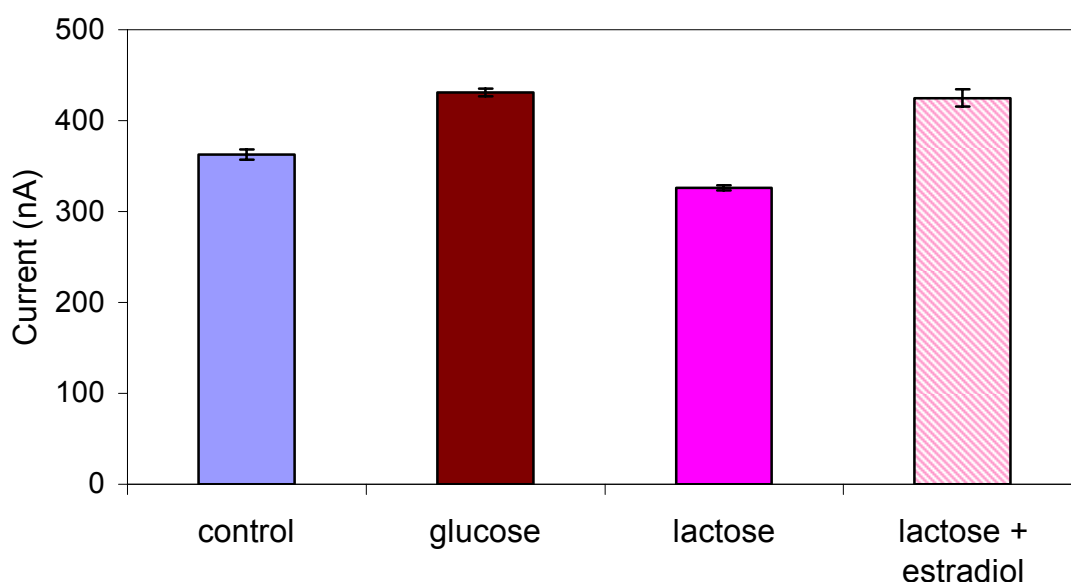
Figure 3.32 Responses of hER *S. cerevisiae* to no substrate (control), glucose (7.5 mM), lactose (7.5 mM) , lactose (7.5 mM)/estradiol (11 nM) mix.

The results in Figure 3.32 show that there was a response to 11 nM estradiol with lactose at 7 hours of incubation. These results led to more rigorous time experiments to identify the ideal incubation time to obtain optimum response to estrogen molecules.

3.5.1 Investigation of incubation time to obtain the dynamic response range

Incubation times were investigated to determine the minimum period that would provide a sufficient dynamic range of hER *S. cerevisiae* responses to 17β-estradiol for analytical purposes (Figure 3.33).
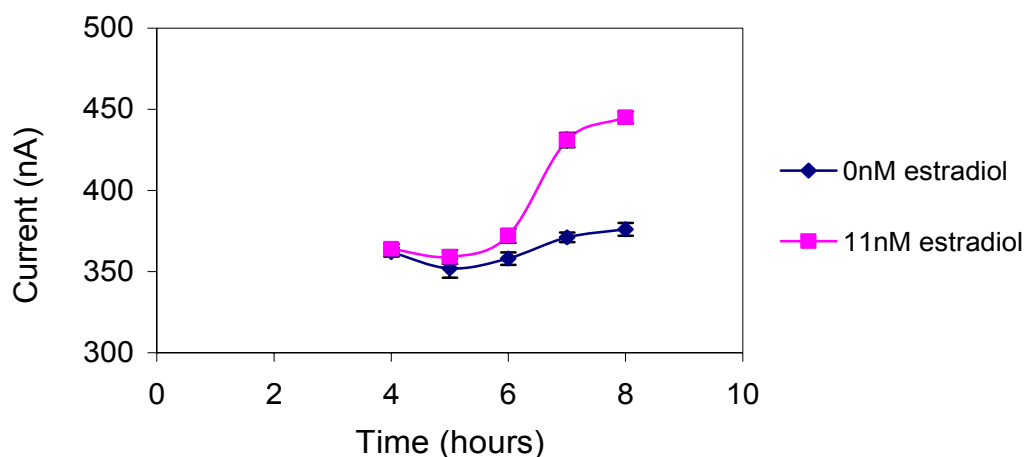
Figure 3.33 Response of hER *S. cerevisiae* to 17β-estradiol at different incubation periods

P-values were obtained by performing ANOVA analysis using raw data (Table 3.12).

Table 3.12 ANOVA Single Factor analysis of hER *S. cerevisiae* time dependent responses to 17β-estradiol.

Incubation period (hours)	P-value
4	3.75×10^{-1}
5	3.37×10^{-2}
6	9.38×10^{-5}
7	6.67×10^{-11}
8	3.03×10^{-11}

The results from an ANOVA Single Factor analysis (Table 3.12) show that the $p > 0.05$ at 4 hours indicating that 17β-estradiol responses were not significantly different statistically to the control response. The response in Figure 3.33 and P-values of the ANOVA Single Factor analysis suggest that hER *S. cerevisiae* responses to 17β-estradiol increased from 5 hours to 8 hours and at 7 and 8 hours, the dynamic range was large. Hence, 8 hours incubation was selected as the incubation period.

The errors that could occur at the very low levels (pM) of 17β-estradiol concentrations used in this study can easily be detected because of the control. The internal control

(without any 17β-estradiol) that had been used for every experiment that investigate the dose dependent 17β-estradiol responses of hER S. *cerevisiae* (Figure 3.34).
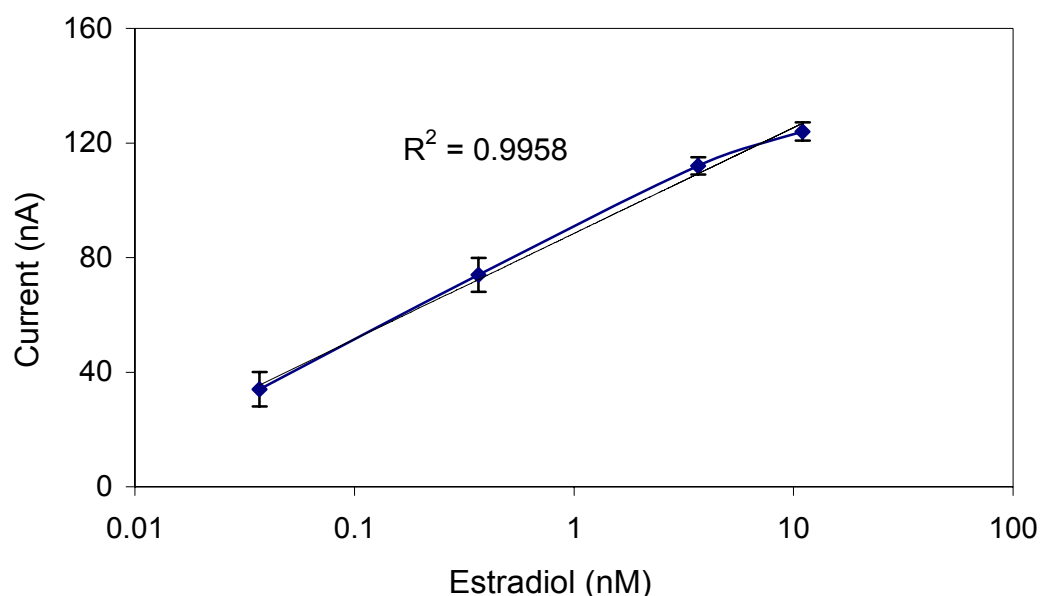
Figure 3.34 Logarithmically related hER *S. cerevisiae* responses to 0.037 nM to 11 nM 17β-estradiol - 8 hours incubation. The endogenous response (0 nM) was subtracted from these responses

P-values were obtained by performing ANOVA analysis using raw data (Table 3.13).

Table 3.13 ANOVA Single Factor analysis of hER *S. cerevisiae* dose dependent responses to 17β-estradiol.

Concentration range (nM)	P-value
0 - 0. 037	1.33×10^{-8}
0.037 - 0.367	2.23×10^{-10}
0.367 - 3.67	7.88×10^{-12}
3.67 - 11	7.62×10^{-6}

The results in Figure 3.34 and Table 3.13 show that hER *S. cerevisiae* exhibited good linear dose dependent response ($R^2 = 0.99$) to the log of 17β-estradiol from 0.037 nM - 11 nM at 8 hours and it was decided to use 8 hours incubation time.

Dose dependent logarithmic responses of hER *S. cerevisiae* to17β-estradiol from 3.67 pM – 22,000 pM were investigated (Figure 3.35a).
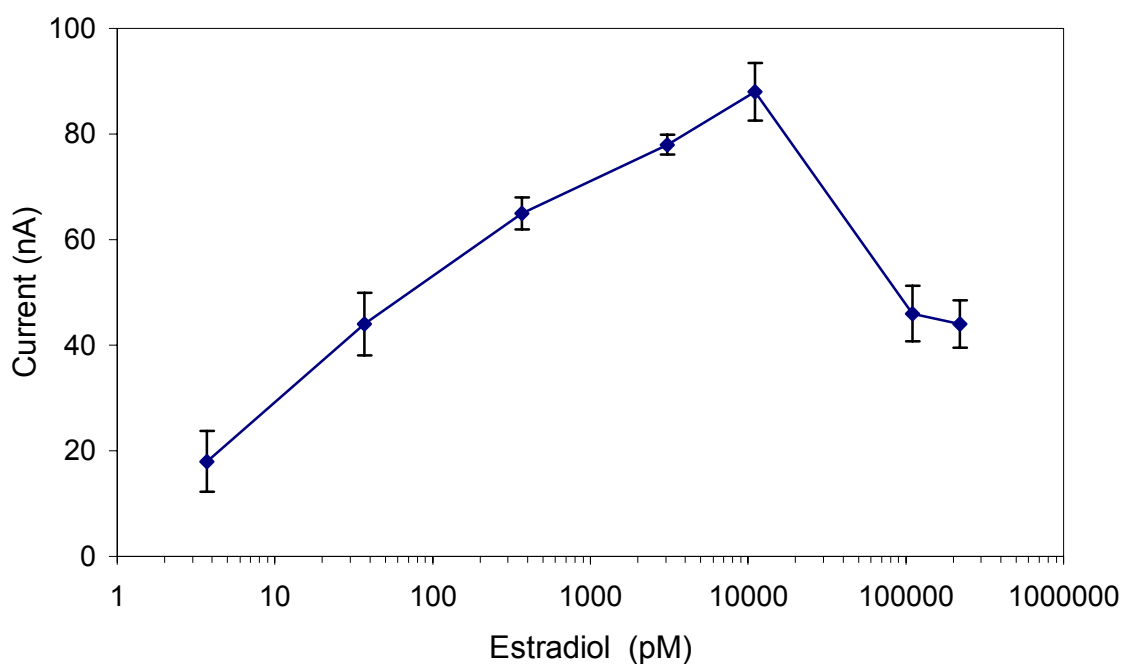
Figure 3.35a Logarithmically related dose-dependent responses of hER *S. cerevisiae* to 3.67 pM to 220,000 pM 17β-estradiol. The endogenous response (0 pM) was subtracted from these responses.

$$y = 14.201 Ln(x) + 61.42$$
$$R^2 = 0.9464$$
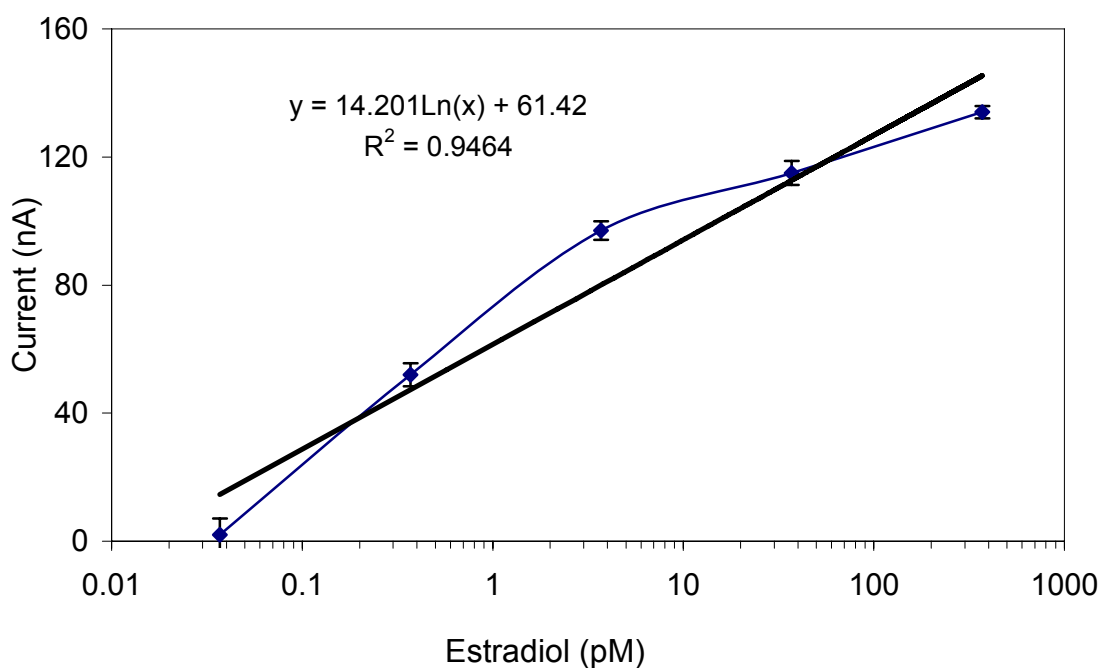
Figure 3.35b Logarithmically related dose-dependent responses of hER *S. cerevisiae* to 0.037pM to 370 pM 17β-estradiol. The endogenous response (0 pM) was subtracted from these responses.

The results in Figure 3.35a show that hER *S. cerevisiae* exhibited dose dependent linear responses to the log of 17β-estradiol from 3.67 pM to 11000 pM after that the responses dropped suggesting that at higher concentration it could be toxic to the cell. Results in Figure 3.35b shows dose dependent linear responses to the log of 17β-estradiol from 0.037 pM to 370 pM. The limits of detection and determination of 0.041 pM (4.1×10^{-14} M) and 0.42 pM (4.2×10^{-13} M) respectively were calculated from the data shown in Figure 3.35b (used the same method described earlier in this section). The EC_{50} value is 1.48 pM (1.48×10^{-12} M) and was calculated from the data presented in Figure 3.35b.

3.5.2 Method used for EC_{50} calculation

EC_{50} value is the measurement of the concentration in dose response curves that gives 50% of the maximum response. There are methods to calculate the EC_{50} value from the equation obtained from the data or directly estimate from the experimental data (Figure 3.35b). The direct estimation of EC_{50} can be done by directly reading from the graph by x-axis interpolation. This is achieved by drawing a horizontal line from the y-axis, at the point, which corresponds to 50% of the maximum response to the point of interception of the concentration-effect curve. In Figure, 3.35b the maximum response of hER *S. cerevisiae* exhibited at 370 nm was 134 nA (where the minimum response is 0 nA) and at 67 nA (50%) of the maximum response a horizontal line from y-axis was drawn at the point of interception of concentration effect curve and read directly on x-axis as the EC_{50} value. The other method is calculating from the equation shown below:

EC_{50}.

The maximum response at 370 pM 17β-estradiol (y) = 134 nA

50% of maximum response = 134 /2 = 67 nA (y)

y = 14.201 Ln(x) +61.42

67 = 14.201 Ln(x) +61.42

14.201 Ln(x) = 67 – 61.42

x = expo (67 – 61.42 / 14.201) = 1.48 pM

EC_{50} = 1.48 x 10^{-12} M.

Investigation of hER *S. cerevisiae* responses to estrogen analogues (natural and synthetic estrogens).

hER *S. cerevisiae* responses to estrogen analogues (Figures 3.36, 3.37 & 3.38). All these experiments with estrogen analogues were performed using the same batch of fresh cells of OD 3.0.
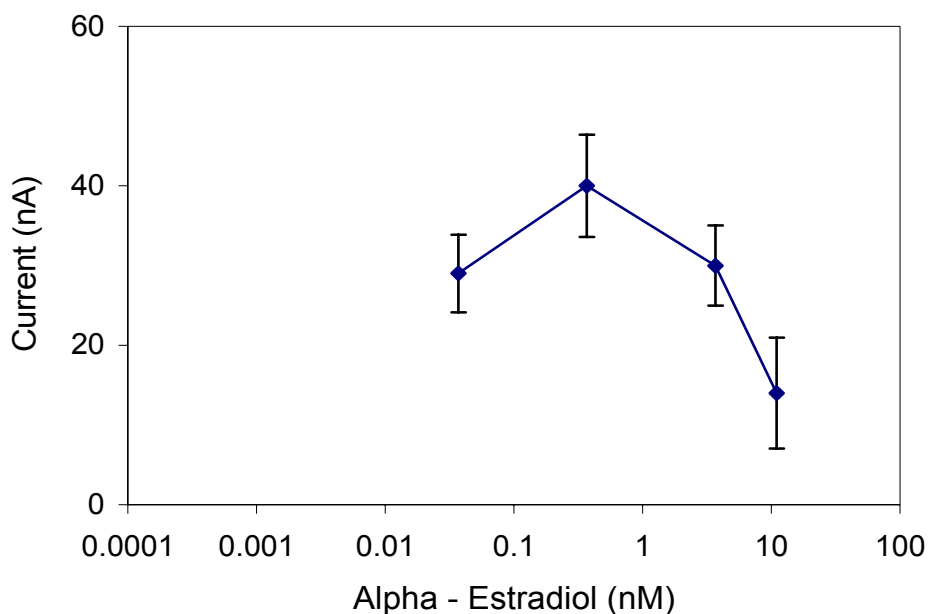
Figure 3.36 Logarithmically related hER *S. cerevisiae* responses to 0.037 nM to 11 nM 17α-estradiol. The endogenous response (0 nM) was subtracted from these responses.

The results in Figure 3.36 show that hER *S. cerevisiae* responses to the log of 17α-estradiol were dose dependent up to 0.367 nM and from 3.67 nM, the response dropped. These results indicate that the yeast cells might be sensitive at these lower concentrations of 17α-estradiol.
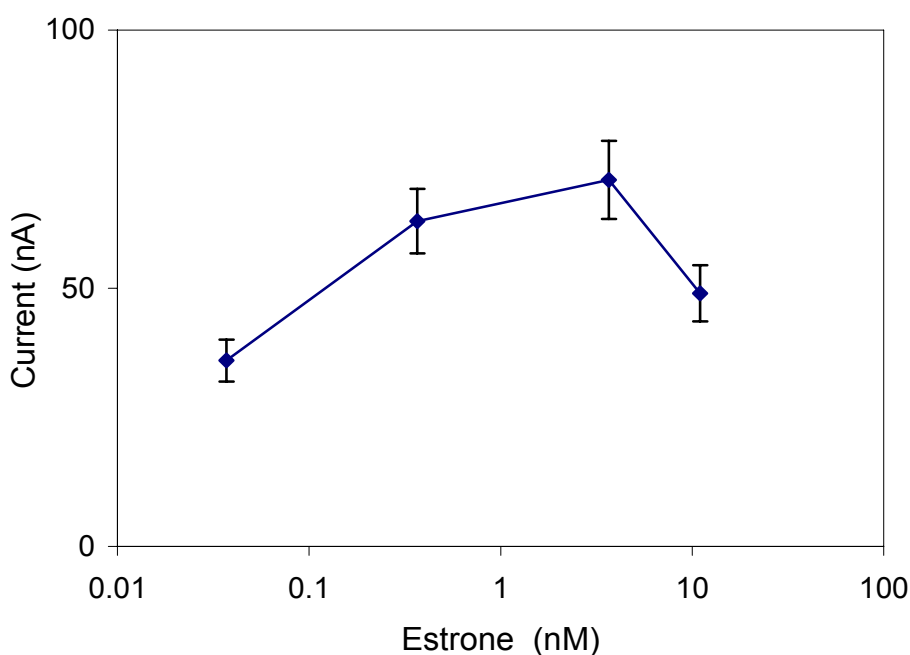
Figure 3.37 Logarithmically related hER *S. cerevisiae responses* to 0.037 nM to 11 nM estrone. The endogenous response (0 nM) was subtracted from these responses.

Results in Figure 3.37 show that hER *S. cerevisiae* responses were linear dose dependent to the log of estrone up to 3.67 nM and from there the response dropped.
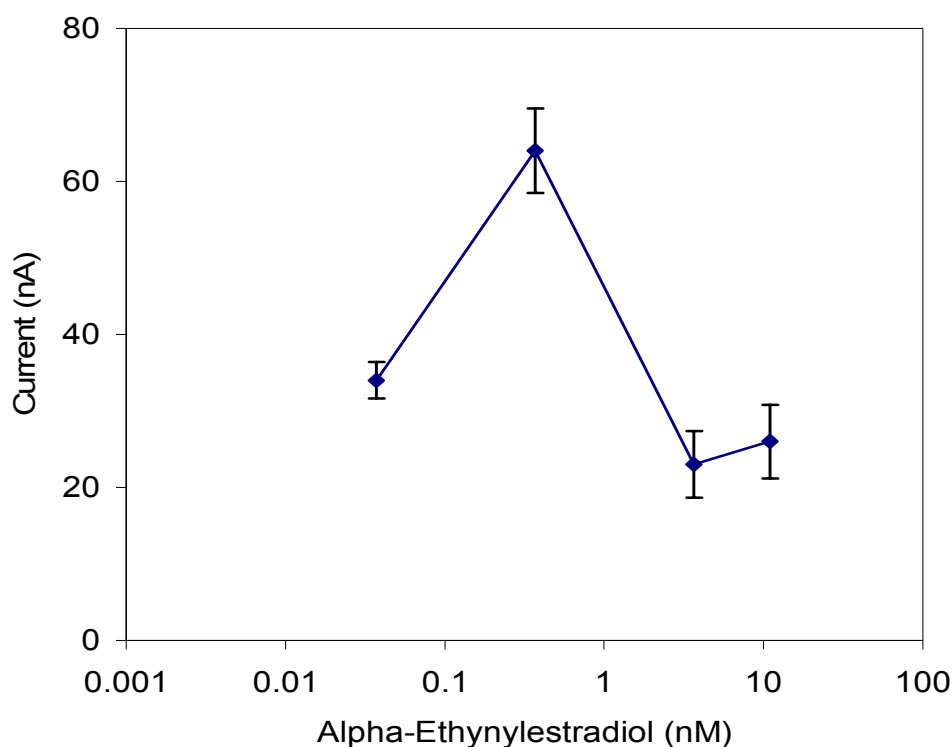
Figure 3.38 Logarithmically related hER S. *cerevisiae* to responses to 0.037 nM to 11 nM 17α-Ethynylestradiol. The endogenous response (0 nM) was subtracted from these responses.

The results in Figure 3.38 show that hER *S. cerevisiae* responses to the log of 17α-ethynylestradiol was relatively higher at lower concentrations of 0.037 nM and 0.367nM than at the higher concentrations of 3.67nM and 11 nM. hER *S. cerevisiae* responses to all estrogen analogues including 17β-estradiol were combined together in summary graph (Figure 3.39).
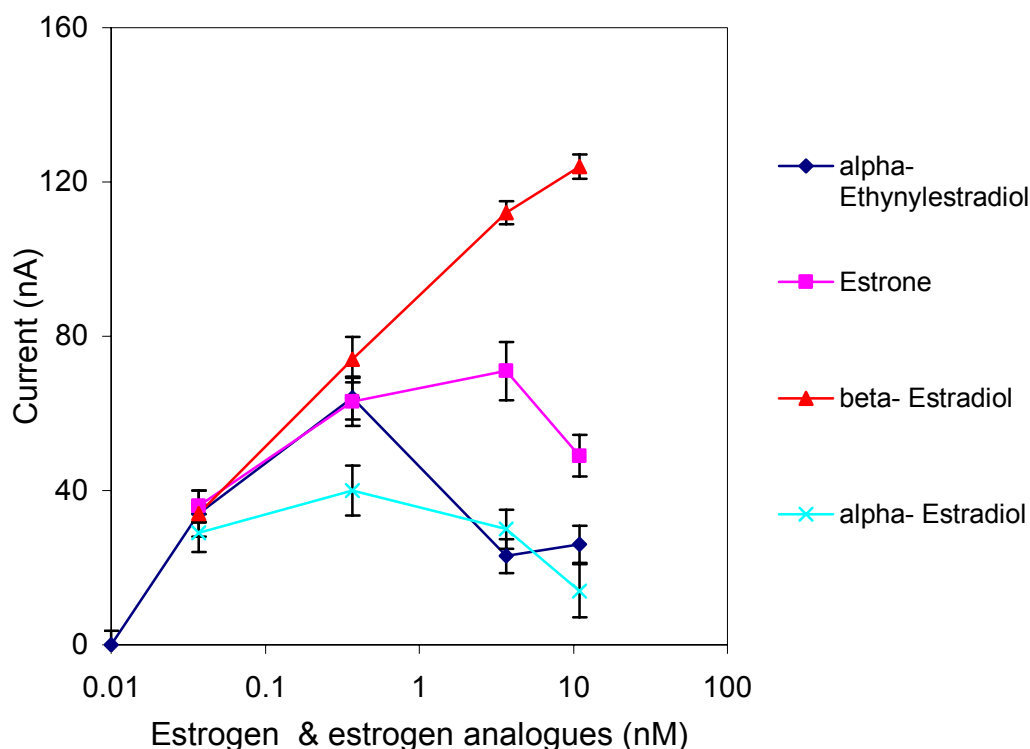
Figure 3.39 hER *S. cerevisiae* responses to estrogen and estrogen analogues (summary graph of hER *S. cerevisiae's* responses).

For the sake of convenience to interpret all these results, the data is presented in summary graph (Figure 3.39) and tabulated in Table 3.14

Table 3.14 Comparison of hER S.*cerevisiae* responses to 17α-estradiol, estrone and α- Ethynylestradiol to 17β-estradiol (activity given as 100% at the concentrations tested).

Substrate	0 nM	0.037 nM	0.367 nM	3.67 nM	11 nM
17β-Estradiol	0%	100%	100%	100%	100%
17α-Estradiol	0%	85%	54%	27%	11%
Estrone	0%	103%	85%	51%	39%
17α-Ehynylestradiol	0%	100%	86%	31%	35%

The results in Figure 3.40 the summary graph show that hER *S. cerevisiae* exhibited good logarithmic response to α- ethynylestradiol and estrone. Results in Figure 3.39 and Table 3.14, suggest that the 17β-estradiol responses were greatest except for estrone at 0.037nm and the 17α-estradiol was the least. 17α-ethynylestradiol and estrone responses were intermediate between 17β & 17α-estradiol except that the estrone response at 0.37nM was greater than 17β-estradiol.

3.5.3 Optimisation of glucose concentration as a blocking agent to block the catabolism of other catabolisable molecules and achieve specificity to estrogens

Glucose was used as a blocking agent to block the catabolism of all the other catabolisable molecules in the sample and achieve specificity to 17β-estradiol (estrogens). Glucose concentration as blocking agent was optimised (Figure 4.40).
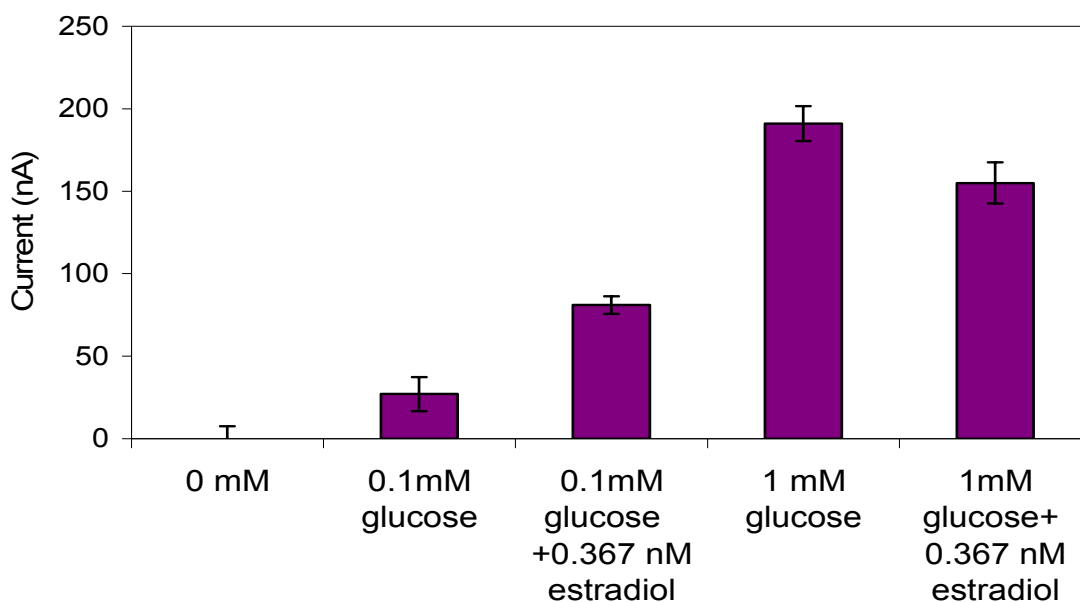
Figure 3.40 hER *S.cerevisiae* responses to glucose and 0.367 nM 17β-estradiol. The endogenous response (0 mM) was subtracted from these responses.

The results in Figure 3.40 show that 0.1 mM glucose is suitable for blocking experiments. At 1 mM glucose it masks the 0.367 nM 17β-estradiol response. 0.1 mM glucose was chosen for in blocking experiments. 17β-Estradiol concentrations using 0.1 mM glucose and increasing estradiol concentrations were investigated to identify the useful 17β-estradiol concentration in blocking experiments (Figure 3.41).
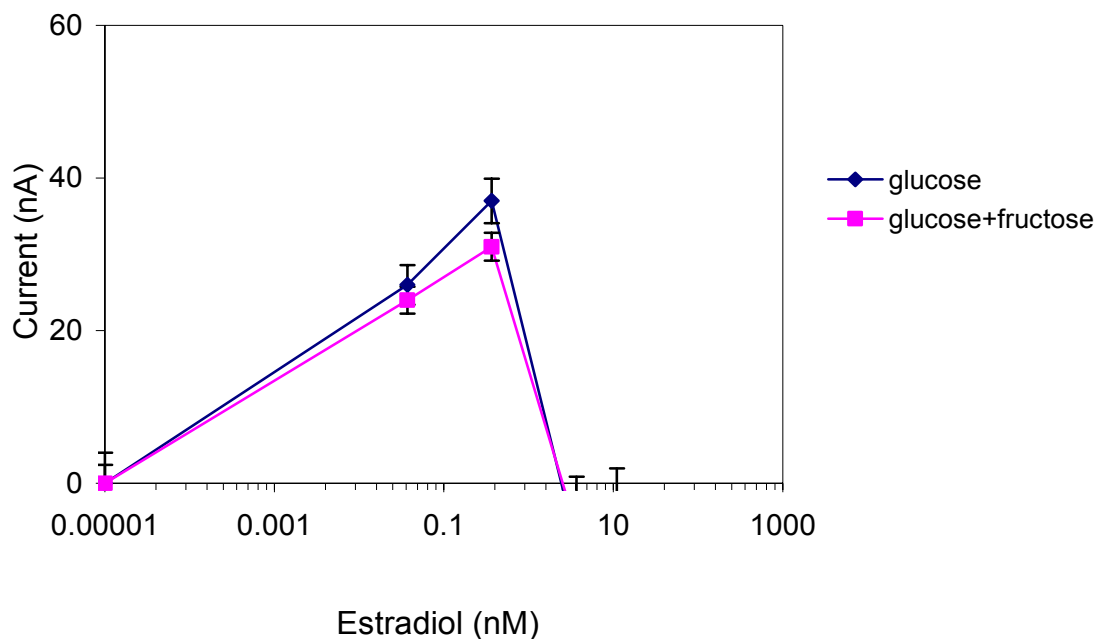
Figure 3.41 Logarithmically related hER *S. cerevisiae* response to increasing 17β-estradiol concentrations in the presence of 0.1 mM glucose and 0.1 mM glucose and 0.1 mM fructose (catabolite repression of fructose by glucose).

The results in Figure 3.41 show that hER *S. cerevisiae* responses in presence of 0.1 mM glucose were dose dependent with log of estradiol up to 0.367 nM above that concentration the signal dropped showing that 0.367 nM 17β-estradiol is useful in obtaining optimal response. It was decided that 0.367nM 17β-estradiol and 0.1 mM glucose and or other catabolisable molecules are to be used for blocking experiments (Figures 3.42 and 3.43).
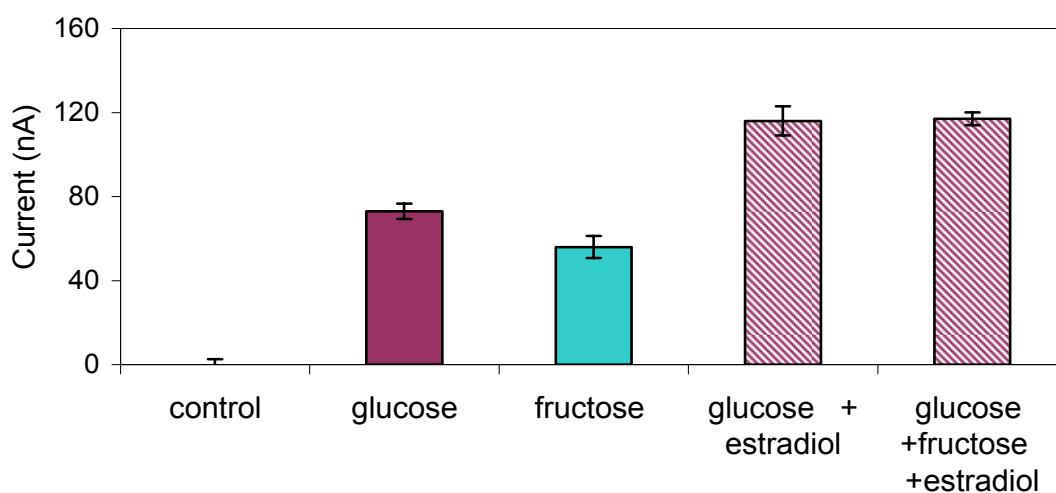
Figure 3.42 hER *S. cerevisiae* responses to 17β-estradiol in the presence of glucose and fructose. Analyte concentrations are glucose, fructose 0.1mM and 17β-estradiol 0.367 nM. The endogenous (control) response was subtracted from these responses
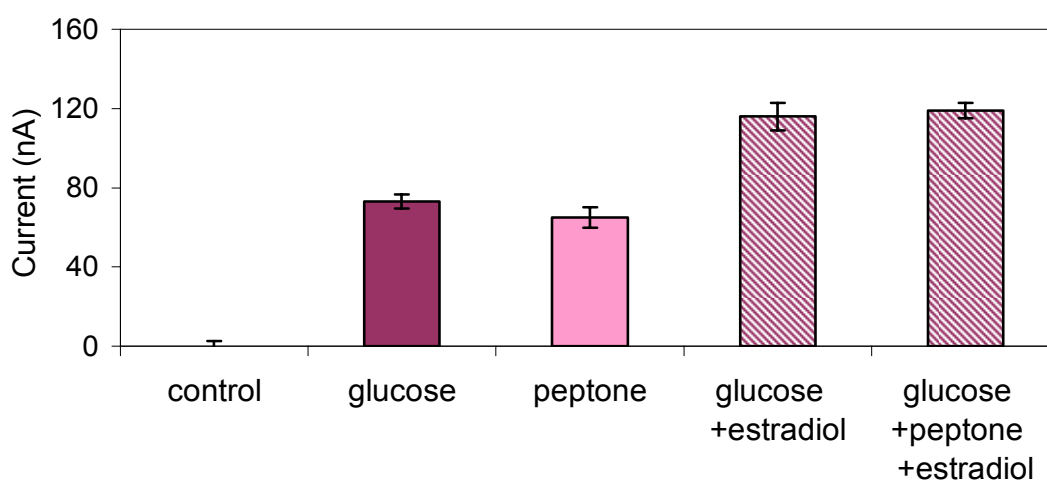
Figure 3.43 hER *S. cerevisiae* responses to 17β-estradiol in the presence of glucose and peptone. Analyte concentrations are glucose 0.1 mM, peptone 0.018% and 17β-estradiol 0.367nm. The endogenous (control) response was subtracted from these responses

ANOVA Single Factor Analysis was performed using raw data (Figures 3.42 & 3.43) and P-values were obtained (Table 3.15).

Table 3.15 ANOVA Single Factor Analysis of hER *S. cerevisiae* responses to 17β-estradiol in the presence of glucose, glucose + fructose and glucose + peptone

Substrate groups	P-value
Glucose + estradiol - glucose + fructose + estradiol	6.65×10^{-1}
Glucose + estradiol - glucose + peptone + estradiol	5.05×10^{-1}
Glucose - glucose + estradiol	7.07×10^{-12}

The results in Figures 3.42 and 3.43 suggest that the glucose + lactose + 17β-estradiol 0.367nM responses were statistically not significantly different to glucose + lactose + fructose +17β-estradiol 0.367nM and glucose + lactose + peptone + 17β-estradiol 0.367nM responses ($p > 0.05$). These results demonstrate that glucose can catabolically repress the responses of other easily catabolisable molecules such as fructose and peptone (a complex mixture of peptides, amino acids and simple carbohydrates) and yet the yeast cells are able to detect the environmentally lower levels of estrogens.

3.5.4 Investigation of the estradiol responses in hER *S. cerevisiae* in the presence and absence of lactose

To confirm the hypothesis that was proposed in Figure 4.5 that hydrolysis of lactose, which is a substrate for β-galactosidase (lac Z reporter gene product) results in glucose and galactose production and augment the catabolism, hER *S. cerevisiae* responses to estradiol in the presence and absence of lactose was investigated (Figure 3.44).
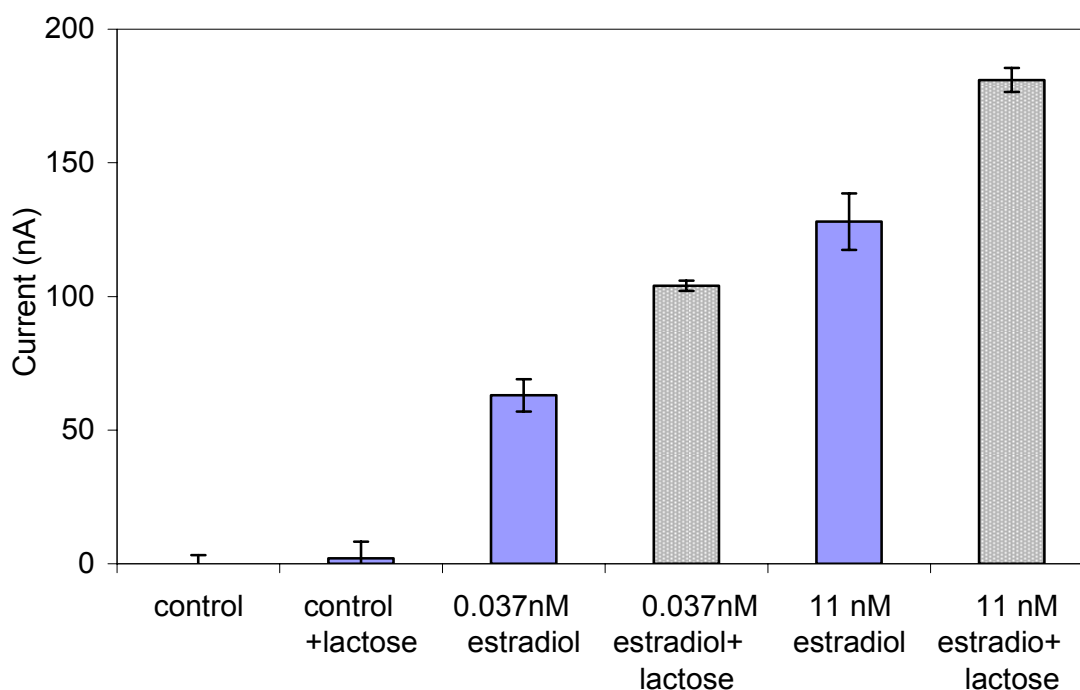
Figure 3.44 Responses of hER *S. cerevisiae* to 0.037 nM and 11 nM 17β-estradiol with and without 7.5 mM lactose. The endogenous response (control) was subtracted from these responses.

The results in Figure 3.44 show hER *S. cerevisiae* responses to 17β-estradiol in the presence and absence of lactose (0.037 nM and 11 nM estradiol responses with and without lactose are bigger than control with and without lactose). These results suggest, the responses could be, one due to the genetically engineered human estrogen receptor and the second could be most likely from the native estrogen receptor of *S. cerevisiae*. The responses observed in hER *S. cerevisiae* show no evidence for a strong lactose dependency, which suggests that the human estrogen receptor is not required for an estrogen response leading to the investigation of wild type *S. cerevisiae*. To confirm the presence of native estrogen receptor wild type *S. cerevisiae* was used.

Objectives

1. Exploit wild type *S. cerevisiae* estrogen binding protein oxidoreductase property to detect estrogens and estrogens analogue.
2. Test well characterised estrogen binding protein of *C. albicans* responses to 17- β-estradiol.

109

3. Exploit glucose catabolite repression property of *S. cerevisiae* to block the catabolism of all other catabolisable molecules and achieve specificity to estrogens in environmental samples.
4. Develop a sensitive estrogen biosensor to quantify environmental estrogens.

3.6 Investigation of wild type *S. cerevisiae* responses to estrogens

Wild type *S. cerevisiae* responses to estrogen (17β-estradiol) with and without lactose was investigated (Figure 3.35).
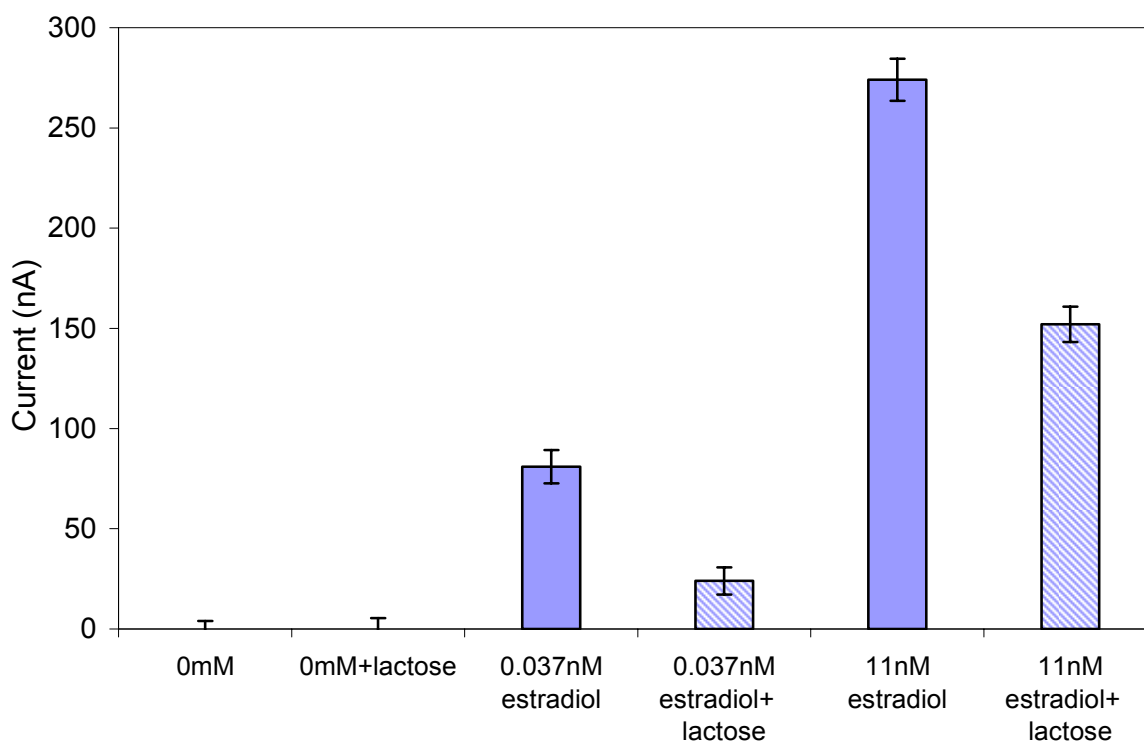
Figure 3.45 *S. cerevisiae* responses to 0.037 nM and 11 nM 17β-estradiol with and without 7.5 mM lactose. The endogenous response (0 mM) was subtracted from these responses.

The results in Figure 3.45 show that *S. cerevisiae* responded to the two estradiol concentrations that were used, both with and without lactose. These results confirm the previous results with hER *S. cerevisiae,* that the response of the native receptor to estrogens is lactose independent (hence, there is no need to add lactose to detect the responses to estrogens in wild type *S. cerevisiae).* The wild type *S. cerevisiae* is known

not to be able to catabolise lactose and, in fact, its presence appears to inhibit the electrochemical responses to estrogens and the reasons for this inhibition are unknown.

3.6.1 Dose dependent responses of wild type *S. cerevisiae* to 17β-estradiol

Dose dependent responses of wild type *S. cerevisiae* to 17β-estradiol were investigated (Figures 3.46a & 3.46b).
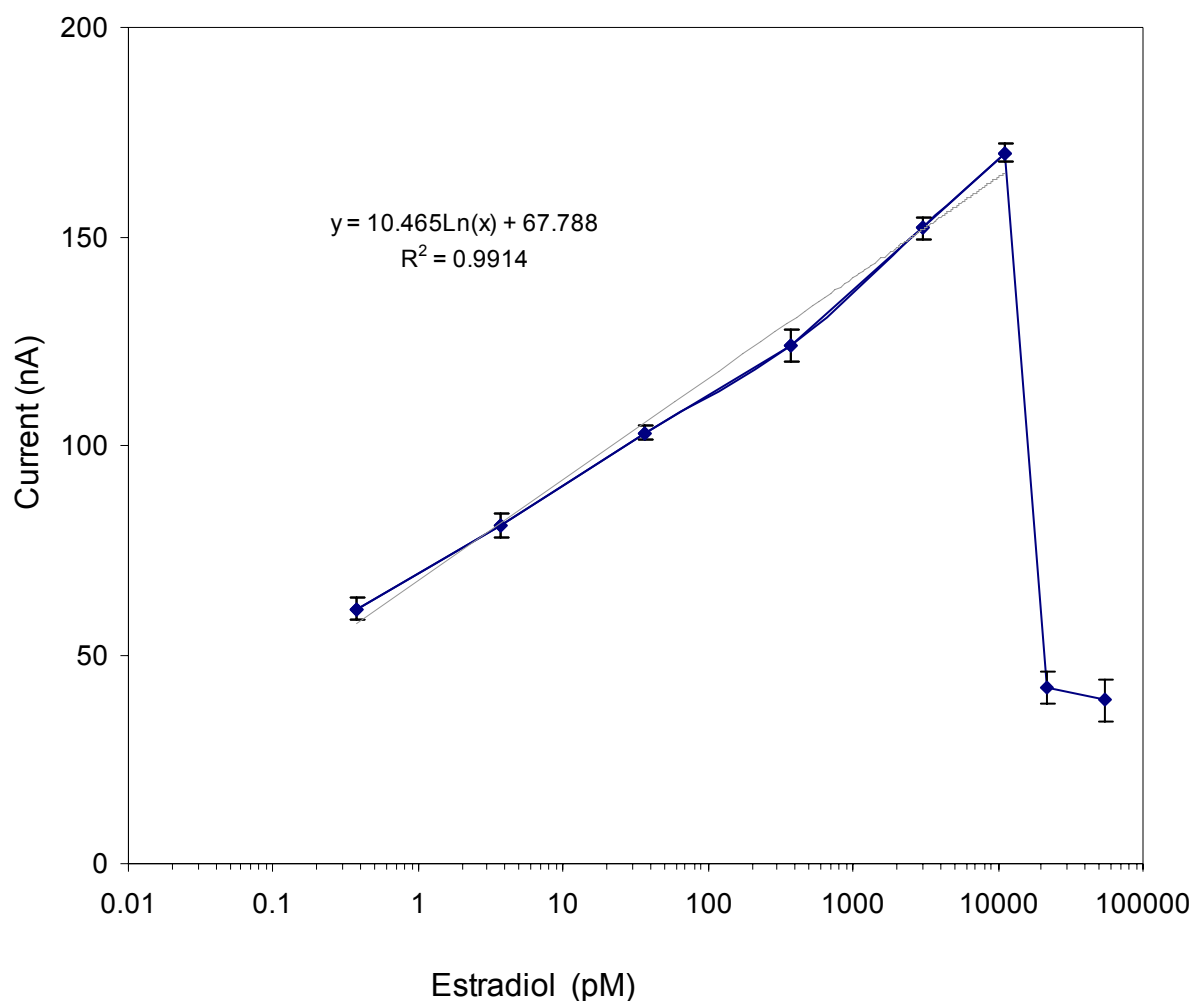
Figure 3.46a Logarithmically related *S. cerevisiae* dose-dependent responses to 17β-estradiol 0.37 pM- 55000pM. The endogenous response (0 pM) was subtracted from these responses.

The results in Figure 3.46a show very good linear dose dependent responses of *S. cerevisiae* to the log of 17β-estradiol from 0.37 pM – 11000 pM. From 11000 pM estradiol concentration, the response dropped dramatically. It is possible that above this concentration, estradiol is toxic to the cell or the exporter protein of the estrogen becomes active and reduces the intracellular estrogen concentration. Data from 0.37 pM

– 11000 pM was used to plot the graph to calculate the limits of detection and determination (Figure 3.46b).
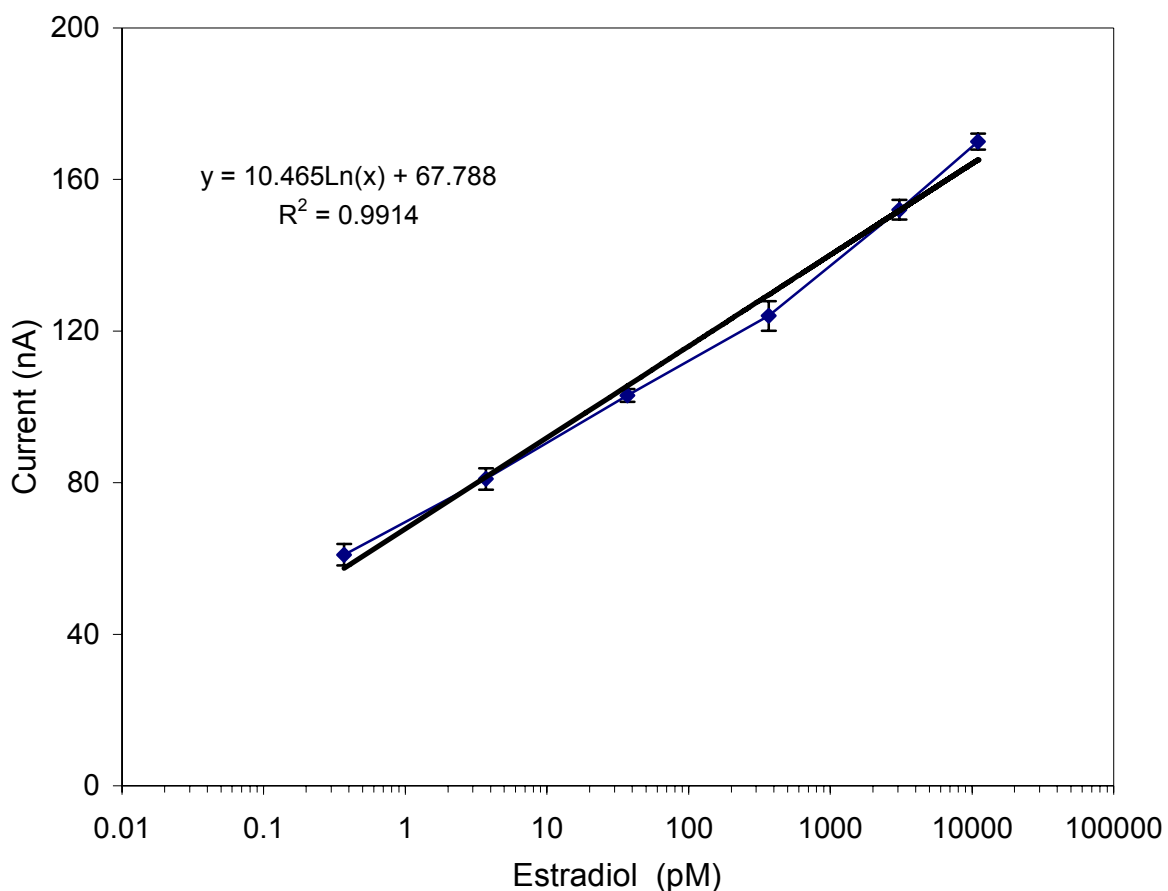
Figure 3.46b Logarithmically related *S. cerevisiae* dose-dependent responses to 17β-estradiol 0.37 pM - 11000pM. The endogenous response (0 pM) was subtracted from these responses. The regression line and the equation were obtained using Microsoft Excel.

The results shown in Figure 3.46b show that *S. cerevisiae* responses were linear dose-dependent to the log of 17β-estradiol from 0.37 pM - 11000 pM. The limits of detection and determination are 0.0036 pM (3.6 x 10^{-15} M) and 0.021 pM (2.1 x 10^{-14} M) respectively were calculated using the data presented in Figure 3.46b. The EC_{50} of 51 pM (5.1x 10^{-11} M) was calculated from the equation shown in the graph (Figure 3.46b).

To minimise the complexity involved in preparing the minimal defined medium which requires the addition of several reagents and vitamins etc, the wild type *S. cerevisiae* responses to 17β-estradiol were explored by substituting the minimal defined medium with YEPD medium (Figure 3.47).
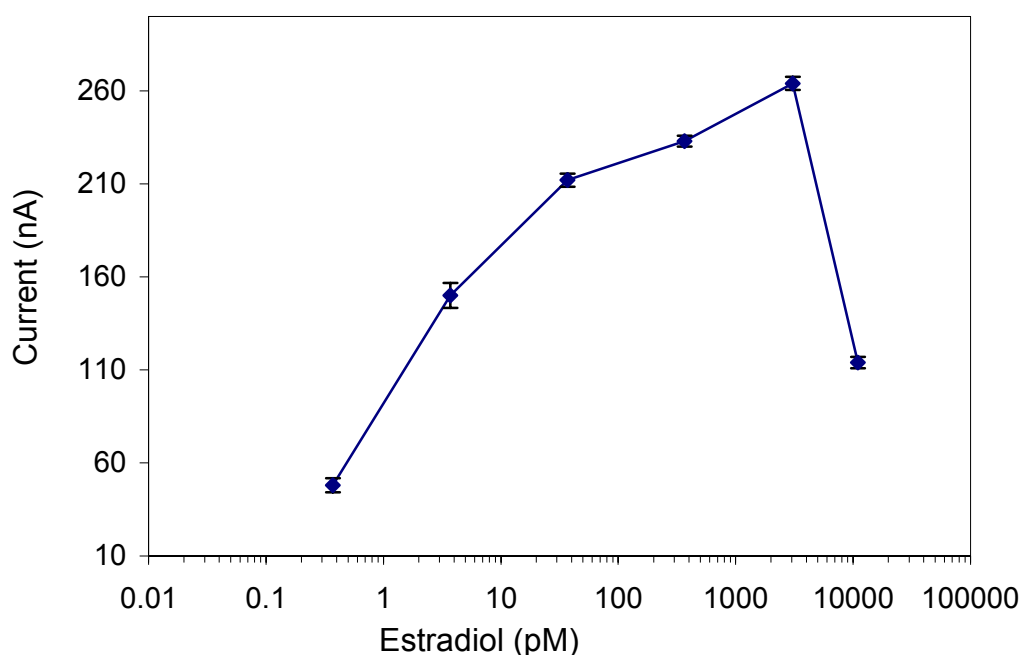
Figure 3.47 Logarithmically related *S. cerevisiae* dose-dependent responses to 17β-estradiol 0.37 pM- 11000 pM in YEPD medium. The endogenous response (0 pM) was subtracted from these responses.

The results in Figure 3.47 shows *S. cerevisiae* exhibited dose-dependent responses to the log of 17β-estradiol from 0.37 to 3670 pM and after that concentration the signal dropped compared to minimal medium where the response dropped from 11000 pM. In addition, it is reported that ingredients in YEPD medium contain estrone and 17β-estradiol (Feldman et al., 1995) and these might interfere with the estrogen analysis, hence it was decided to use minimal medium for subsequent experiments.

The well characterised *Candida albicans* estrogen binding protein's responses to 17β-estradiol were investigated (Figure 3.48).
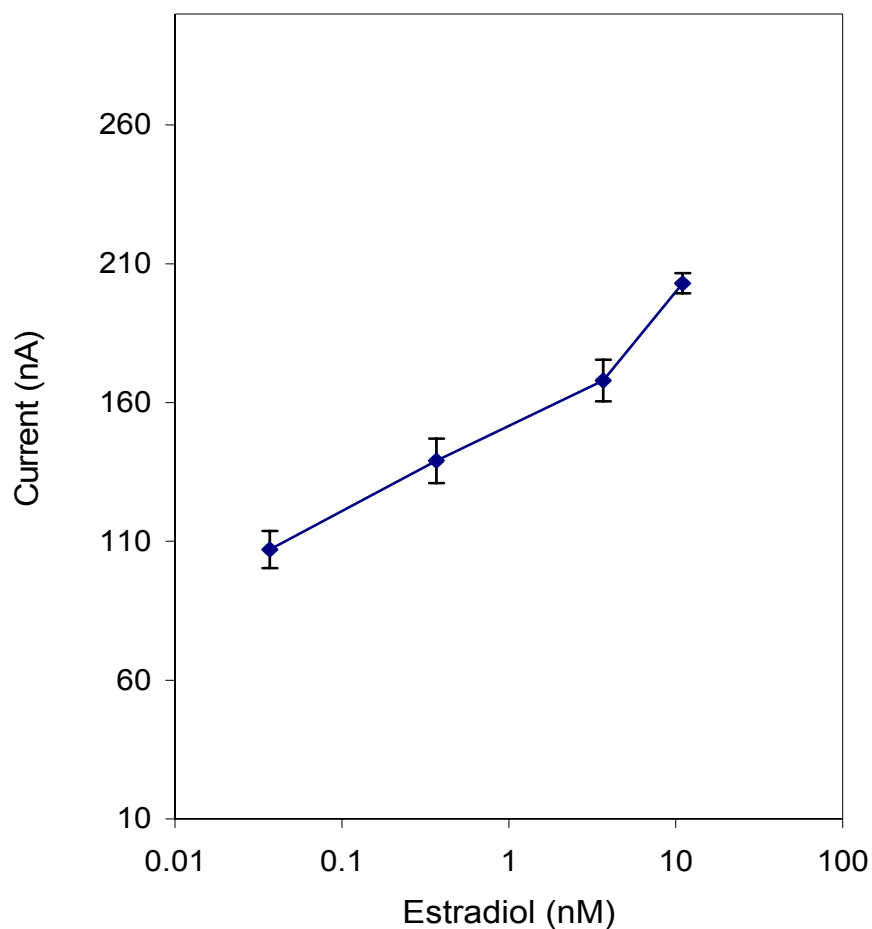
Figure 3.48 Logarithmically related *C. albicans* dose-dependent responses to 17β-estradiol 0.037 - 11 nM. The endogenous response (0 nM) was subtracted from these responses.

The results in Figure 3.48 show that *C. albicans* exhibited dose-dependent responses to the log of 17β-estradiol, which were similar to *S. cerevisiae*. These results indicate that estrogen binding protein in these two organisms might be playing a role in estrogen binding and generating subsequent electrochemical responses. To attribute a role to estrogen binding protein, the generality of the binding affinities of estrogen binding protein to other estrogen and estrogen analogues in *S. cerevisiae* were tested (Figures 3.49, 3.50 & 3.51). All these experiments were performed using the same batch of fresh cells of OD = 3.0.
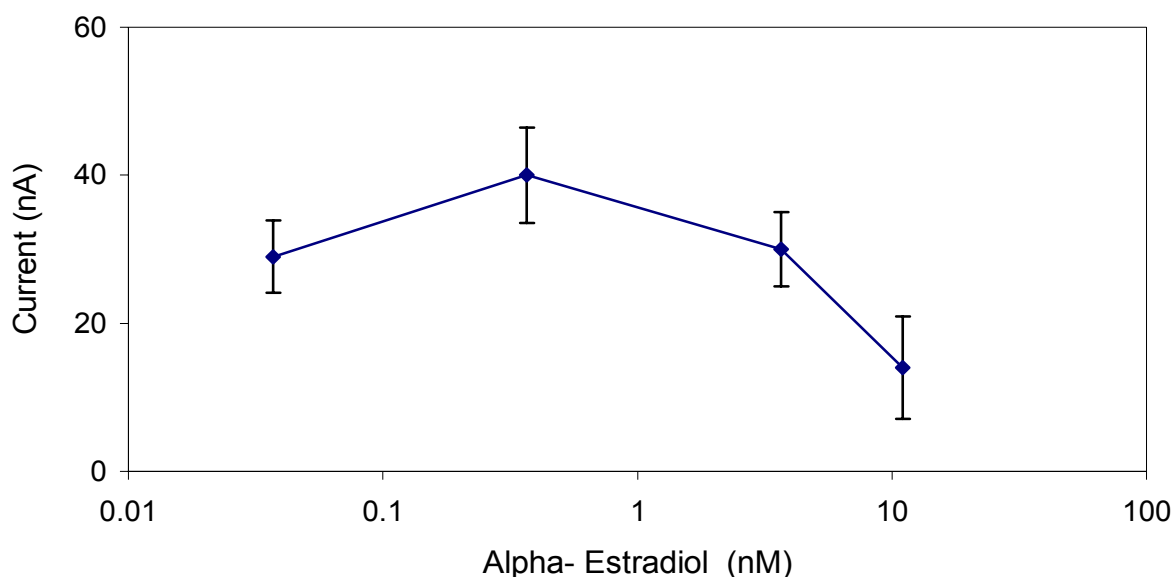
Figure 3.49 Logarithmically related *S. cerevisiae* responses to 0.037 - 11 nM 17α-estradiol. The endogenous response (0 nM) was subtracted from these responses.

The results in Figures 3.49 show the dose dependent responses of *S. cervisiae* to the log of 17α-estradiol from 0.037 to 0.367 nM. Above 0.367 nM concentrations the responses dropped.
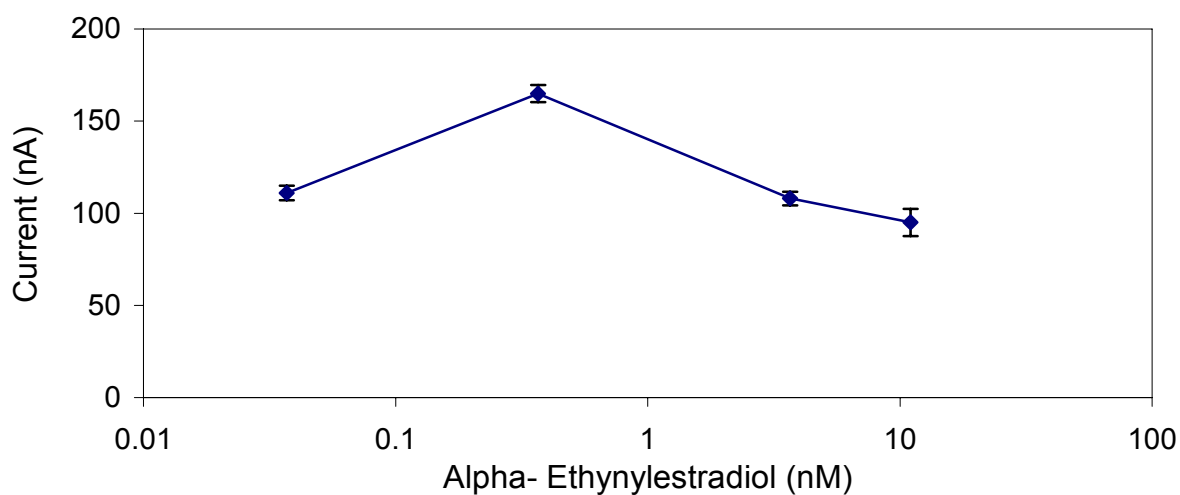
Figure 3.50 Logarithmically related *S. cerevisiae* responses to 0.037 - 11 nM 17α-ethynylestradiol. The endogenous response (0 nM) was subtracted from these responses.

The results in Figure 3.50 show that *S. cerevisiae* responses to the log of 17α-ethynylestradiol was relatively higher at lower concentrations of 0.037 nM and 0.367nM than at the higher concentrations of 3.67nM and 11 nM.
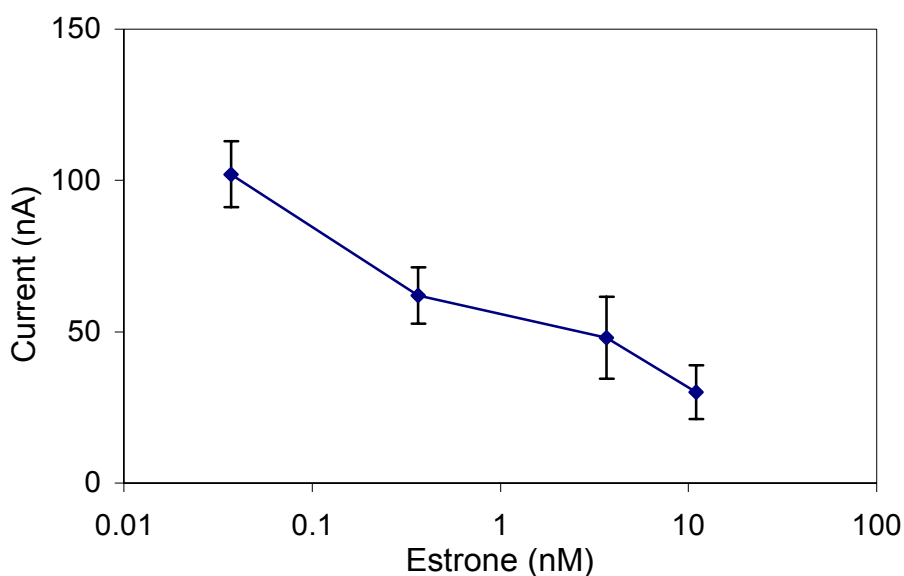
Figure 3.51 Logarithmically related *S. cerevisiae* responses to 0.037 - 11 nM estrone. The endogenous (0 nM) was subtracted from these responses.

The results in Figure 3.51 show that estrone responses in *S. cerevisiae* are different from other estrogen analogues and of hER *S. cerevisiae*'s responses. The signal dropped from 0.037 nM onwards. It is possible to get linear range at lower concentrations than 0.037 nM. This aspect was not pursued further because of the time constraint and this information is not very critical as these experiments were performed to check the generality of the binding affinities of the estrogen binding protein to estrogens and estrogen analogues other than 17β-estradiol, which were already demonstrated in these studies. *S. cerevisiae* responses to all estrogen analogues including 17β-estradiol were combined together in summary graph (Figure 3.52).
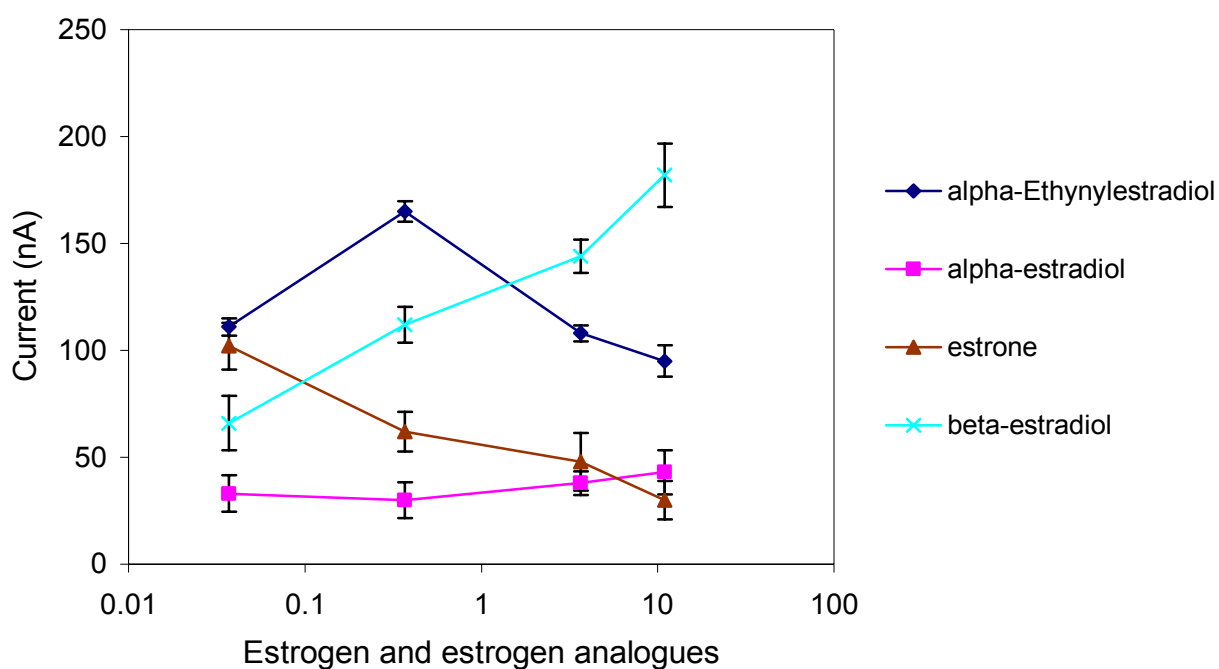
Figure 3.52 Logarithmically related *S. cerevisiae* responses to estrogen and estrogen analogues (Summary graph of *S. cerevisiae* responses).

The results in Figure 3.52 show the responses of wild type *S. cerevisiae* to estrogens and estrogen analogues, suggesting the existence of a native estrogen receptor system operating in the organism.

For the sake of convenience, to interpret the results, the data presented in summary graph (Figure 3.52) are tabulated in Table 3.16.

Table 3.16 Comparison of *S. cerevisiae* responses to 17α-estradiol, estrone and α-ethynylestradiol activities to 17β-estradiol (activity given as 100% at the concentrations tested)

Substrate	0nM	0.037nM	0.367nM	3.67nM	11nM
17β-Estradiol	0%	100%	100%	100%	100%
17α-Estradiol	0%	50%	27%	26%	24%
Estrone	0%	135%	55%	33%	17%
17α-Ethynylestradiol	0%	140%	132%	75%	48%

The results summarised in Table 3.16 demonstrate that the synthetic estrogen 17α-ethynylestradiol is more potent than 17β-estradiol at lower concentrations i.e. 0.037 and 0.367 nM, it is less potent at higher concentrations of 3.67 and 11 nM. The next potent estrogenic substance is estrone which is more potent than 17β-estradiol at 0.037 nM, but less potent at higher concentrations. Estrone could probably have given a linear response at much lower concentrations, e.g., in the 0.00037 - 0.037 nM range and perhaps could be even more potent than 17β-estradiol at these lower concentrations. The least potent estrogenic substance is 17α-estradiol at all concentrations tested.

Blocking experiments were performed using 0.1 mM glucose (the same concentration used for hER S. *cerevisiae,* Figure 3.40) to observe the effect of increasing 17β-estradiol concentrations on the signal size and also to confirm 0.367 nM 17β-estradiol, that was used for hER S. *cerevisiae* was a useful concentration for these blocking experiments (Figure 3.53).
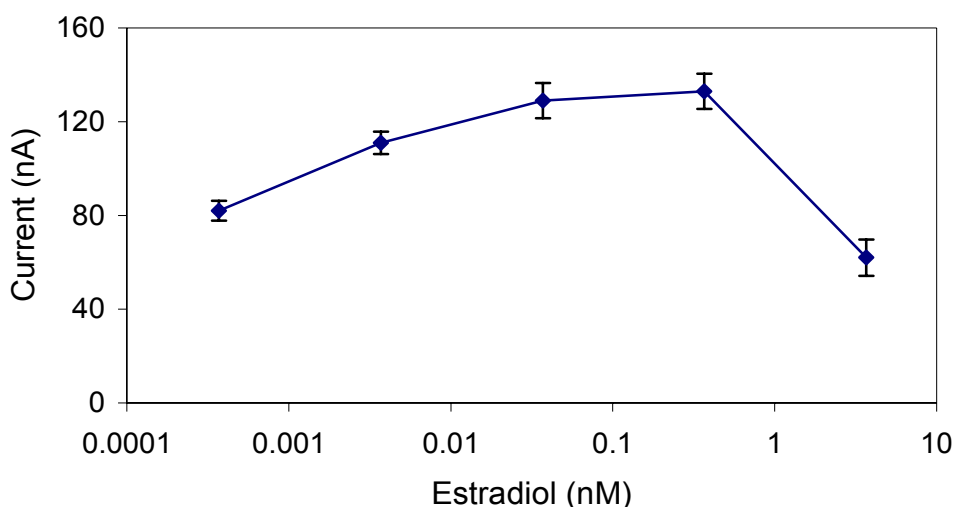
Figure 3.53 Logarithmically related *S. cerevisiae* response to 0.00037 - 3.67 nM 17β-estradiol using glucose (0.1 mM) as catabolic blocking agent. The endogenous response (0 nM) was subtracted from these responses.

The results in Figure 3.53 demonstrate that 0.367 nM 17β-estradiol in the presence of 0.1 mM glucose has shown the maximum response above which concentration the response dropped, confirming that the concentration that was used in blocking experiments with hER *S. cerevisiae* (0.367 nM) was the concentration resulting in maximum response. Glucose 0.1 mM and 17β-estradiol 0.367 nM was used for blocking experiments with wild type *S. cerevisiae* (Figures 3.54 & 3.55)*.*
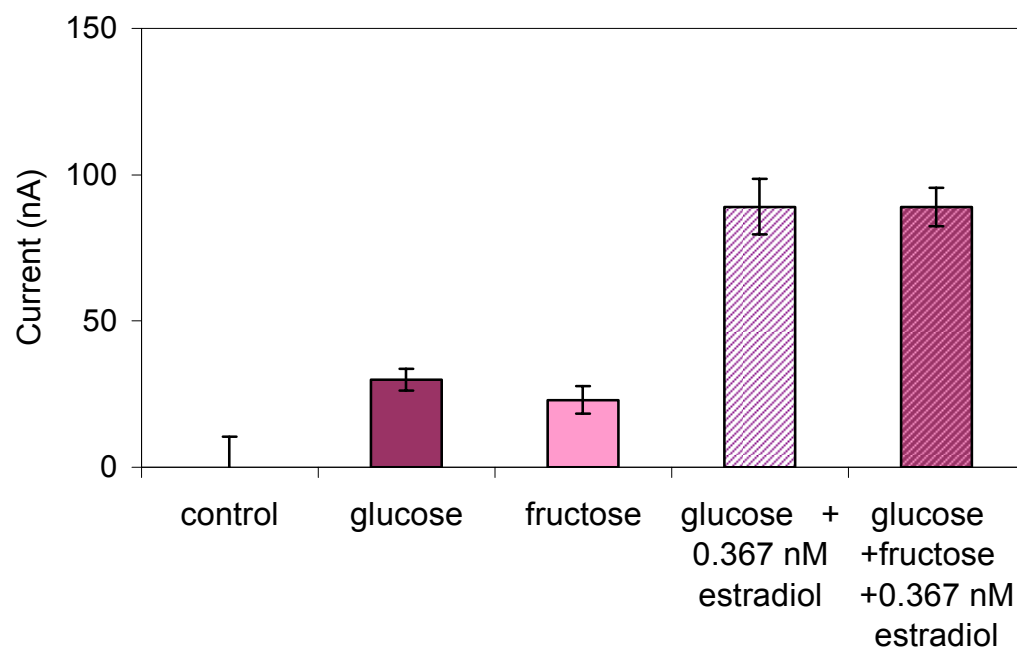
Figure 3.54 *S. cerevisiae* responses to 0.1 mM glucose, 0.1 mM fructose, 0.1 mM glucose and 0.367 nM 17β-estradiol and 0.1 mM glucose, 0.1 mM fructose and 0.367 nM 17β-estradiol. The endogenous response (control) was subtracted from these responses.
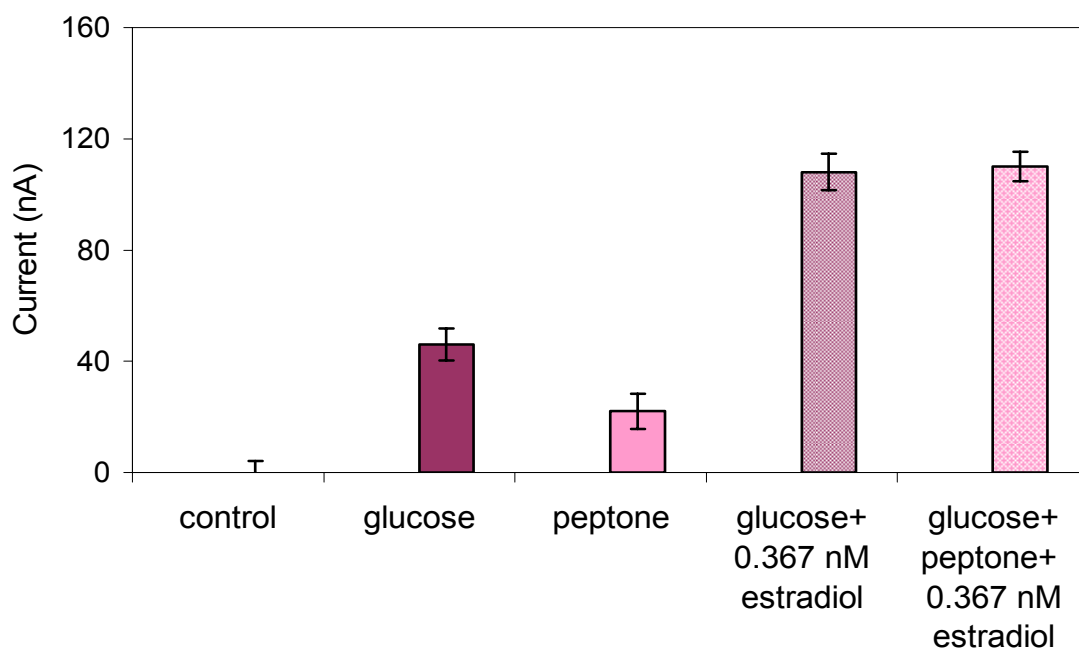
Figure 3.55 *S. cerevisiae* responses to 0.1 mM glucose, 0.018% peptone, 0.1 mM glucose and 0.367 nM 17β-estradiol and 0.1 mM glucose, 0.018% peptone and 0.367 nM 17β-estradiol. The endogenous response (control) was subtracted from these responses.

P-values were obtained by performing ANOVA analysis using raw data from Figures 3.54 and 3.55 (Table 3.17).

Table 3.17 ANOVA Single Factor Analysis of *S. cerevisiae* responses to 0.367 nM estradiol with glucose, glucose + fructose and glucose + peptone

Substrate groups	P-value
Glucose + estradiol - glucose + fructose + estradiol	9.09×10^{-1}
Glucose + estradiol - glucose + peptone + estradiol	1.72×10^{-1}
Glucose - glucose +estradiol	3.84×10^{-10}

Results in Figures 3.54 and 3.55 and Table 3.17 demonstrate that the responses to 0.1 mM glucose and 0.367 nM 17β-estradiol were not significantly different statistically from the response to 0.1 mM glucose, 0.1 mM fructose and 0.367 nM 17β-estradiol ($p > 0.05$) and 0.1 mM glucose 0.018% peptone and 0.367 nM 17β-estradiol response ($p > 0.05$). Glucose efficiently blocked the catabolic responses of the simple sugar fructose and complex mixture peptone, which were similar to the results that were obtained with hER *S. cerevisiae*.

3.6.2 Investigation of the effect of incubation time on *S. cerevisiae* responses to 17β-estradiol

Although 8 hours of incubation time was used to detect 17β-estradiol responses in wild type *S. cerevisiae*, it is important to identify the minimum incubation time that is required to detect the responses to 17β-estradiol. Wild type *S. cerevisiae* is devoid of human estrogen receptor (hER) and may take less time to respond to 17β-estradiol which would reduce the assay time. The minimum incubation time that is required to detect the wild type *S. cerevisiae* responses to 17β-estradiol was investigated (Figure 3.56).
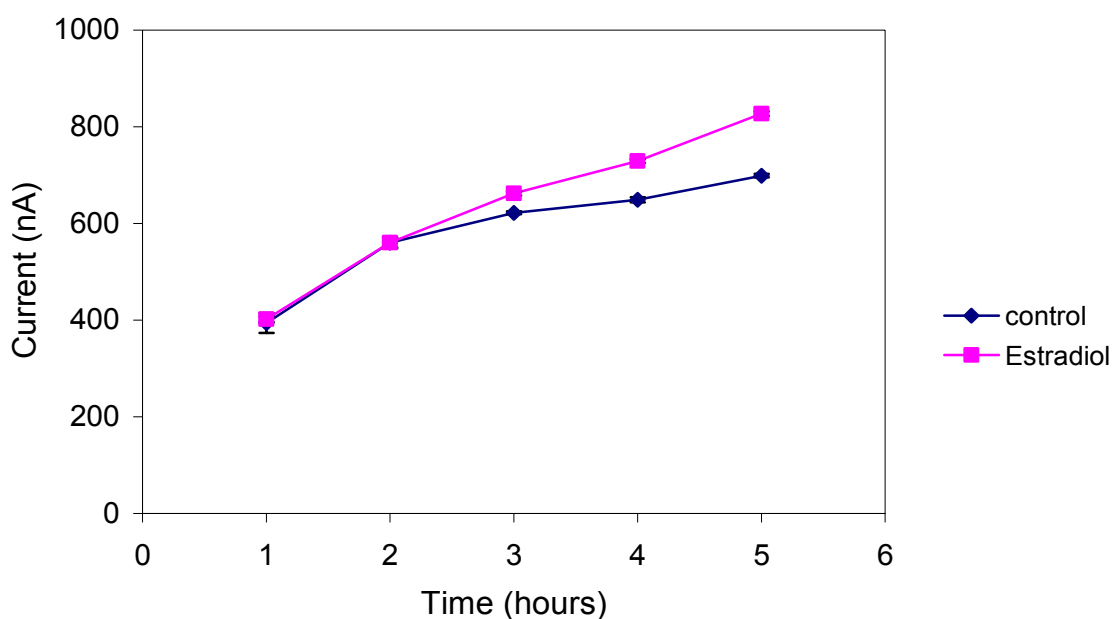
Figure 3.56 *S. cerevisiae* time- dependent (1-5 hours) responses to 17β-estradiol

P-values were obtained by performing ANOVA analysis using raw data from Figure 3.56 (Table 3.18).

Table 3.18 ANOVA Single Factor Analysis summarising *S. cerevisiae* time dependent responses with and without 17β-estradiol.

Time	P-value
1 hour	9.39×10^{-2}
2 hours	6.06×10^{-1}
3 hours	5.16×10^{-9}
4 hours	4.45×10^{-11}
5 hours	1.84×10^{-13}

P-values for different time points in Table 3.18 show that the two-hour responses of *S. cerevisiae* with and without 17β-estradiol are not significantly different statistically to control responses. The difference between the control and 17β-estradiol starts showing from three hours and the difference becomes more prominent between the control and 17β-estradiol as the time increases. Although the one-hour p-value < 0.05 shows that the control and 17β-estradiol responses are statistically different, it is unlikely that the one hour response is real because the two hour p-value > 0.05 shows the control and 17β-estradiol responses are statistically similar. A significant difference between the two responses is not detected until the three hour incubation.

3.6.3 The non-interference of phenol with 17ß-estradiol's response

Estrogen binding protein has a weak binding affinity to phenol and other phenolic compounds compared to very high affinity of old yellow enzyme. It is very important to check the effect of phenol on the response of 17β-estradiol as phenol and phenolic compounds are known environmental contaminants. They also compete for the binding site on the estrogen binding protein. Phenol was used at very higher concentrations than 17β-estradiol (Figure 3.57).
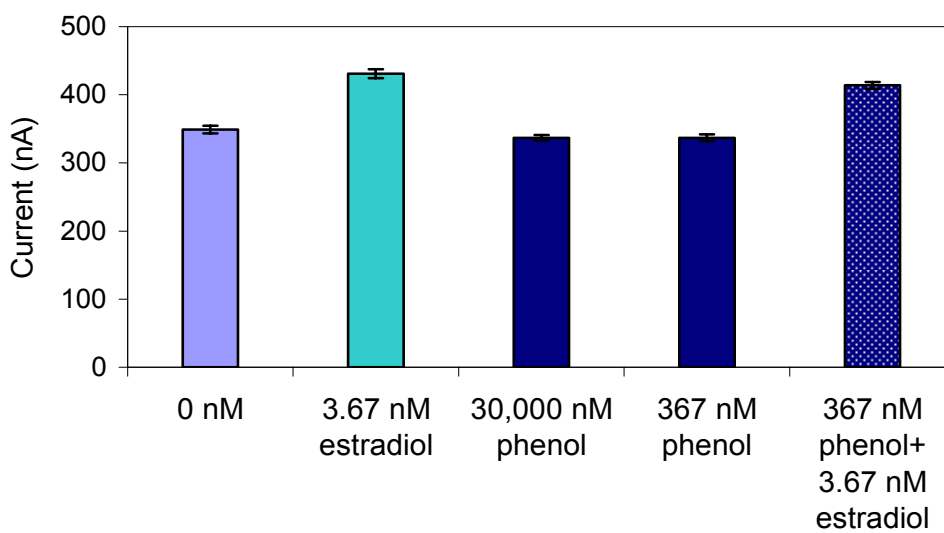
Figure 3.57 *S. cerevisiae* response to phenol and phenol plus 17β-estradiol.

The results in Figure 3.57 show that the phenol responses at both the concentrations used were less than the endogenous response (338 nA vs 349 nA) and the p-value of < 0.05 suggesting that the difference was significantly different statistically and phenol was inhibitory. The same inhibitory effect was observed in 367 nM phenol + 3.67 nM 17β-estradiol resulting in reduced response compared to the 17β-estradiol 3.67 nM response alone (415 nA vs 431 nA) and p-value of < 0.05. This phenol inhibitory effect was relative to the endogenous response and 17ß-estradiol response was still detectable in the presence of very high concentrations of phenol suggesting that the inhibitory effect of phenol is not interfering greatly with the 17β-estradiol response.

3.6.4 Environmental samples: whole-cell estrogen bioassay: selection of dilutions for environmental sample analysis

Owing to the constraint of number of tubes that can be used in each experiment and the system limitation of responses dropping above 11 nM estradiol concentration in samples, it was decided to use different dilutions such as 1:7, 1:70 and 1:700 for environmental samples.

Environmental sample 1 - Trickling Filters effluent from Wastewater Treatment Plant (WTP), Christchurch

Trickling Filters effluent was supplied by the staff of WTP, Christchurch. As the total estrogenic compounds in samples were unknown, the samples were diluted to 1: 7, 1:70 and 1:700 to get the readings within the detection range (Figure 3.58a).
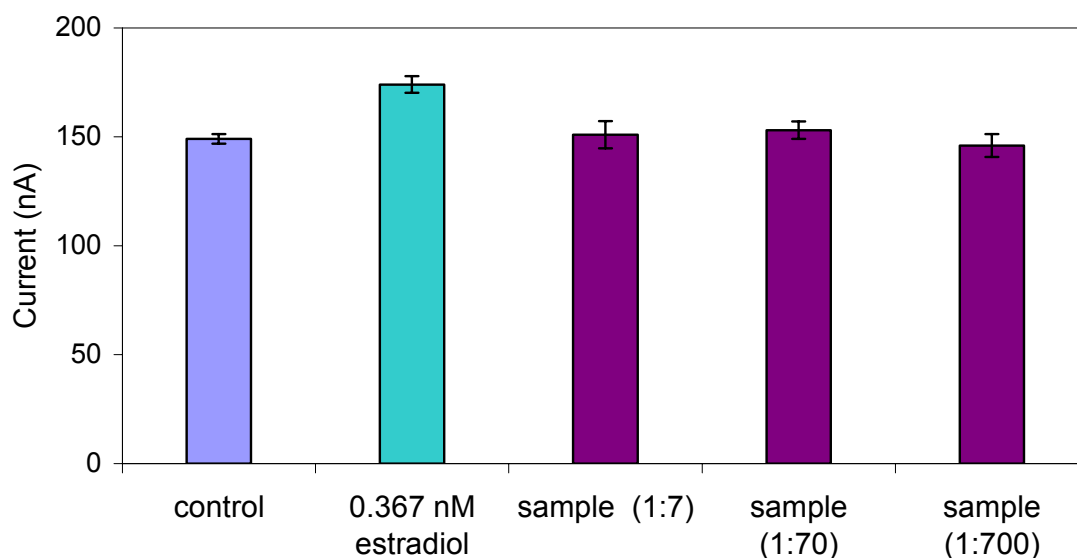
Figure 3.58a Double mediated electrochemical responses of estrogenic molecules in Trickling Filters effluent (sample): *S. cerevisiae* based whole-cell estrogen bioassay.

The results in Figure 3.58a show that there were no estrogenic compounds detected with this estrogen bioassay in Trickling Filters effluent. The estrogen bioassay method was verified by spiking the Trickling Filters effluent (1:7) with 0.367 nM 17β-estradiol (Figure 3.58b).
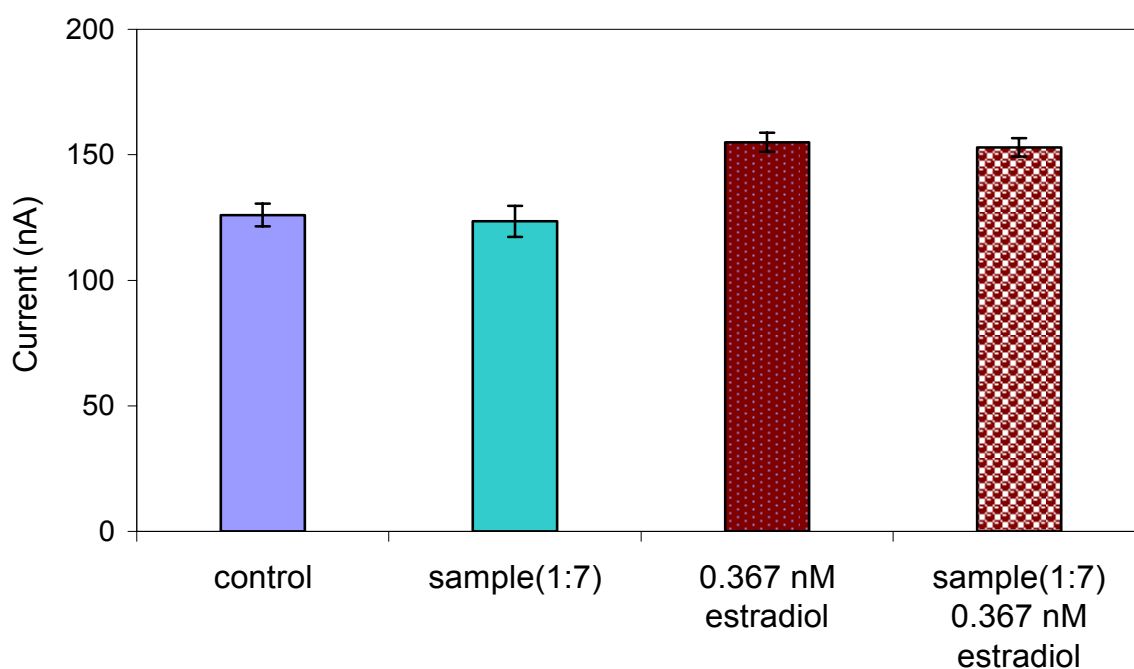
Figure 3.58b Spiked 17β-estradiol response in Trickling Filters effluent (sample)

The results in Figure 3.58b confirmed that the method was able to detect 0.367 nM 17β-estradiol in effluent. $P > 0.05$ of 0.367 nM 17ß-estradiol and spiked effluent sample with 0.367 nM 17ß-estradiol show that they are not significantly different statistically and confirmed the previous result (Figure 3.58a) of not detecting any estrogenic compounds in the effluent. It was decided to use influent from Trickling Filters to detect estrogenic compounds.

Environmental sample 2 –Trickling Filters influent from WTP, Christchurch

Trickling Filters influent is expected to have some estrogenic compounds and 1:70 and 1:700 dilution were used for analysis. Glucose 0.1mM was used to block the catabolism of all other catabolisable molecules in the sample (Figure 3.59).
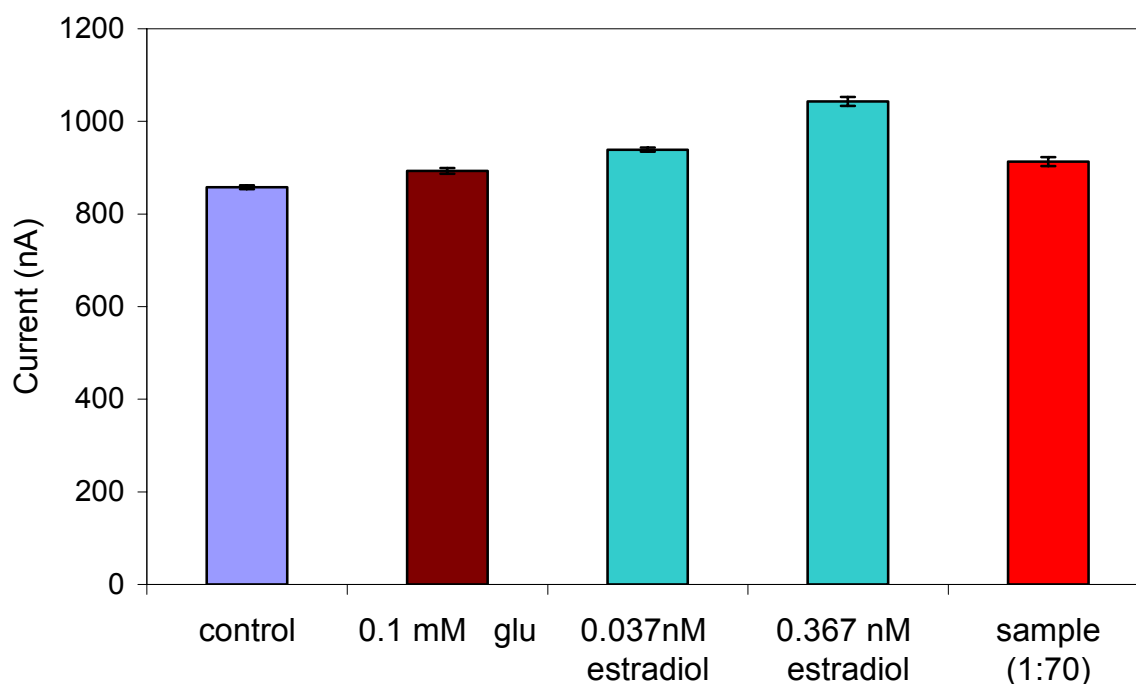
Figure 3.59 Double mediated electrochemical responses of estrogenic molecules in influent (sample) from Trickling Filters (WTP): *S. cerevisiae* based whole-cell estrogen bioassay.

The results in Figure 3.59 show that estrogenic compounds were present in influent. The ability of influent to reduce TMPD was checked, the values are insignificant (>1 nA). Estrogenic responses were detected in influent in both the dilutions, 1:70 appears to be working and this was used for calculations. The reasons for choosing this dilution will be discussed shortly.

Table 3.19 ANOVA Single Factor Analysis of influent sample from Trickling Filters (WTP).

Substrate groups	P-value
Control - 0.1 mM glucose	3.7×10^{-8}
0.1 mM glucose - 0.037 nM 17β-estradiol	$4{,}98 \times 10^{-12}$
0.1 mM glucose - 0.367 nM 17β-estradiol	3.1×10^{-17}
0.037 nM - 0.367 nM 17β-estradiol	2.76×10^{-15}
0.1 mM glucose - Sample (1:70)	3.58×10^{-5}

Dose response data for 17β-estradiol

The means of the determinations for the 0.037 and 0.367 nM estradiol responses in Figure 3.59 were used to calculate a dose response function. Assuming the linear response as in, for example, figure 3.46a & b, the following function was obtained

$y = 45.085Ln(x) + 195.19$ which will be used to quantify the total estrogenic compounds in the environmental sample. This aspect will be discussed in the latter part of this section.

3.6.5 Quantitation of glucose in Trickling Filters influent from WTP (Christchurch)

Because the added glucose blocks responses to all other catabolisable molecules, it was reasoned that any contribution to the estrogen signal could only come from glucose present in the sample. To eliminate the glucose contribution to the estrogen responses, the glucose in the environmental sample will need either to be removed prior to the estrogen quantitation or quantified and the result used to correct the total estrogen and sample glucose response. In these studies, the glucose in environmental sample is quantified and used to correct the estrogen responses. The response due to the presence of glucose in Trickling Filters influent (data from Figure 3.59) and has been estimated using the glucose oxidase enzymatic activity and electrochemical technique (Figure 3.60) described in Chapter 2

.
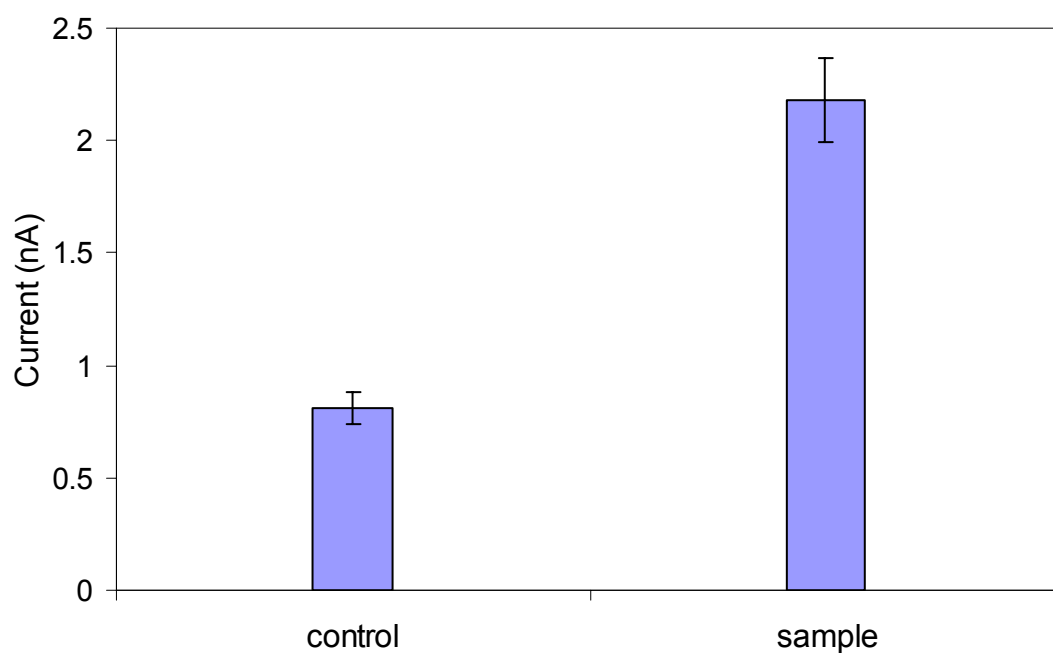
Figure 3.60 Glucose dependent electrochemical responses in Trickling Filters influent (1:5 dilution).

The results in Figure 3.60 show the electrochemical response obtained in influent is due to the presence of glucose, which has been used to calculate the concentration of glucose in influent to correct the estrogenic responses in influent, which will be discussed in the method of calculating the estrogenic compounds in influent (3.6.5).

An experiment to obtain a glucose calibration curve using the glucose oxidase enzyme was performed to calculate glucose concentration in environmental samples (Figure 3.61).
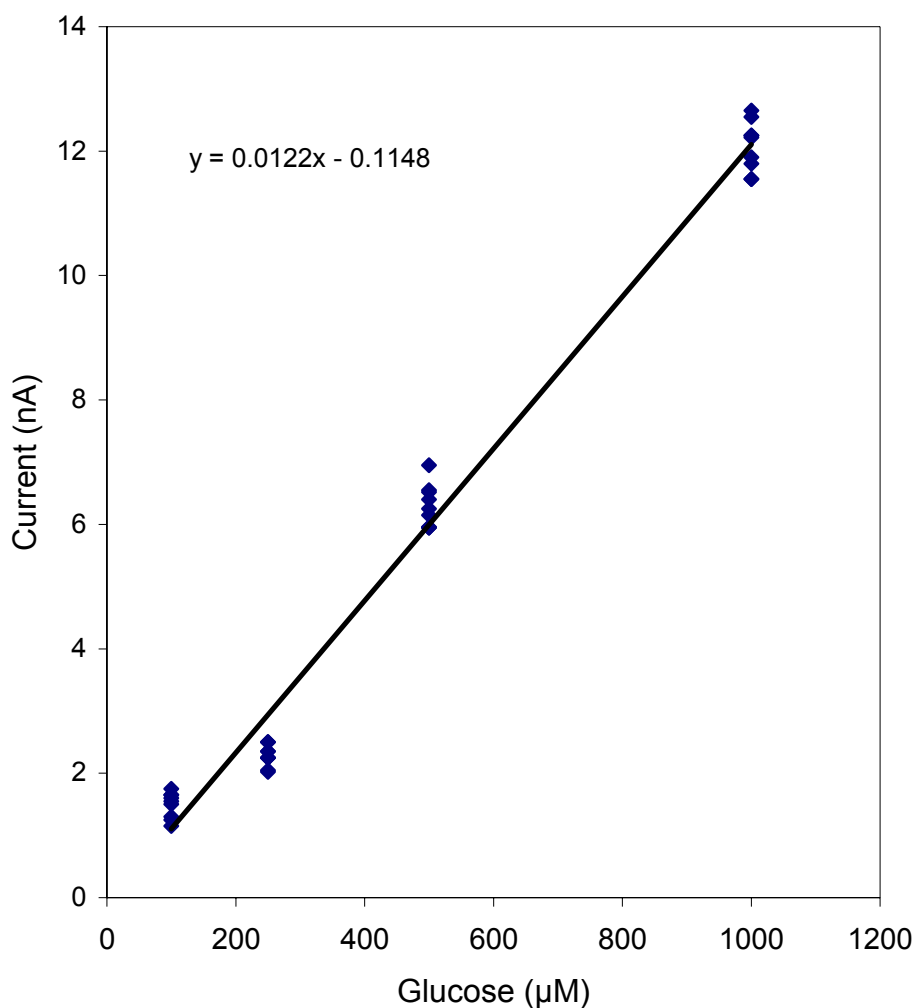
Figure 3.61 Glucose standard calibration curve using Glucose oxidase enzyme (GOX).

The results presented in Figure 3.61 show that there is a linear glucose response range for calibrating glucose in environmental samples (Daniel, 2003 & Skoog & Nieman, 2004).

It is important to calculate the response of *S. cerevisiae* to 0.1 mM glucose and subtract that value from the responses to environmental samples (correction factor) to quantitate total estrogenic compounds in the samples. The response of 0.1 mM glucose was calculated from the data presented in Figure 3.60a. The control endogenous response was subtracted from 0.1 mM glucose response and the correction factor was estimated as 0.34 by averaging the data of 0.1 mM glucose. This correction factor was used to calculate the response due to glucose that is present in the influent. Although single

point glucose calibration is not ideal, it is acceptable in BOD (biological oxygen demand) measurements (Dr. Bruno Lange commercial BOD sensors, Germany).

3.6.6 Method used for the calculation of estrogenic compounds in Trickling Filters influent from WTP

I. Calculation of glucose concentration in the Trickling Filters influent sample: The glucose in the influent was calculated using glucose linear response with glucose oxidase enzyme (line of slope) described in Chapter 3 and the glucose in the samples was calculated by the following method:

$y = 0.0122x – 0.1148$ (from Figure 3.61 glucose calibration curve)

$y = 1.37$ nA (response from glucose in the sewage sample in 1:5 dilution (see Figure 3.63)

$0.0122x = 1.37 + 0.1148$

x (glucose concentration in influent) $= 1.37 + 0. 1148 / 0.0122 = 121.70 \mu M$

$121.70 \mu M \times 5$ (dilution factor) $= 608.52 \mu M$

Glucose concentration in Trickling Filters influent $= 608.52 \mu M$

Glucose concentration in Trickling Filters influent (1:70) $= 608.52 / 70 = 8.69 \mu M$

II. Calculation of the response that is produced due to the presence of 8.69 μM glucose that is present in the Trickling Filters influent sample (1:70)

$y = 0.34 \ x$ (calculated by using the data from Figure 3.61a by subtracting 0.1 mM glucose response from endogenous response). Although the single point glucose calibration is not ideal it has been used in BOD sensors in Germany (Dr. Bruno Lange commercial BOD sensors)

$y = 0.34 \times 8.69 = 2.95$ nA

III. Calculation of the total estrogenic molecules (EEQ) in Trickling Filters influent from Christchurch (WTP)

$y =$ sample response (1:70) – (control response + response due to endogenous glucose).

$y = 913 - (892 + 2.955) = 18.05$ nA

$y = 45.085 \ln (x) + 195.19$ (equation obtained from 17ß-estradiol dose response data shown in Figure 3.59).

$18.05 = 45.085 \text{Ln} (x) + 195.19$

Ln (x) = 18.05 – 195.19 / 45.0851 = -3.92790

x = 0.01968 nM x 70 (1:70 dilution) = 1.37 nM

The total estrogenicity of estrogenic compounds expressed as EEQ - estradiol equivalent concentration (Korner et al., 2001) = 1.37 x 272 (molecular weight of 17β-estradiol) = 375 ng/L.

This system detected total estrogenic compounds 375 ng/L in the Trickling Filters influent which seems to be within the range of the reported value of 385 ng/L in influent of Wastewater Treatment Plant (Table 4.3) from Netherlands using ER-binding assay (Murk et al., 2002). It is well known fact that the total estrogenicity of estrogenic compounds vary according to the bioassay method used (Murk et al., 2002), location, population and the number of people using the contraceptive pills and hormone therapy (Leusch et al., 2006) and thus the value obtained by this estrogen bioassay using 1:70 dilution is likely to be correct.

Precision of the estrogen bioassay

These measurements were made based on the estimated equation may have associated with the measurement errors that were obtained from the linear regression which accumulate in final calculations, especially when there is not a good fit. This error can have a significant effect on the precision of final calculations although it will not affect the actual estimates. It is not uncommon for certain dilutions not to working in bioassays depending on the assay limitations. In this study, it has been demonstrated that above 11 nM 17β-estradiol, the responses dropped dramatically, so 1:7 dilution could be very low and also this response is logarithmically related (the response is not 10 times more or less in relation to the dilution factor) and when the response was substituted in the formula and multiplied with the appropriate dilution factor the total estrogenic compounds were either lower (1:7) or higher (1:700) than the dilution (1:70) that has always appeared to be working in this bioassay.

Validity of the estrogen bioassay

The Trickling Filters influent was stored at -20ºC and analysed five times for total estrogenic compounds at different times during storage to check the reproducibility of the assay system and to check if there was any loss of estrogenic activity over time during storage as reported by Murk et al. (2002) to validate this bioassay method. The experiments were performed in exactly the same way each time as described earlier

using appropriate controls and the total estradiol equivalent concentration of different estrogenic compounds in Trickling Filters influent was calculated (Figure 3.62).
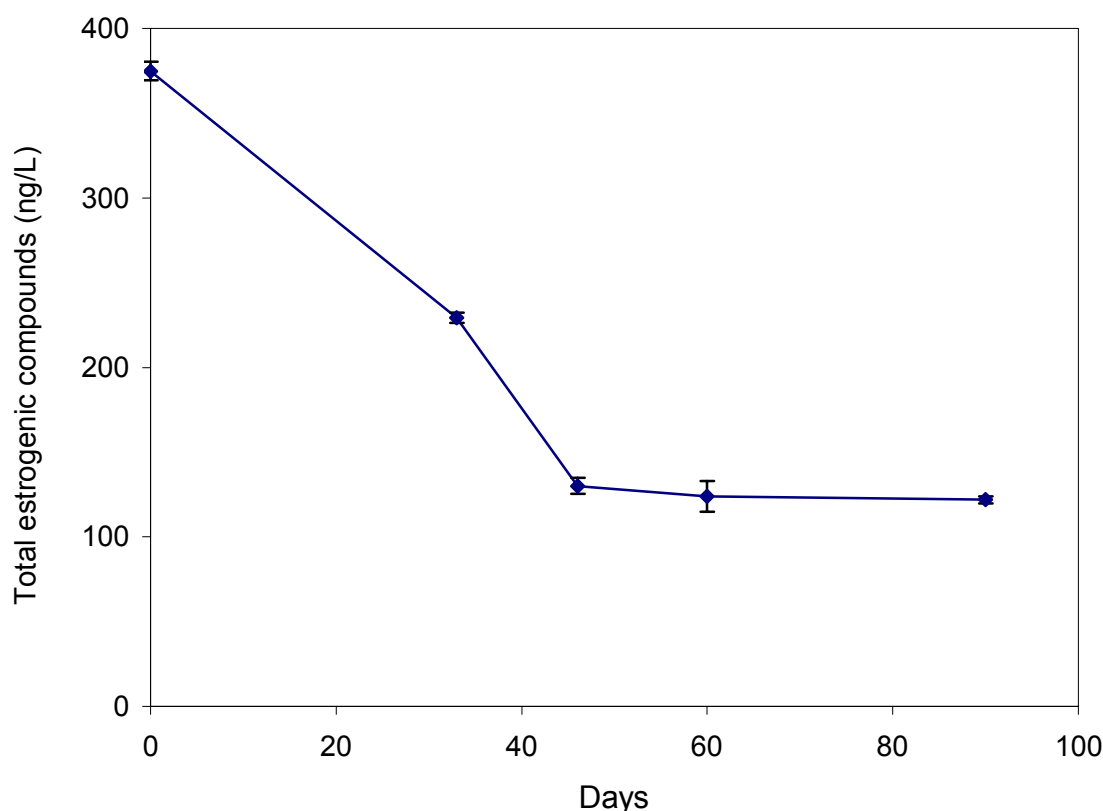
Figure 3.62 The loss of estrogenic activity in Trickling Filters influent (1:70 dilution) during storage over period.

The results presented in Figure 3.62 show that the estrogen responses in Trickling Filters influent dropped steadily over time for up to 46 days and then stabilised. This could be due to the loss of estrogenic activity of estrogenic compounds over a period during storage, even at $-20^{\circ}C$. This observation is consistent with the results of Murk et al. (2002). To check the robustness of the assay method different environmental samples were tested and quantitated the total estrogenic compounds.

Environmental sample 3 – Avon River Water
Avon River water in Christchurch was tested for total estrogenic compounds. It was not expected to have estrogenic compounds in this river water as there are no diaries,

131

agricultural or sewage effluents are mixing. Hence, lower dilutions were used (Figure 3.63).
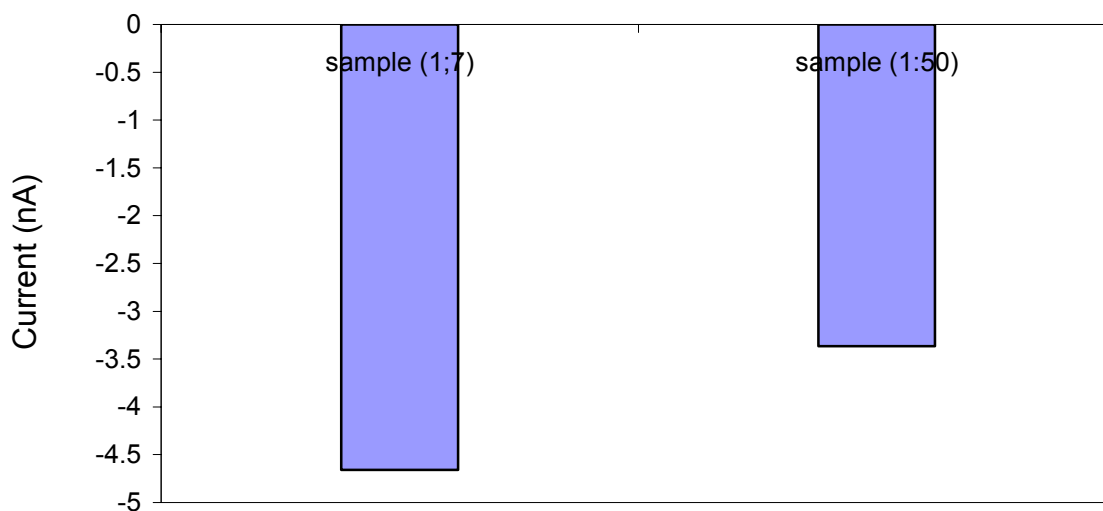
Figure 3.63 Double mediated electrochemical responses of estrogenic molecules in Avon River water (sample): *S. cerevisiae* based estrogen whole-cell bioassay.

The results in Figure 3.63 show that there were no estrogenic compounds detected through this system in the Avon River water. Glucose was quantified using the same method described earlier and was found to be 365 μM glucose in Avon River water.

The two rivers, Halswell River (Site A) Christchurch and Tai Tapu River, Canterbury regions that flow through dairy farmland were also analysed. It was suspected that these rivers would contain estrogens because of the presence of cows.

Environmental Sample 4 – Halswell River water

Halswell River water (1:70 dilution) was used for quantifying the estrogenic compounds (Figure 3.64).
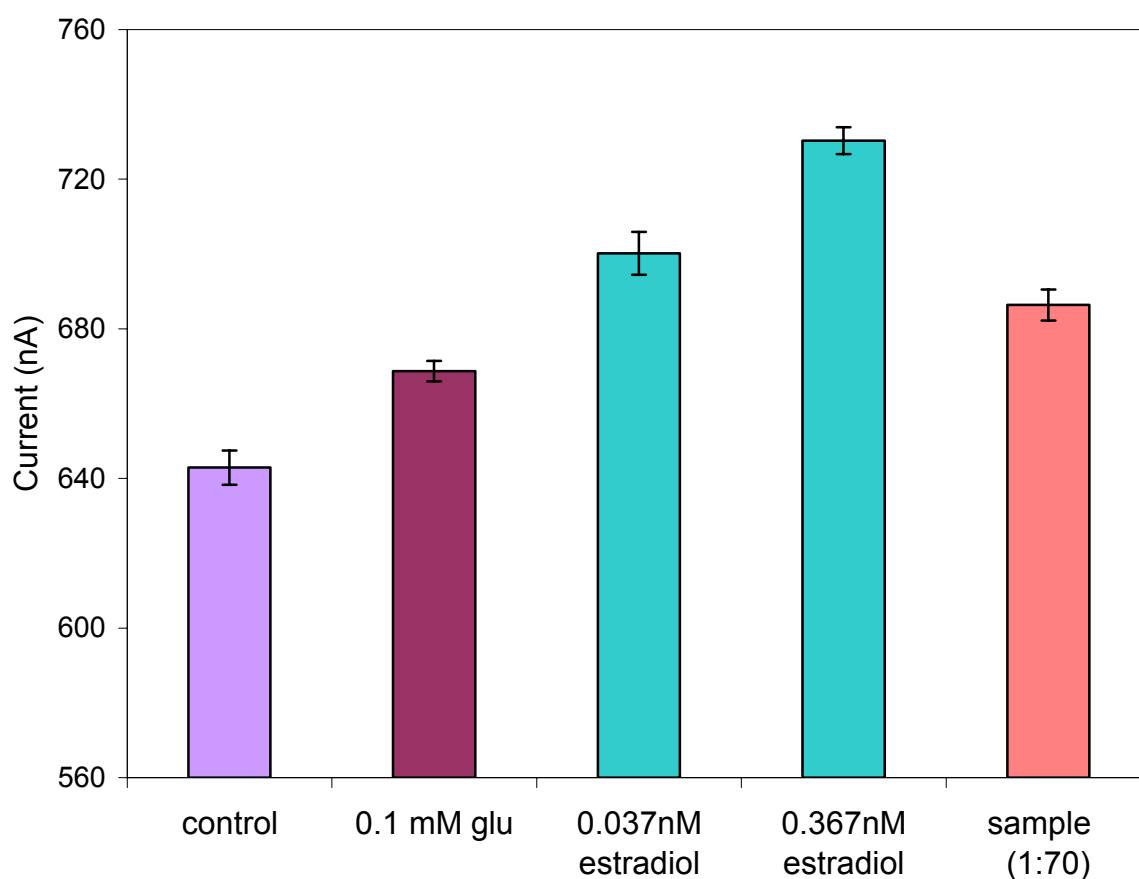
Figure 3.64 Double mediated electrochemical responses of estrogenic molecules in Halswell River (Christchurch) water (sample). *S. cerevisiae* based estrogen whole-cell bioassay.

The results in Figure 3.64 show the estrogenic responses in Halswell River water. Using the data presented in Figure 3.64 *S. cerevisiae* responses to 17β-estradiol have been used to calibrate estrogenic compounds in the sample.

Table 3.20 ANOVA Single Factor Analysis of Halswell River water (sample)

Substrate groups	P-value
Control- 0.1 mM glucose	1.33×10^{-10}
0.1 mM glucose - 0.037 nM 17β-estradiol	2.21×10^{-9}
0.1 mM glucose - 0.367 nM 17β-estradiol	1.3×10^{-17}
0.037 nM - 0.367 nM 17β-estradiol	1.12×10^{-8}
0.1 mM glucose - Sample (1:70)	1.19×10^{-8}

3.6.7 Calculation of the total estrogenic compounds in Halswell river water

The glucose concentration was 204 μM and the response due to the 2.91 μM glucose in 1:70 dilution was 0.758 nA. These were measured using the same method described earlier in section 3.6.6. The total estrogenic compounds were calculated using the 17β-estradiol dose response data and the equation obtained from the data presented in Figure 3.64. The total estrogenicity of the different estrogenic compounds in Halswell River water expressed as equivalent to 17β-estradiol concentration (EEQ) is 207 ng/L.

Environmental Sample 5 – Tai Tapu River water

Tai Tapu River water (Canterbury region) was tested for total estrogenicity of different estrogenic compounds using 1:70 dilutions (Figure 3.65).
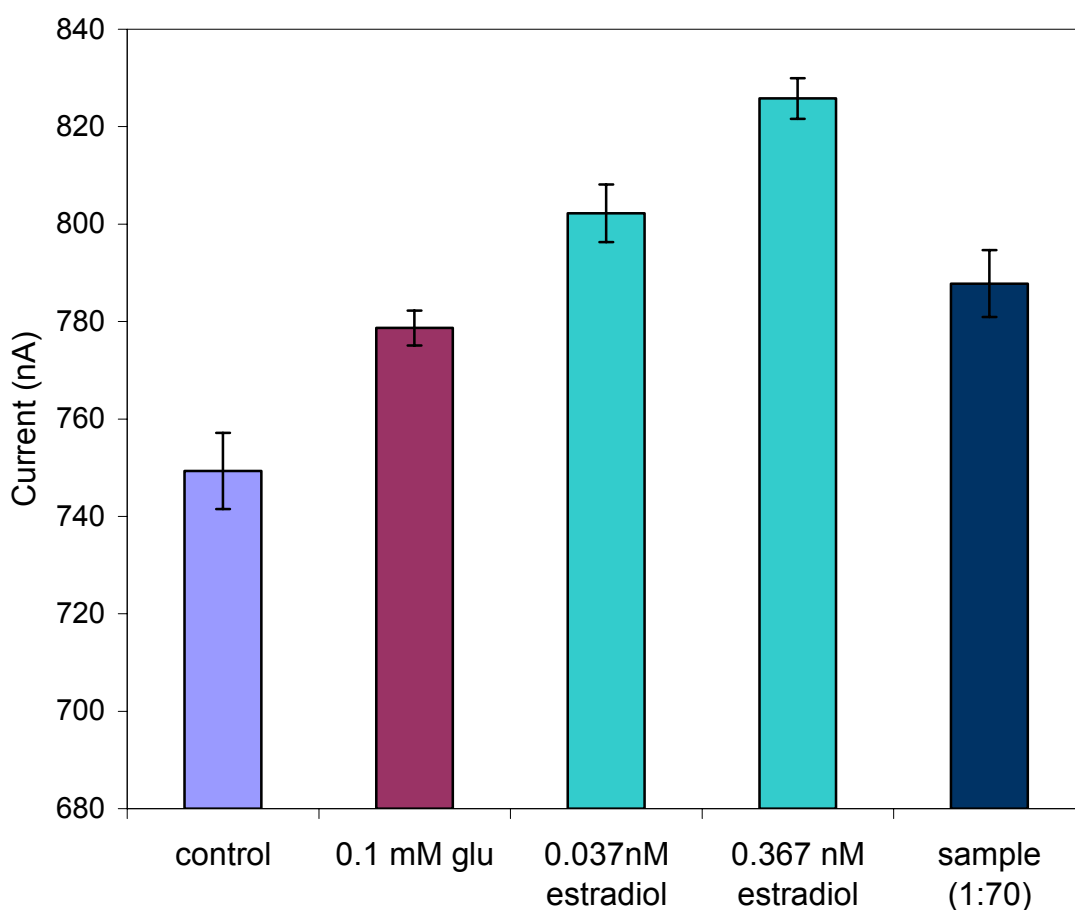
Figure 3.65 Double mediated electrochemical responses of estrogenic molecules in Tai Tapu River (Canterbury) water (sample). *S. cerevisiae* based estrogen whole-cell bioassay.

The results presented in Figure 3.65a show that the estradiol dose dependent response and estrogenic responses in both dilutions of Tai Tapu River water. *S. cerevisiae* responses to 17β-estradiol have been plotted for calibrating estrogenic compounds in the sample (Figure 3.65).

Table 3.21 ANOVA Single Factor Analysis of Tai Tapu River water (sample)

Substrate groups	P-value
Control- 0.1 mM glucose	7.93×10^{-6}
0.1 mM glucose - 0.037 nM 17β-estradiol	8.51×10^{-7}
0.1 mM glucose - 0.367 nM 17β-estradiol	7.96×10^{-12}
0.037 nM - 0.367 nM 17β-estradiol	3.71×10^{-8}
0.1 mM glucose - Sample (1:70)	2.5×10^{-3}

3.6.8 Calculation of total estrogenic compounds in Tai Tapu River water

Glucose concentration was 297 μM and the response due to the glucose in 1:70 dilution measured using the method described earlier in section 3.6.6 were 4.24 μM and 1.244 nA respectively. The total estrogenic compounds were calculated by 17β-estradiol dose response data using the data presented in Figure 3.65 and obtained the equation. Total estrogenicity of estrogenic compounds in Tai Tapu River water expressed as equivalent to 17β- estradiol concentration (EEQ) is 29 ng/L

All these results, especially the results presented in Figure 3.62, suggest that the estrogen whole-cell bioassay developed using wild type *S. cerevisiae* is valid in quantifying estrogenic compounds in environmental samples. These environmental samples have not been tested by standard instrumental analysis such as GC-MS analysis to make the valid comparisons of the estrogen bioassay and check for the accuracy because of cost (each sample quoted $2,500 by the Cawthron Institute, Nelson) and in any case it has been reported that the instrumental analysis such GC-MS results in over estimating the values by a factor of 2 to 4 higher than the values obtained by bioassays (Korner et al, 2001).

CHAPTER 4

DISCUSSION

4.0 Molecular characterisation of *A. adeninivorans*: Identification of self-splicing intron of 25S rDNA by Polymerase Chain Reaction (PCR)

A. adeninivorans LS3 strain was provided by Professor G. Kunze, Glatersleben, Germany. The restriction map of the 25S rDNA localising self-splicing intron is schematically represented below:
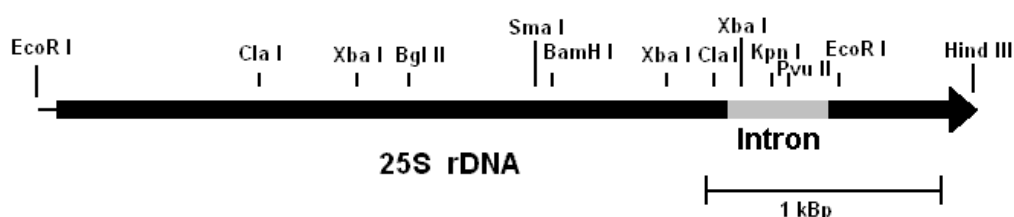
Figure 4.1 Restriction map of 25 S rDNA representing the 410 bp intron.
(From - Rosel & Kunze 1996).

The results presented in Figure 3.1 show that the Polymerase Chain Reaction has amplified the desired DNA fragments. The controls (negative) for the two sets of primers, Int-I and Int-II, where the DNA sample was absent, did not amplify any fragments (well.nos.1 & 2). The two PCR samples consisted of Int-II primers (well.nos. 5 & 6) were amplified the 600 bp desired fragment harbouring the unique intron of 410 bp. The two PCR samples consisting of Int-I primer (wells 7 and 8), amplified the desired longer fragment of 3719 bp fragment only in well no.8. The reason for not amplifying the 3719 bp fragment in well number 7, could be owing to the insufficient concentration of DNA. These results confirm the authenticity of the *A. adeninivorans* strain that was obtained from Professor Kunze, Germany and was used as a biocomponent in the biosensor.

4.1 Effect of nitrogen sparging on the efficiency of mediator oxidation of cellular redox molecules

Oxygen competes with the mediators for cell redox compounds, and its presence decreases the mediator oxidation efficiency of cellular redox molecules resulting in smaller responses compared to nitrogen sparging, which efficiently removes oxygen. Figure 3.3 shows that in the non-sparged samples the responses were approximately

47% - 81% smaller than the sparged samples (Table 3.1). These results confirm that nitrogen sparging and one-hour incubation gives sufficient response range for analysis and hence, it was decided to use one-hour incubation with nitrogen sparging. However, low oxygen or oxygen deprivation during nitrogen sparging shifts the yeast from respiratory mode to fermentative mode.

4.2 Mediator interactions to detect yeast catabolic responses: Mediated electrochemical detection

Mediated electrochemical detection of catabolism in bacteria can be performed with single hydrophilic mediator such as potassium ferricyanide (ferricyanide) or with a double mediator system comprising a lipophilic and hydrophilic mediator. In contrast, yeast catabolism can be best monitored by using double mediator systems (Baronian et al., 2002 & Zhao et al., 2005).

The results in Figures 3.4 to 3.7 shows that in all the four yeast species tested, substrate dependent catabolic responses (electrochemical responses) were observed with both single and double mediator systems, both in the presence and absence of glucose. Using the double mediator system comprising ferricyanide as the hydrophilic mediator and menadione as the lipophilic mediator, the response was 24-36 times greater for all three yeast except for *R. rubra* where the response was approximately 15 times greater than it was with the single mediator ferricyanide (Table 3.2). The double mediator system comprising ferricyanide and TMPD as the lipophilic mediator resulted in 38-80 times greater responses than those from the single mediator (ferricyanide) in all three yeast with the exception of *R. rubra* where the response was approximately 16 times larger. In *T. cutaneum,* all responses were smaller than those detected in the other three yeast species were. This may be attributed to the relatively slow growth rate of this organism compared to those of the other three yeast tested.

In *A. adeninivorans* and *T. cutaneum,* the use of a ferricyanide + TMPD double mediator system resulted in a bigger response than with the ferricyanide + menadione double mediator system both with and without glucose. In contrast, the two organisms *S. cerevisiae* and *R. rubra* using ferricyanide + menadione + glucose resulted in a bigger response than ferricyanide + TMPD without glucose, suggesting not only some similarities but also some differences exist among these species. These are unrelated to the classification (phylum) of these yeast belong to, as *A. adeninivorans* and

R. rubra belong to Basidiomycota and *S. cerevisiae* and *T. cutaneum* belong to Ascomycota.

It is known that ~ 4% of TMPD supplied is in the reduced form and is oxidised by ferricyanide and contributes to the overall responses (Baronian et al., 2002) but this does not completely explain the differences seen between the menadione and TMPD double mediator responses in all the four yeast species tested. These results show that without glucose the ferricyanide/TMPD double mediator system gives overall a bigger response than the ferricyanide/menadione double mediator system in all the four yeast species tested. In all of these experiments, lipophilic mediators were not used alone because their low aqueous solubility limits the transfer of electrons from cell to electrode and this will affect the magnitude of the response, i.e. a lower dynamic range is available for measurement.

Use of the binary mixtures comprising of the hydrophilic mediator ferricyanide and lipophilic mediators TMPD and PES (phenazine ethosulphate) as a secondary mediator augmented the substrate conversion efficiency in the prokaryote *Escherichia coli* in a concentration dependent manner (Pasco et al., 2005). They have shown in *E. coli*, using the double mediator system of ferricyanide and TMPD or ferricyanide and PES, that the substrate conversion efficiency increased. The substrate conversion increased from 23% - 43% for the double mediator system comprising of ferricyanide + TMPD and 33% - 43% for the ferricyanide + PES double mediator system respectively (Pasco et al., 2005). These are several fold smaller compared to yeast (Table 3.3). In contrast, the other lipophilic mediators: menadione, benzoquinone, N, N-tetramethyl phenylenediamine (N-TMPD) show that an increase in the concentration of these mediators decreased the substrate conversion efficiency. These results show that while in bacteria, the use of a single hydrophilic mediator produces responses of useful magnitude and the addition of second mediator in particular TMPD, enhances the percentage of substrate conversion efficiency, this is in contrast the situation in yeast where the double mediator response is several fold higher than the single mediator response (Table 3.3).

4.2.1 Interaction of the mediators with prokaryotes and eukaryotic cells
The mechanism of single mediator interaction in prokaryotes is schematically described in the next page.

Proposed mechanism of single hydrophilic mediator interactions in prokaryote
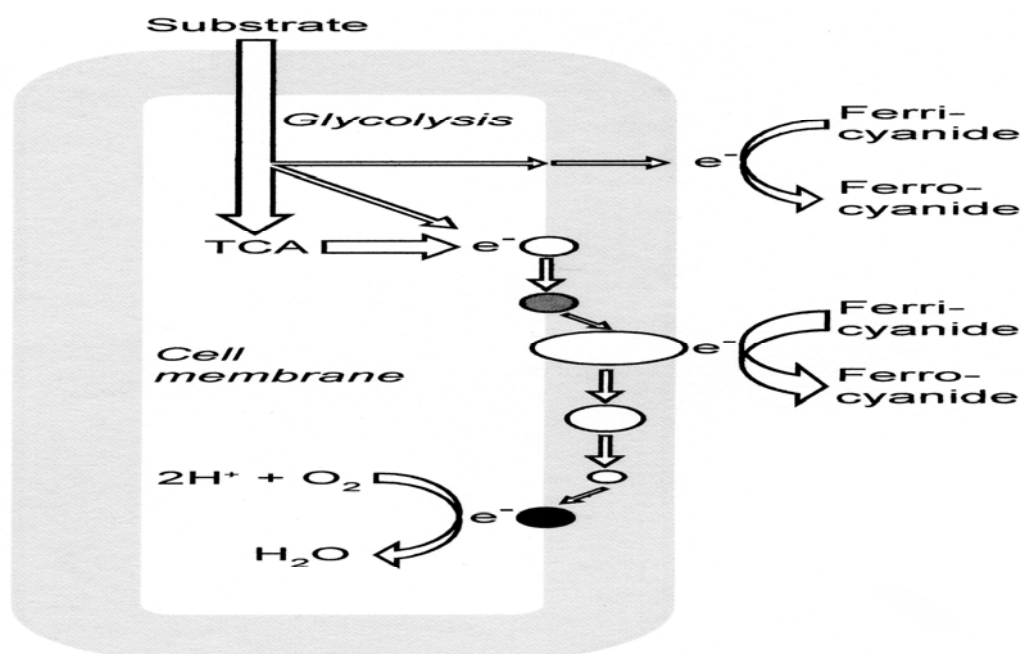
Figure 4.2 Diagram of hydrophilic mediator potassium ferricyanide interaction with prokaryotic cell.
(From - Baronian, 2006).

Because the components of catabolic pathways in prokaryotes and eukaryotes are located differently and this will affect the interaction of the mediator with the catabolic and anabolic redox compounds. The removal of electrons by a hydrophilic mediator from prokaryotic cells is relatively simple, because at least some of the redox reactions are accessible from outside the cell membrane.

The respiratory electron transport molecules in prokaryotic cells are located in the cell membrane and are accessible from the periplasm can be oxidised by the hydrophilic mediators. By using blocking agents, it has been demonstrated that 90% of the reduction of ferricyanide arises from the electron transport molecules in *E. coli* (Ramsey & Turner, 1998). Although the glycolytic pathways are located within the cytoplasm, the reduction of ferricyanide occurs by the interaction of ferricyanide with trans-plasma membrane electron transport enzymes (tPMET) such as ferrireductase enzyme. Membrane spanning NADH dehydrogenases act as potassium ferricyanide reductases and couple potassium ferricyanide reduction to the intracellular oxidation of NADH/NADPH.

The use of single hydrophilic mediator is not sufficient to measure the eukaryotic organism's catabolic responses because the catabolic pathways such as the TCA cycle, which occurs inside the mitochondria, are not accessible to the hydrophilic mediator. The mechanism of the double mediators' interaction in eukaryotic cell is schematically described below (Figure 4.3)

Proposed mechanism of double mediator interactions in eukaryotes
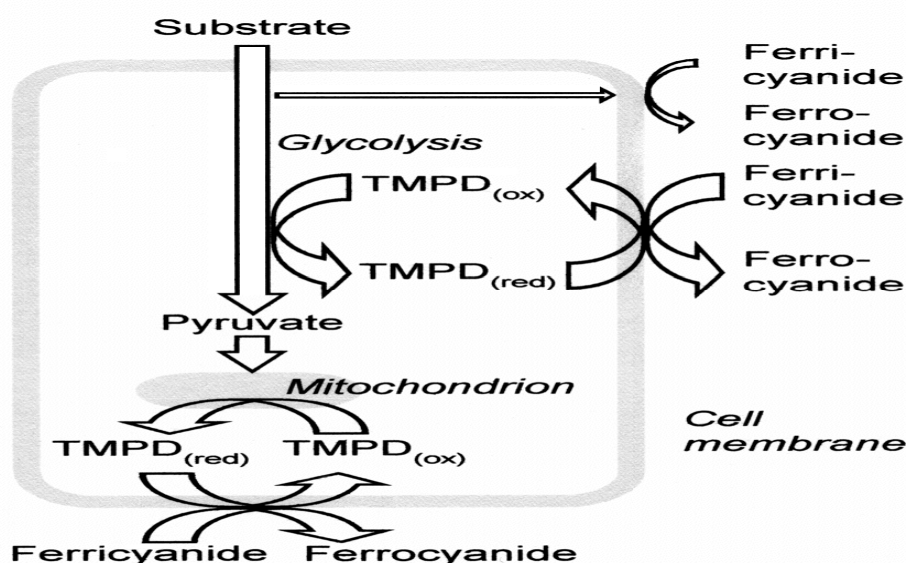
Figure 4.3 The interaction of a double mediator system with a eukaryotic cell. A small quantity of ferricyanide is reduced via the NADH: Fe^{3+} reductase system
(From - Baronian, 2006).

The use of a single mediator in eukaryotic cells results in small responses (Ly & Lawen, 2003; Baronian et al., 2002; Trosok, Driscoll & Luong, 2002, 2001; Caterall et al., 2001; Morris et al., 2001). These responses are also most likely to be due to the interaction of ferricyanide with the externally facing part of the trans-membrane NADH: Fe^{3+} reductase enzyme. In contrast, lipophilic mediators can cross the cell and mitochondrial membrane and access internal redox molecules such as NADPH resulting from catabolism (glycolysis and TCA cycle). However, the use of lipophilic mediators alone to monitor the catabolism is not ideal because their behaviour in an aqueous environment is not conductive to their detection by conventional electrochemical techniques. The double mediator system using hydrophilic and lipophilic mediators

overcomes this problem and allows efficient detection of both cell membrane and internal redox events in eukaryotes.

4.2.2 Proposed mechanism for the double mediated electrochemical signal detection

- A small amount of ferricyanide is directly reduced by trans-plasma membrane electron transport mechanisms (tPMET).

- TMPD crosses the cell membrane and oxidises reduced catabolic molecules such as NADPH and NADH. The reduced TMPD shuttles the electrons to the cell surface where it is oxidised by ferricyanide.

- The reduced ferricyanide is quantified by voltammetry; the amount of reduced ferricyanide represents the amount of catabolism occurring because of the yeast response to the specific catabolite used (Baronian, 2006).

4.2.3 Origins of single and double mediator responses in yeast

In order to gain insights into the origins of the responses experiments were performed with ageing cells (Baronian et al., 2002).They report that the origin of single mediator response is not certain but they suggest that the ageing in *S. cerevisiae* cells in the absence of glucose will become increasingly derepressed catabolically with time and will be fully functional in respiratory pathway. When glucose is added to the starved cells, the cells will be in respiratory mode and it is likely that the redox active species produced during respiration will diffuse out through the cell membrane reducing ferricyanide and augmenting the signal of single mediator responses in ageing cells. It is now known however, that that the redox products from respiration cannot cross the cell membrane and reduce ferricyanide and the mechanism involves NADH from glycolysis reducing a trans-membrane electron transport protein (tPMET) which in turn reduces the external ferricyanide. They found that the ageing of the cell had a different and dramatic effect on glucose responses to single and double mediator systems in *S. cerevisiae*. They also found that the response to glucose in a single mediator system increased dramatically with age whereas with double mediator systems using ferricyanide + menadione the response dropped, suggesting that the origins of these responses are different. In the double mediator response, the menadione reduction either occurred by NADPH produced in the pentose phosphate pathway or in the electron transport chain (Rabinowitz et al., 1998). The glucose concentration that has been used in growth and incubation is above the catabolite repression pathway of *S. cerevisiae*

(Walker, 1998) and hence, the organism operates in the fermentative mode, suggesting that it could be that the reduction of menadione is from the pentose phosphate pathway and not by the electron transport system. However, the glycolytic enzyme inhibitor, iodoacetate, inhibited not only the exogenous glucose metabolism but also endogenous metabolism in *S. cerevisiae* (Zhao, Wong & Yung, 2005). In contrast, the pentose phosphate pathway inhibitor, epiandrosterone, only caused a 4% reduction of total response, suggesting that the main pathway is glycolytic pathway for glucose metabolism in *S. cerevisiae*.

In our laboratory, some blocking experiments were performed with *S. cerevisiae* using dicoumarol, which blocks the transfer of electrons from NADH to menadione. These results show that the percentage of change of signal with single mediator ferricyanide response was greater than with double mediator comprising ferricyanide/menadione, suggesting that hydrophilic mediator ferricyanide reduction occurs mainly through the NADPH dehydrogenases. Another blocker, oxamate, which blocks the transfer of electrons from NADH to pyruvate, was also used with single and double mediators with *S. cerevisiae*. As expected both single and double mediator responses were raised with oxamate, supporting the model that we proposed in Figure 4.3. All of these blocking experiments (personal communication with Baronian and Robson) suggest that a substantial proportion of intracellular menadione reduction is due to NADPH, which is consistent with the reported values (Rabinowitz et al., 1998).

The relatively small responses achieved with yeast and a single mediator is generally not useful in biosensor development because of the need for larger response: noise ratios, which the double mediator system provides.

4.2.4 Glucose response range of *A. adeninivorans*
The initial experiments for the glucose dependent catabolic response of free
A. adeninivorans cells in Figure 3.8 show that the linear logarithmic responses were from 0.1mM to 7.5 mM glucose.

4.2.5 Free *A. adeninivorans* cells catabolic responses to glucose: ageing effect
The effect of ageing on *A. adeninivorans* free cell responses was investigated. Results in Figure 3.9 show that responses dropped not only in glucose treated cells but also in the control cells up to four days. These results clearly show that the free cells lose activity much faster with ageing (within days). The reason for this loss of activity observed in

free cells could be the cells are dying (even at 4ºC), the cells metabolic rate is dropping, or both.

4.2.6 Immobilised *A. adeninivorans* cells in 'Lentikats'

Biosensors using immobilised cells as the biocomponent have advantage of retaining the cells within a matrix, which permits their recovery and subsequent reuse. *A, adeninivorans* cells were successfully immobilised in 'Lentikats' and cells were viewed under bright field microscope (Figure 3.10). It is important to standardise the 'Lentikat' numbers to get useful response range. 'Lentikats' numbers 20 and 100 were used to test the glucose dependent catabolic responses of immobilised *A. adeninivorans*. Results presented in Figure 3.11 show that using 100 'Lentikats' gave larger glucose dependent catabolic responses compared to 20 'Lentikats'. It was decided to use 100 'Lentikats' in subsequent experiments.

The results in Figure 3.12 show that in 16 days the activity loss for 7.5 mM glucose treated cells was 14% and for control cells 11%. This is a much slower rate of activity loss than that seen in free cells where the activity loss in 3 days was 16% in 7.5 mM glucose cells and 12% in the control cells (Figure 3.9). This indicates that the 'Lentikat' hydrogel matrix provides an environment that prolongs cell activity. The same batch of 'Lentikats' were used experimentally for another 3 weeks. This study showed that *A. adeninivorans* cells immobilised in 'Lentikats' retain sufficient activity for experimental purposes for up to 2 months. This includes the time (20 days) from the day "Lentikats" were prepared to standardise the number to be used in each experiment (Figure.3.11) plus 16 days (Figure.3.12), and they were used for another 3 weeks for subsequent experiments (Figures 3.13 & 3.15).

4.2.7 The detection limit of glucose

Results in Figure 3.13 show that although there were very good linear logarithmic responses to low concentrations from 0.001 – 0. 75 mM glucose, there was a larger increase in the responses of *A. adeninivorans* between 0.001- 0.08 mM glucose (0.079 mM gives 107 nA than over concentrations from 0.08 – 0.75 mM glucose (0.67 mM gives 71 nA). These results are consistent with the results of Yin, Hatton & Brown (2000) who related the rate of glucose uptake, and therefore availability for catabolism, to the concentration of glucose in the cells environment. The results, plotted in Figure 3.14 show glucose responses over the entire concentration range using data from different experiments. It appears that *A. adeninivorans* exhibited log linear responses

over a wide glucose concentration range. A single experiment covering concentrations over the whole was not done because of the limitations of the apparatus used for these experiments. It has facility (15 taps) to sparge nitrogen in 15 tubes only (see Figure 2. 3). Because different batches of yeast cells respond differently to the same concentration of glucose, the data from the three experiments do not align perfectly; however, the trend over the concentration range is evident. The limits of detection and determination of glucose were calculated using very low glucose (μM) concentrations presented in Figure 3.15 and are 0.236 μM (2.36×10^{-7} M) and 3.58 μM (3.58×10^{-6} M) respectively.

4.3 Development of *A. adeninivorans* based biosensor to detect priority environmental contaminants: "IDEA" concept

4.3.1 Gallic acid

The responses of *A. adeninivorans* cells were investigated using cells grown in minimal medium that contained gallic acid (pre-conditioned cells) or glucose as a sole carbon energy source. The results in Figure 3.16 show that cells were incubated with gallic acid, glucose or no substrate (control) in the incubation suspension. The pre-conditioned cells that were grown in gallic acid responded significantly differentially to gallic acid than to glucose. The glucose grown cells also responded to gallic acid more strongly than to glucose. These results demonstrate that *A. adeninivorans* can utilise gallic acid as a sole carbon source, but gallic acid grown and glucose grown cells did not exhibit a differential response. This suggests that the gallic acid degradative enzymes are always present in the cells and pre-conditioning with gallic acid has no effect on the induction of this degradative enzyme pathway.

Gallic acid is known to have anti-fungal activity (Dowd, Duvick & Rood, 1997), however *A. adeninivorans* has an unusual ability to metabolise gallic acid. In presence of glucose, the response of gallic acid pre-conditioned cells was less than the endogenous response suggesting gallic acid might be interfering with the glucose metabolism. This is consistent with a report by Scalbert (1991). He reported the direct action of tannins (gallic acid is the hydrolysis product of tannic acid) on the microbial metabolism through inhibition of oxidative phosphorylation of the substrate (Scalbert, 1991). It is likely that the gallic acid as tannins has an inhibitory effect on the oxidative phosphorylation of glucose that interferes with the basic catabolism. This could have resulted in the inhibitory effect on addition of glucose in gallic acid pre-conditioned

cells. This aspect was not investigated further because *A. adeninivorans* did not respond differentially with gallic acid.

4.3.2 Naphthalene

A. adeninivorans can use naphthalene as a sole carbon and energy source, although growth is slow and an incubation period of 3 days was required to grow sufficient cells to perform the experiments. Figure 3.17 shows that the *A. adeninivorans* response was maximum at log of 0.1 mM naphthalene, but at higher concentrations, the response dropped indicating that naphthalene at higher concentrations might be toxic to the cells. To precisely identify the detection limit, naphthalene responses between 0.09 to 0.5 mM were investigated. The results in Figure 3.18 show that there was a dose dependent response to the log of naphthalene from 0.09 - 0.2 mM, from there the response dropped dramatically (data above 0.2 mM naphthalene not shown). The calculated limits of detection and determination are 60 μM (6 x 10^{-5} M) and 317 μM (3.17 x 10^{-4} M)) respectively. The detection limit required for environmental analysis is between 4 - 40 nM (0.4 – 4 x10^{-8} M, Lloyd-Jones, 2002) which was significantly less than the detection limit achieved by this system and is not useful for environmental applications.

The responses of *A. adeninivorans* cells grown in minimal medium with either naphthalene or glucose are shown in Figure 3.19. ANOVA Single Factor analysis was performed using the data obtained from Figure 3.19 and presented in Table 3.4. The response of naphthalene grown cells to naphthalene was, relative to the control (p < 0.05), larger than the response to glucose, while the control and glucose responses were statistically similar (p > 0.05). This indicates that cells pre-conditioned in naphthalene are able to metabolise naphthalene at a faster rate than glucose grown cells can. Glucose grown cells do however show a small response to naphthalene relative to the control, p-value of < 0.05 (but compared to glucose response it was small). This suggests that the cells of *A. adeninivorans* must possess some constitutive naphthalene degradative enzymes, which enable them to use it as an energy source. These results suggest that the "IDEA" rationale, i.e., the differential response, only partially applies to *A. adeninivorans* cells pre-conditioned on naphthalene. It is possible that while pre-conditioning the cells with naphthalene completely induces the naphthalene degradative pathway enzymes, some of the enzymes will be also available in glucose grown cells.

These studies do not completely support the "IDEA" concept that pre-conditioning cells with the target contaminant molecules induces specific degradative enzymes as glucose

grown cells are shown to have some ability to metabolise naphthalene. Additionally, the detection limit that could be achieved was 60 µM, which is not sensitive enough for environmental detection purposes. Gas phase analysis of naphthalene in a whole-cell assay is possible but the 10-fold reduction achieved by Werlen et al. (2004) would result in a detection limit of about 50 nM which is still above the detection limits required of 4 and 40 nM (Lloyd-Jones, 2002). Because the "IDEA" concept was not viable, the gas phase analysis of naphthalene was not pursued in this study

4.3.3 Morphological changes in A. adeninivorans cells grown in naphthalene

It is well known that environmental stress and nutritional stress can affect yeast growth rate, morphology and pathogenicity. *S. cerevisiae* cells can change their morphology under nutrient limitation (Gangiano, Bauer & Pretorius, 2002). They reported that nitrogen limitation caused diploid *S. cerevisiae* cells to form pseudo-hyphae whereas glucose limitation caused haploid *S. cerevisiae* cells to undergo invasive growth. The naphthalene metabolic pathway is shown in Figure 1.3 (p-26) and the genes involved in naphthalene metabolism are explained (p-26). The morphological changes that are associated with nutrient limitation were investigated. *A. adeninivorans* cells grown in minimal medium with naphthalene as a sole carbon source were compared to the cells grown on minimal medium with glucose and observed morphological changes that occurred. These are shown in Figures 3.20 and 3.21. The morphology of the cells grown in naphthalene appears to be more oval compared to glucose grown cells, which were more elongated. This change in morphology may be related to the poor growth of *A. adeninivorans* in a minimal medium with naphthalene as a sole carbon source.

4.3.4 Di-butyl phthalate

A. adeninivorans can utilise di-butyl phthalate as a sole carbon source. Results presented in Figure 3.22 show that the organism show linear response to the log of DBP from 0.025 – 0.075 mM. The limits of detection and determination achieved by the double mediated electrochemical method are 25 µM (2.5×10^{-5} M) and 50 µM (5×10^{-5} M) respectively. The detection limit that was achieved by this method is still above the detection limits required of 33 nM (3.3×10^{-8} M, Lloyd-Jones, 2002).

The responses of *A. adeninivorans* cells that were grown in minimal medium with di-butyl phthalate or glucose as a sole carbon source are shown in Figure 3.23 and the ANOVA Single Factor analysis performed using that data is presented in Table 3.5.*28A.*

adeninivorans cells grown in the presence of di-butyl phthalate as a sole carbon source responded more to di-butyl phthalate than to glucose. The ANOVA Single Factor analysis of *A. adeninivorans* cells grown in presence of di-butyl phthalate as a sole carbon source shows that the control response (no substrate) and glucose response are significantly not different statistically ($p > 0.05$). The glucose grown cells however show no response to di-butyl phthalate. These results suggest that pre-conditioning the cells with di-butyl phthalate induces the degradative enzymes responsible for phthalate that were absent in unconditioned (glucose grown) cells. These results support the "IDEA" concept (Baronian, Gurazada & Thomas, 2005).

4.3.5 Yeast metabolism of di butyl Phthalate.

The complex degradative pathway of di-butyl phthalate has been reported (Eaton and Ribbson, 1982). Di-butyl phthalate grown microbial cells express all the metabolic enzymes leading to oxaloacetate and pyruvate, which are explained in detail (section 1.10.5, p- 28 first para). The oxaloacetic acid and pyruvate produced from di-butyl phthalate catabolism enters into central metabolic pathway via "tricarboxylic acid cycle" (TCA) and is shown in next page (Figure 4.4).

Glucose
↓
Glucose-6 Phosphate
↓
Fructose-6-Phosphate
↓
Glyceraldehyde-3-Phosphate
→ NADH
↓
3 Phosphoglycerate
↓
Phosphoenolpyruvate
↓
Di-butyl phthlatate → Oxaloacetate ← Pyruvate

TCA CYCLE / Mitochondria:
Oxaloacetate — Isocitrate
NADH
Malate   **TCA CYCLE**
Fumarate
NADH   NADPH   NADH
Succinate ← α Ketoglutarate
Mitochondria

O2 → H2O
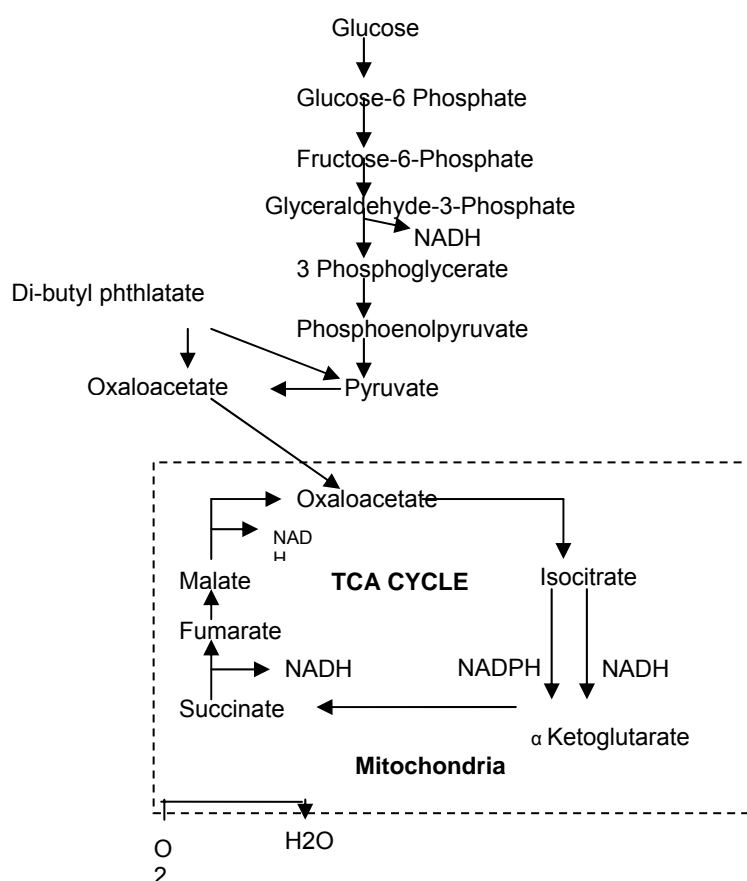
Figure 4.4 Yeast metabolic chart, depicting di-butyl phthalate metabolites entering central metabolic pathways

4.3.6 Depression of di-butyl phthalate response in *A. adeninivorans* by glucose

During the course of this study an interesting observation was made, the response of the di-butyl phthalate grown *A. adeninivorans* cells to glucose was less than the control ($p < 0.05$, Table 3.5), whereas the response of the glucose grown cells to di-butyl phthalate was same as ($p > 0.05$) as the control response (Figure 3.23). It was a very interesting observation that the presence of glucose depressing the di-butyl phthalate pre-grown *A. adeninivorans* cells response less than endogenous response. This was investigated further. In an experiment where the di-butyl phthalate grown cells were incubated with di-butyl phthalate 0.075 mM/glucose 0.075 mM mix, the response was less than the di-butyl phthalate response, but more than the control (endogenous) response (Figure 3.24) i.e. even in the presence of di-butyl phthalate, glucose still acts to depress the response expected from the phthalate. These results indicate that the presence of glucose depresses catabolism both at endogenous level and in the presence of a known substrate (DBP), suggesting that the presence of glucose depresses the basal catabolic rate in cells grown in di-butyl phthalate. It is very well known in the literature that when glucose is

present in the sample it represses all the other catabolic pathways; this is known as glucose catabolite repression. All the genes that are responsible for glucose repression well characterised in yeast (Gancedo, 1998) and are explained earlier in sections 1.14 and 1.14.1 (p37 – 39). In this particular instance the glucose depression phenomenon is unique and is different from the glucose catabolite repression phenomenon, it is termed as glucose depression. The mechanism of this glucose depression is unknown and it is difficult to even suggest how it may function. These observations were made after one hour of incubation. Further investigations of this glucose depression of catabolism in di-butyl phthalate grown cells were performed to establish whether this depression was concentration dependent by keeping the di-butyl phthalate concentration constant (0.075 mM) and varying the glucose concentration. Figure 3.25 shows that the repression was more prominent at 0.075 mM glucose and it depresses even endogenous response. This depression was concentration dependent and vanished at concentrations of 1 and 3 mM glucose (Figure 3.25). These results indicate that glucose must be lower than 1 mM for this depression mechanism to function. To identify the lowest concentration of glucose responsible for this depression the glucose range of 0.035 - 0.1 mM was used. Results in Figure 3.26 show that the repression was more prominent at 0.035 mM glucose concentration. These results show that the glucose depression was seen between 0.035 mM - 0.1 mM. Based on these observations, it was decided to use 0.035, 0.075 and 0.1 mM glucose concentrations in my subsequent work.

4.3.7 Effect of incubation time on the glucose depression phenomenon: possibility of glucose transporter genes induction

It was speculated that the glucose depression phenomenon is related to the glucose transporter genes induction/expression mechanism creating a need to identify the time when the glucose depression starts and ends. At one hour, the depression is seen and experiments were done to investigate how long it takes the depression phenomenon to disappear. An experiment was initially performed at two and three hours of incubation and the results are presented in Figure 3.27. At two and three hours of incubation, the di-butyl phthalate response was also not observed. These results suggest that depression of the di-butyl phthalate response occurs between 0 - 1 hour of incubation and after one hour the depression of the di-butyl phthalate response disappears. These results suggested that the glucose depression phenomenon might be related to the glucose transporter genes expression. An investigation of the effect of different incubation times (15, 30 and 45 minutes) on the depression effect was then carried out. Experiments were performed with di-butyl phthalate (0.075 mM), a mix of constant di-butyl phthalate

(0.075 mM) and increasing glucose concentrations of 0.035 mM, 0.075 mM, and 0.1 mM. Results in Figure 3.28 show that at 15 min the *A. adeninivorans* cells did not respond to di-butyl phthalate itself. But results at 30 minutes and 45 minutes incubation which are presented in Figures 3.29 and 3.30 and Tables 3.9 and 3.10 show that the cells responded to di-butyl phthalate and the glucose depression was seen at all three glucose concentrations, i.e., 0.035, 0.075 and 0.1 mM. The glucose depression was more prominent at 0.035 mM glucose concentration at both 30 and 45 min incubation. All these results suggest that the phenomenon of glucose depressing di-butyl phthalate response starts after 15 minutes is still seen at 30 and 45 minutes and remains up to one hour. After one hour, this phenomenon disappears. The glucose depression of the di-butyl phthalate response and to some extent the basal catabolism itself observed in *A. adeninivorans* could presumably relate to the function of the cells at the gene expression level.

Yeast are very sensitive to low glucose <0.01% (5 mM) and will modulate its gene expression depending on the glucose concentrations (Yin, Hatton & Brown, 2000). The same group reported that the glucose transporter genes in yeast are controlled by two sets of genes (Yin et al., 2003). These glucose transporters are differentially expressed at different glucose concentrations. They reported that at 0.1% (55 mM) glucose transcription of hexose transporters, genes *HXT2* and *HXT4* were activated and expressed, whereas *HXT1* remained low. In contrast at 4% (2.2 M) glucose concentrations *HXT1* is activated and expressed , whereas *HXT2* and *HXT4* remain low (Ozcan, Dover & Johnston, 1998). At 0.01% (5 mM), i.e., very low glucose concentration, which is above the lowest concentrations used in this study (0.035 mM glucose); gluconeogenic mRNAs are repressed (Yin et al., 2003). At these different glucose concentrations, distinct signalling pathways are active (Ozcan, Dover & Johnston, 1998; Ozcan et al., 1996). The results of glucose depression of di-butyl phthalate suggest that it could be possible that at 0.035 mM glucose, some of the glucose transporter genes (*HXT*) are induced and expressed. The gene expression starts after 15 min and reaches maximum between 30 - 60 min, peaking at 45 min when the glucose is transported inside the cell and interferes with the catabolic enzymes of di-butyl phthalate and depressing its response. It is assumed that the gene expression shuts off after 1 hour, so that the glucose depression is relieved. mRNA analysis however would be required to confirm this explanation (Baronian, Gurazada & Thomas, 2005).

The experiments with glucose and di-butyl phthalate confirm the interference of glucose with the di-butyl phthalate response. Interference from other substrates can be a problem for whole-cell sensor. Interference in a *Saccharomyces* based biosensor was reported (Mascini & Memoli, 1986). There are however, examples in the literature where specificity in cell-based sensors has been achieved. For example, a sensor was described that was designed to detect lactate in blood samples using *Hanansula anomala* as a sensing cell (Racek & Musil, 1987). The response of the sensing cells to blood glucose was completely blocked by the addition of sodium fluoride to the sample; the purpose of this addition was to achieve specificity to lactate.

Although attempts were made in these studies to overcome the problem of interference by pre-conditioning (growing) the yeast cells with the contaminant substance as sole energy source, the interference of the di-butyl phthalate signal by glucose has been demonstrated in this study (Baronian, Gurazada and Thomas, 2005). These results imply that these biosensors most probably have an application only when there is a single contaminant molecule in the sample but not mixtures.

4.3.8 Localising the glucose depression phenomenon

To localise the metabolic pathway that is responsible for the occurrence of glucose depression of the di-butyl phthalate response, an experiment was performed using only the hydrophilic single mediator, potassium ferricyanide. Ferricyanide can interact with the externally facing part of the trans-membrane NADH: Fe^{3+} reductase enzyme. Figure 3.31 shows that in the single mediator experiments, *A. adeninivorans* responded to di-butyl phthalate and glucose depression of the di-butyl phthalate response was observed at all three glucose concentrations (0.035, 0.075 and 0.1 mM). The glucose depression pattern was almost identical to the double mediator system. Interpretation of this result locates the depression of the di-butyl phthalate in the glycolytic pathway because the ferricyanide that has been reduced could only be reduced by NADH from glycolysis (NADH produced in the mitochondria cannot exit the mitochondria). However, combining the single mediator and the gene induction experiments, the glucose depression phenomenon might not be completely attributed to cytoplasmic metabolic flux but also could be due to the nuclear export of the induced glucose transporters gene products such as *HXT*.

Although this research has shown that a yeast-based biosensor can detect di-butyl phthalate, its application as an environmental sensor has limitations of a higher

detection limit than that required for environmental analysis and interference of other catabolisable molecules in the sample with the di-butyl phthalate response. This may be premature comment as only di-butyl phthalate was tested in this research and it is worth pursuing the other phthalates to verify these observations. Although there are, methods to control interference from other catabolisable molecules, such as, use of catabolic inhibitors, those could inhibit specific intermediate reactions in the central catabolic pathways. This aspect was not pursued further and the IDEA approach to biosensor development was abandoned because the same limitations with bacteria were found by Lincoln Ventures Limited (personal communication, Dr. Neil Pasco). As a result the research was re-focused on the application of the double mediated detection method to improve an existing yeast estrogen screen (YES) bioassay using genetically modified hER *S. cerevisiae.*

4.4 Modification of the current optical detection system for yeast estrogen screen (YES) using hER *S. cerevisiae* with the double mediated electrochemical detection system

The current YES assay uses genetically modified hER *S. cerevisiae* to quantify estrogens. An optical detection system is used to detect the response of the cells to estrogen. It takes 72 hours for sufficient colour to develop to allow a reading. The steps involved in the optical detection system were described in detail in (Figure 1.4) and will be discussed in detail in later part of this section. In this study, the chromogenic substrate chlorophenol red-β-D - galactopyranoside (CPRG) used in the optical system was replaced with lactose and the double mediated electrochemical detection technique was used to detect the response of the cells to increasing glucose resulting from the presence of estrogen.

4.4. 1 Estrogen whole-cell bioassay using hER *S. cerevisiae*

Results in Figure 3.32 show the catabolic responses of hER *S. cerevisiae* to glucose, lactose and estrogen mix, and no substrate, at 7 hours incubation. There are strong responses to glucose and to the lactose/17β-estradiol mix at 7 hours of incubation, but not at 1 and 4 hours incubation (data not shown). There was no response to lactose alone (*S. cerevisiae* does not produce the enzymes required for lactose catabolism). The estrogen concentration used in this experiment was 11 nM, the maximum concentration used for calibration of an optical YES system (Baronian, Gurazada and Thomas, 2005). These preliminary results indicate that mediated electrochemistry can detect the increase in catabolism that occurs in hER *S. cerevisiae* in response to the presence of estrogen

and lactose. Wild type *S. cerevisiae* is unable to catabolise or transport lactose and the insertion of *lac Z* reporter fusion enables the cells to produce β-galactosidase enzyme which hydrolyses lactose and lactose analogues. However, the β-galctosidase enzyme is produced only when estrogen is present in the cell environment. There is no lactose permease gene in hER *S. cerevisiae* to transport lactose inside the cell and presumably, the degradation of lactose is extracellular (Baronian, Gurazada & Thomas, 2005). *S. cerevisiae* transformed with a fungal β-galactosidase gene (*lac A*) from *Aspergillus niger* is known to secrete up to 40 % of the enzyme produced by the modified cells (Kumar et al., 1992).

4.4.2 Proposed mechanism of estrogen sensing and generating electrochemical signal in hER *S. cerevisiae*

After the estrogen molecule binds to the estrogen receptor (hER), the receptor attains a form that is capable of binding to the estrogen responsive elements in the expression plasmid inducing the reporter gene *lac Z,* resulting in β-galactosidase enzyme production. This is secreted outside the cell, i.e. the same process that occurs in the optical system. The difference between the optical and mediated electrochemical system is that the secreted β-galactosidase in the mediated electrochemical detection system cleaves the lactose to produce glucose and galactose with the glucose being transported inside the cell resulting in increased catabolism and increasing the electrochemical signal. Whereas, in the optical system, the secreted β-galactosidase cleaves the yellow chromogenic substance CPRG to produce a red coloured molecule that is measured by spectrophotometry (Figure 1.4). The reason for the very long detection time of 72 hours for the optical detection system is assumed the rate of the β-galactosidase interaction with the chromogenic substrate involved in colour production.
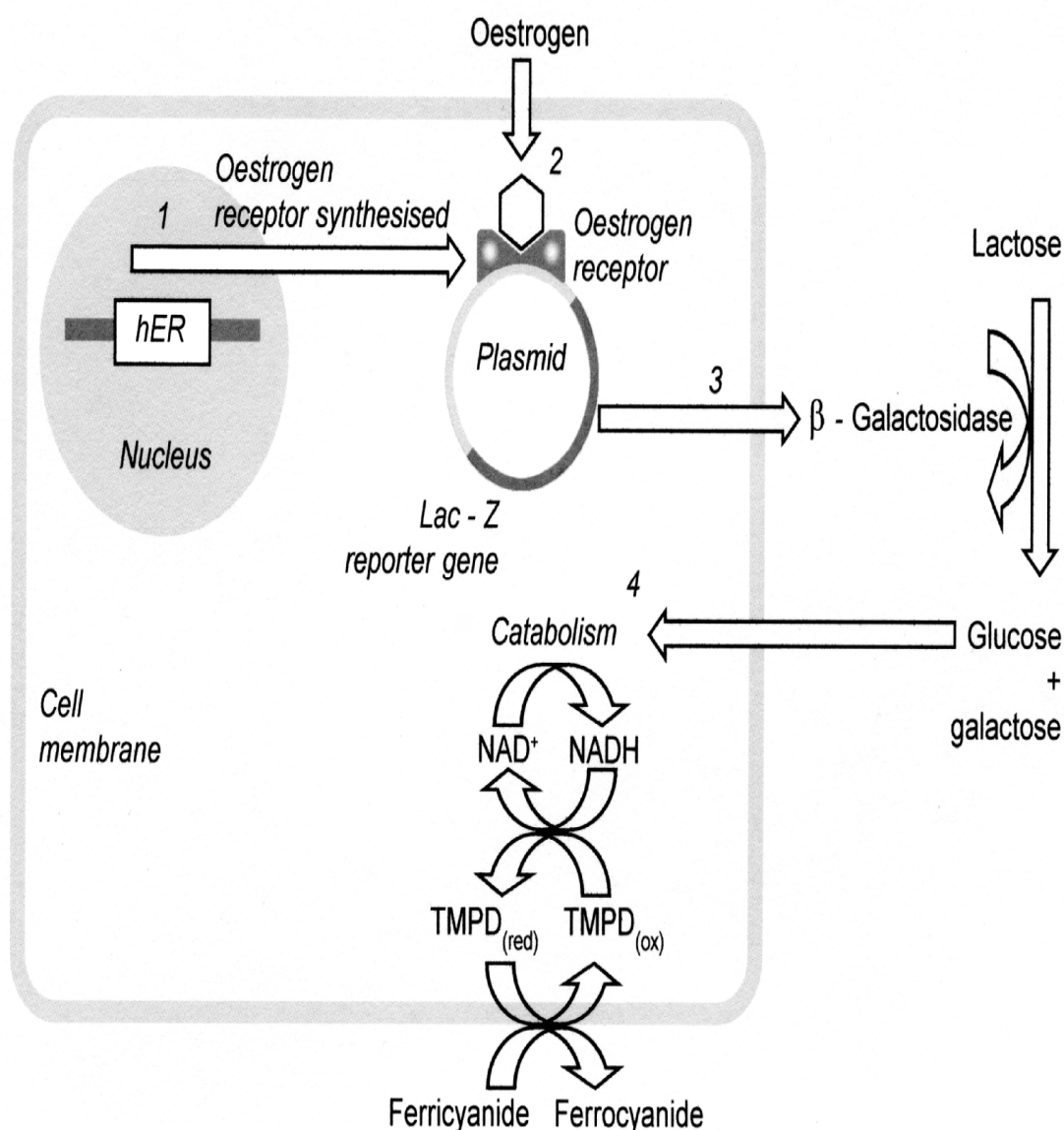
Figure 4.5 Proposed mechanism of estrogen sensing and double mediated electrochemical detection by hER *S. cerevisiae.*

(From – Baronian, 2006)

4.4.3 Electrochemical detection time

Time-dependent 17β-estradiol responses of hER *S. cerevisiae* (Figure 3.33 & Table 3.12) suggest that the response is detectable at 5 hours ($p < 0.05$) in the ANOVA Single Factor analysis the maximum response occurring between 7- 8 hours. Hence, it was decided to use an eight-hour incubation period in all subsequent experiments. Results in Figure 3.34 show that hER *S. cerevisiae* dose dependent linear responses to the log of 17β-estradiol were from 0.037 nM to 11 nM.

4.4.4 Dose-dependent responses of hER *S. cerevisiae* to 17β-estradiol

The dose-dependent logarithmic response of hER *Scerevisiae* in Figure 3.35a shows that the lowest concentration of 17β-estradiol that was detected using this system is at 3.7 pM. The limits of detection and determination were calculated by the blank-value procedure described in section 3.3.3 and were calculated from Figure 3.35b. The limit of detection is 0.041 pM (4.1×10^{-14} M) and the limit of determination is 0.42 pM (4.2×10^{-13} M). The EC$_{50}$ value is 1.48 pM (1.48×10^{-12} M).

The limits of detection, determination and EC$_{50}$ achieved by mediated electrochemical detection are significantly lower than many other reported bioassay values obtained using genetically modified S. *cerevisiae*. The limits of detection, determination and EC$_{50}$ values achieved by using other yeast estrogen screens are compared and tabulated in the next page (Table 4.1).

Table 4.1 Comparison of values of EC_{50} (M), limit of detection(a) and (b) limit of quantitation, incubation period (and, where stated or possible to calculate, additional time required to complete the assay) for some yeast estrogen assays. ND – Not done.

Estrogen assay	EC_{50} (M) 17β-estradiol	(a) limit of detection M, 17β-estradiol (b) limit of quantitation [method where given]	Incubation period (h)/ Additional assay time (h) [excluding cell preparation]	Reference
E-Screen Cell proliferation assay	5.3×10^{-12}	(b) $1.2 \pm 0.2 \times 10^{-12}$	144/4	Schultis and Metzger (2004)
Receptor binding (RB) assay hERα/β	ND	(b) α $7.5 \pm 0.5 \times 10^{-9}$ β $3.9 \pm 0.5 \times 10^{-9}$	2/1.5	Schultis and Metzger (2004)
Yeast estrogen screen (YES) hERα/ERE/lac-Z	2.4×10^{-10}	(a) 7.3×10^{-12}	72/1.5	Routledge and Sumpter (1996) Sanseverino et al. (2005)
YES (modified)	$1.13 \times 10^{-10} - 5.64 \times 10^{-10}$	ND	45	De Boever et al. (2001)
LYES	5.8×10^{-11}	(a) $(4.1 \pm 0.03) \times 10^{-12}$ [DIN 32 645]	7	Schultis and Metzger (2004)
YEPKB1/ YRPE2	ND	(a) $< 7 \times 10^{-14}$ 1.0×10^{-14}	8/0.1-6 18/0.1-6	Klein et al. (1994) Coldham et al. (1997)
(hER/GAL4 + lacZ)	1.5×10^{-9}	ND	4/<8	Rehmann et al. (1999)
ER+LYES 8127	ND	ND	18/-	Kang, et al. (2002)
yEGFP/ERα yEGFP/ERβ	5×10^{-10} 6×10^{-11}	ND	24/- 4/-	Bovee et al. (2004)
BLYES	$(4.4 \pm 1.1) \times 10^{-10}$	(a) 4.5×10^{-11}	6/	Sanseverino et al. (2005)
YTH system (hERα, lac Z)	ND	(a) 0.11×10^{-11}	18/-	Schwartz-Mittelman et al. (2005)
hERα pG1/ER(G)	5×10^{-10}	(a) 3×10^{-11}	2.5/ <8	Leskinen et al. (2005)
RMY/ERα-ERE	ND	ND	0.5/-	Wozei et al. (2005)
hER/GAA/phyK	ND	(a) $1.83 \times 10-11$ $3.66 \times 10-11$	30/-	Hahn et al. (2006)
Electrochemical detection- YES	1.48×10^{-12}	(a) 4.1×10^{-14} (b) 4.2×10^{-13}	7/2	current study

Estrogen assays are based on *S. cerevisiae* except hER/GAA/phyK (Hahn et al., 2005) is based on *A. adeninivorans*.
(From – Baronian et al., *Biosensors and Bioelectronics* 2007 Submitted)

Abbreviations used in Table 4.1: BLYES-estrogen-inducible lux-based bioluminescent receptor; ER- estrogen receptor; ERE- estrogen responsive elements; hERα- human estrogen receptor lac-Z- β-galactosidase structural gene; LYES- lyticase and triton X treatment in yeast estrogen screen; GAL 4- yeast transcription activator; YEPKB1- plasmid carrying CUP1 metallothionein promoter fused to human estrogen receptor cDNA; YRPE2 – plasmid carrying frog vitellogenin estrogen responsive element and yeast iso-1-cytochrome promoter fused to β-galactosidase structural gene; YTH – yeast two hybrid system; yEGFP – yeast enhanced green fluorescent protein; phyK- *Klebsiella*-derived phytase reporter gene; GAA- *A. adeninivorans*-derived glucoamylase (GAA) promoter which had been modified by the insertion of estrogen-responsive elements, RMY- An estrogen-sensitive *S. cerevisiae* RMY/ER-ERE with human estrogen receptor (ERα) gene and the *lacZ* gene

4.4.5 Comparison of existing yeast estrogen bioassays with the double mediated electrochemical detection system

In the current YES optical detection system, the β-galactosidase enzyme produced in response to the presence of estrogens in samples is secreted into the medium and cleaves chlorophenol red-β-D-galactopyranoside (CPRG) producing a colour change from yellow to red. This requires 72 hours of incubation. However, Kumer et al. (1992) reported that 40% of the β-galactosidase produced in *S. cerevisiae* transformed with *lacZ* and *lac A* (which encodes for lactose permease gene from *Aspergillus niger*) is secreted. This may partially explain the 72 hours of detection in YES assays that lack the lactose permease gene (*lac A*).

Recently a modified version of the YES assay, the lysis yeast estrogen screen assay (LYES) was developed which includes a digestion of the membrane with the enzyme lyticase and triton X (Shultis & Metzger, 2004). Using this technique the detection time could be reduced to 7 hours. The authors claim that the LYES assay sensitivity is an order of magnitude better than the original YES assay. They state that digesting the cell membrane with the lyticase enzyme increase the cell permeability and enhance the secretion of the β-galactosidase enzyme that is produced by hER *S. cerevisiae*.

In the double mediated system, the time experiment shows that the minimum time required for the *lac Z* gene induction in response to 17β-estradiol and the production of the β-galactosidase enzyme is 4-5 hours. The process of *lac Z* gene induction and subsequent enzyme production is the same in all these systems, i.e., YES, LYES and the double mediated systems; the difference in the detection time depends on the substrate that has been used in these three systems, the chromogenic substrate or lactose. In the electrochemical detection mechanism (as proposed in Figure 4.5), the enzyme produced

is secreted and hydrolyses the lactose. It would seem that the rate of enzyme conversion of the substrate and/or the sensitivity of the detection system account for the differences in detection times seen in these three systems.

Several estrogen assays have been developed by other researchers using *lac Z* reporter gene and an optical system with varying detection limits and times (Table 4.1). *S. cerevisiae* ER+ LYS 8127 was used and 18 hours incubation time was required for the detection of their binary estrogen mixtures (Kang, Cho & Lee, 2002). An assay was developed using a construct expressing a fusion protein from the human estrogen binding domain and GAL4- DNA binding domain linked with the *lac Z* reporter gene to enhance the sensitivity and reduce the incubation time to 2 hours with substrate (Rehmann, Schramm & Kettrup, 1999). This was followed by the lysis of the cells and incubating with the chromogenic substrate o-nitrophenyl-ß-D-glactopyranoside for another 2 hours. The EC_{50} value however, is 1-2 orders of magnitude greater than the yeast estrogen assay and three orders of magnitude greater than the E-screen assay (Rehmann, Schramm & Kettrup, 1999).

A *S. cerevisiae* based assay system comprising two plasmids containing CUP1 metallothionine promoter fused to human estrogen cDNA (YEPKB1) and frog vitellogenin estrogen responsive element and yeast iso-1-cytochrome promoter fused to the β-galactosidase structural gene (YRPE-2) was reported to have a limit of detection of $< 7 \times 10^{-14}$ M (0.07 pM). The incubation period for this assay was 12-14 hours.

Recently an electrochemical detection method was developed for studying the effects of the estradiol induced dimerisation of the human estrogen receptor α (hERα) expressing a *lac Z* reporter gene (Schwartz-Mittleman et al., 2005). This assay is based on modified *S. cerevisiae* with a yeast two-hybrid system (YTH). The yeast two-hybrid system is a very powerful technique to identify protein interactions such as dimirisation (Wang et al., 1995).This YTH based assay involves a redox product of β-galactosidase activity via protein-protein interaction. Eukaryotic transcriptional activators such as GAL4 consist of two individual domains, the DNA binding domain (DBD) and the activation domain (AD). The DBD recognizes a specific DNA sequence such as Estrogen responsive elements (ERE). The AD coordinates the assembly of the elements required for transcription and enables RNA polymerase II to transcribe a specific reporter downstream of the DBD domain. An ERα monomer is fused to the DNA binding

domain (DBD) derived from the transcription factor (GAL4), and the second monomer is fused to the activation domain of the same transcription factor. When both fused receptors are co-expressed in yeast, ER dimerisation induced by estradiol leads to the reconstitution of the GAL4 transcription factor measured as β-galactosidase activity. β-galactosidase cleaves p-aminophenyl β-D-galactopyranoside to p-aminophenyl, which can be detected electrochemically.

Fluorescence assays were developed using the existing *S. cerevisiae* optical system and the authors claimed a reduction in time from 20 hours for the optical system to 30 minutes for fluorescence assays by using the RMY/ER-ERE strain (Wozei et al., 2005). This strain produces β-galactosidase enzyme on exposure to 17β-estradiol. The yeast cells were incubated with the sample for 20 hours followed by the cell lysis and then the colour change was measured to quantify the enzyme. The authors claim an assay time of 30 minutes ignoring the incubation time of 20 hours. A photometric detection system was developed to monitor estrogens by using genetically modified *A. adeninivorans* cells carrying hER and a *Klebsiella* derived phytase (phyK) reporter gene under the control of an *A. adeninivorans* derived glucoamylase (GAA) promoter which had been modified by insertion of the estrogen-responsive elements (Hahn et al., 2006). They achieved a detection limit of 1.83×10^{-11} M and a limit of determination of 3.66×10^{-11} M (Table 4.1).

Compared to the other *lac Z* based yeast estrogen bioassays, the limits of detection, and determination and the EC_{50} values of the double mediated system are a significant improvement. This system is simple and needs no special equipment and trained staff. In addition, this system also offers the sensitivity equivalent to mammalian and tissue-bioassays requiring equipment that is more sophisticated and trained staff.

4.4.6 hER *S. cerevisiae* responses to estrogens and estrogen analogues

After standardising the incubation time and sensitivity range of 17β-estradiol, hER *S. cerevisiae*'s responses to other estrogens and estrogen analogues were tested in this research to validate the double mediated detection assay to quantify estrogenic molecules. The range used for 17α-estradiol, estrone and 17α-ethynylestradiol analysis was from 0.037 nM to 11 nM. Data from Figures 3.36, 3.37 & 3.38 suggest that the response was larger at lower concentrations (0.037 nM and 0.367 nM) than at higher concentrations (3.67 nM and 11 nM) for 17α-estradiol and 17α-ethynylestradiol. Estrone exhibited a dose-dependent response up to 3.67 nM but at 11 nM, the signal

dropped. Figure 3.39 summarises hER *S. cerevisiae* responses to estrogens and estrogen analogues suggested that 17β-estradiol exhibited dose-dependent response up to 11 nM. Table 3.14 summarises the responses of all estrogen and estrogen analogues tested. Table 3.14 suggests that 17α-estradiol has the lowest binding affinity, i.e., least potent, especially at the higher concentrations. Estrone is the next most potent estrogen compared to 17β-estradiol. At 0.037 nM, estrone is more potent than 17β-estradiol. 17α-Ethynylestradiol is the intermediate between estrone and 17α-estradiol. At 3.67 nM concentration of 17α- estradiol and estrone, the responses were found to be 27 % and 51% of the signal from the same concentration of 17β-estradiol. These values obtained in this research are close to those established in an equimolar competitive binding assays system (Bovee et al., 2004). They tested various natural and synthetic hormones using yeast enhanced green fluorescent protein (yEGFP) as a reporter gene to detect the estrogenic activities. In their system, the 17β-estradiol show the dose response curve from 0.2 nM to 80 nM, for 17α-estradiol from 50 nM to 600 nM, estrone 4 nM to 1000 nM and 17 α- ethynylestradiol 0.4 nM to 60 nM. These results show that the responses to estrogen and estrogen analogues begin at much higher concentrations than they do in the electrochemical detection system (0.37 pM) used in this study. There is no clear explanation for these differences, however, these differences could be because the yeast responses are more sensitive at the lower levels compared to their sensitivity at higher concentrations especially for 17α-estradiol and 17α-ethynylestradiol and Bovee's system was unable to detect at these concentrations. It has been suggested that these molecules could be toxic at higher concentrations (Dr. Toshihiro Horiguchi, Environmental Research Institute, Tsukuba, Japan, personal communication, 2005). However, this does not explain the results observed using different detection techniques with hER *S. cerevisiae* assay.

4.4.7 Development of estrogen whole-cell bioassay using hER *S. cerevisiae* exploiting glucose catabolite repression.

The validity of using hER *S. cerevisiae* and the mediated electrochemical detection system detecting estrogens in environmental samples is possible only if other catabolisable molecules are blocked in non-extracted samples to achieve specific responses to estrogens. The blocking experiments with glucose suggest that blocking of other catbaolisable molecules catabolism can be achieved. Results summarised in Figure 3.40 demonstrated that a useful glucose concentration to suppress catabolic reactions to other substrate and yet permit the detection of 17β-estradiol response was 0.1 mM. At higher glucose concentration, (1 mM) the estradiol response is masked by

the response to the glucose. Hence, 0.1 mM glucose was used in subsequent blocking experiments. In the above experiment, the 17β-estradiol concentration used was 0.367 nM. The usefulness of the blocking method was investigated by using different 17β-estradiol concentrations with 0.1 mM of glucose concentration. Results in Figure 3.41 show that the method worked up to 0.367 nM but from 3.67 nM, the response dropped. Hence, concentrations of 0.1 mM glucose and 0.367 nM 17β-estradiol were selected for subsequent blocking experiments. Figures 3.42 and 3.43 show that the presence of 0.1 mM glucose in the incubation mixes could efficiently block responses to fructose and peptone. In these experiments, fructose and peptone are used because fructose is a simple monosaccharide and it is the next preferable sugar to glucose and is a good competitor to test the efficiency of glucose blocking its catabolic responses. Peptone (casein hydrolysate) is a broad mixture of amino acids, peptides and simple carbohydrates and is very useful for testing the ability of glucose to simultaneously block the catabolism of different types of molecules. An ANOVA Single Factor analysis of these results (Table 3.15) gives a p-value < 0.05 for glucose and glucose plus 17β-estradiol showing with high probability that they are significantly different statistically and that the 17β-estradiol response could be detected in the presence of glucose. The p-value > 0.05 for glucose + 17β-estradiol and glucose + fructose + 17β-estradiol is indicating that they are not significantly different statistically and hence, having the similar response, i.e. glucose blocked efficiently the catabolic responses of fructose. Similarly glucose + 17β-estradiol and glucose + peptone + 17β-estradiol, p-value > 0.05 indicating that they are not significantly different statistically and hence, having the similar response, i.e. glucose blocked efficiently the catabolic responses of peptone. Similar blocking experiments were performed with more complex mixtures of substrates including carbohydrates, amino acids and peptides in soy peptone. The blocking effect of glucose was demonstrated, p-value > 0.05 indicating that the response of glucose + 17β-estradiol is not significantly different statistically to the response of glucose + soy peptone + 17β-estradiol.

All these results demonstrate that 0.1mM glucose can efficiently block hER *S. cerevisiae* catabolic responses in the other catabolites including easily assimilable substrates like fructose and peptone, which are simple mixtures of amino acids, peptides catabolised using mitochondrial enzymes. The peptone used was a casein hydrolysate, an undefined mixture of protein digestion products that include amino acids and peptides. Figure 3.33 suggests that the cells do not produce glucose from lactose until

about 4 - 5 hours of incubation time and therefore it is necessary to add external glucose 0.1 mM to block the responses to other catabolites present in the sample at the beginning of incubation. The glucose blocking experiments give the system the specificity required to analyse environmental samples, which are invariably complex mixtures of molecules. Finally, the application of the double mediated electrochemical detection system should be able to be applied to any yeast assay that results in the production of a catabolisable molecule, for example, any assay system that produces the β- galactosidase enzyme.

In the course of my studies with hER *S. cerevisiae,* a lactose-independent response to 17β-estradiol was discovered.

4.4.8 hER *S. cerevisiae* responses to 17β-estradiol in the presence and absence of lactose

Results in Figure 3.44 show the responses of hER *S. cerevisiae* to 17β-estradiol with and without lactose. Although the response with lactose is larger than the response without lactose, it was expected that there would not be any response in the samples without lactose. These results are very interesting and unexpected. As the *lac Z* reporter gene needs lactose as a substrate to act and generate electrochemical signal in hER *S. cerevisiae* these results suggest that in addition to genetically modified hER sequences in *S. cerevisiae* there must also be a native estrogen 'receptor' that can also bind estrogen and produce an electrochemical signal. Thus, the signal observed in hER *S. cerevisiae* trials with lactose is probably the response to two receptors i.e. one from a native 'receptor' and the other from the genetically modified human estrogen receptor. Because our detection system sensed two estrogen dependent responses in hER cells and therefore produce composite results, further work with the hER assay was abandoned and the estrogen sensing mechanism in wild type *S. cerevisiae* was further investigated.

4.5 Wild type *S. cerevisiae* responses to estrogens (17β-estradiol)

Experiments using wild type S. *cerevisiae* with and without lactose were performed to confirm the presence of an estradiol response in wild type cells and to demonstrate the redundancy of lactose in this technique. Results in Figure 3.45 indicate that the wild type *S. cerevisiae* show a good response to 0.037 nM and 11 nM 17β-estradiol with and without lactose. The response without lactose was 4 and 1.5 times larger at 0.037 nM and at 11 nM respectively. The reason(s) for the repression of the by 7.5 mM lactose are unknown. These results confirm the hypothesis that in hER *S. cerevisiae* the responses

that were detected to estrogens were from dual receptors; one is due to the lactose dependent genetically modified hER and the second to a lactose independent native estrogen receptor-like molecule.

Results in Figures 3.46a & b clearly demonstrate that *S. cerevisiae* show dose-dependent linear responses to the log of 17β-estradiol from 0.37 pM to 11000 pM. The calculated limit of detection and determination and EC_{50} are 3.6 fM (3.6 x 10^{-15} M) and 21 fM (2.1 x 10^{-14} M) and 51 pM (5.1 x10^{-11} M) respectively, which are very low detection limits compared to those reported for most of the yeast estrogen assays using genetically modified yeast, which have been already, discussed (Table 4.1). The dramatic decrease of the signal at concentrations higher than 11 nM 17β-estradiol observed in this study suggest that is probable that 17β-estradiol at higher concentrations is toxic to the cell and is discussed in detail in the later part of this section. However, less dramatic decrease of the signal at higher concentrations than 11 nM using other detection systems using genetically modified organisms have been reported (Bovee et al., 2004).

Simplifying the growth medium by cultivating the *S. cerevisiae* in a YEPD medium rather than a minimal defined medium was performed. Results in Figure 3.47 show that *S. cerevisiae* responded to the log of 17β-estradiol from 0.37 pM – 3067 pM in a dose-dependent manner, but the response dropped from 3067 pM suggesting that in cells grown in YEPD experience 17β-estradiol toxicity at a lesser concentration, than that in cells grown in minimal medium. This is probably because Bacto-peptone, which is one of the ingredients in YEPD medium, contains both 17β-estradiol and estrone (ng/L concentrations) and has more amount of estrone than 17β-estradiol (Feldman & Krishnan, 1995). Yeast can efficiently convert estrone that is present in Bacto-peptone to 17β-estradiol over time leading increased intracellular levels of 17β-estradiol (Feldman & Krishnan, 1995). Since the YEPD medium leads to 17β-estradiol accumulation over time, it was decided to continue to use a minimal medium to avoid this external source of 17β-estradiol contamination that could confuse the results.

4.5.1 *Candida albicans* dose dependent response to 17β-estradiol

The dose dependent responses of *C. albicans* to 17β-estradiol were investigated. Results in Figure 3.48 show *C. albicans* dose-dependent responses to the log of 17β-estradiol from 0.037 nM - 11 nM, strengthen the hypothesis that the estrogen binding protein may be playing a major role for these responses. The estimated size of estrogen binding

77protein in *S. cerevisiae* (60- 70 Kda) is different from that of well-characterised estrogen binding protein (46 Kda) of *C. albicans.* Because of the difference in size of the estrogen binding protein in these two organisms, a question is raised about the mechanism that operates in *S. cerevisiae* and *C. albicans*, which could well be a different or a common mechanism. It would be very interesting to see if the estrogen binding protein of *S. cerevisiae* has binding site/affinity for other estrogens and estrogen analogues so that the assay can be used to detect any estrogenic compounds in general other than 17β-estradiol, which is the case in environmental samples.

4.5.2 *S. cerevisiae* response to estrogen and estrogen analogues

Results in Figures 3.49, 3.50 & 3.51 demonstrate *S. cerevisiae* responded not only to 17β-estradiol, but also to other estrogens and estrogen analogues. These responses were summarised in Figure 3.52 and Table 3.16 and gave roughly similar profiles except for estrone. Estrone is more potent than 17β-estradiol only at 0.037 nM; and its trend is different from other estrogen analogues tested. Estrone could probably have given a more linear response range if tested below 0.037 nM range or the sample dilution would have overcome this. 17α-Ethynylestradiol although more potent than 17β-estradiol between 0.037 and 0.367 nM, it is 25 – 52% less potent at higher concentrations i.e. 3.67 and 11 nM. The least potent estrogen analogue is 17α –estradiol that is 50-86% less potent than 17β-estradiol at all the concentrations. These results demonstrate that 17α-estradiol is the least potent estrogen of all estrogen and estrogen analogues tested. All these results suggests that *S. cerevisiae* responses are more sensitive to the lower levels of estrogen (0.037 - 0.367 nM) and might be toxic at higher concentrations (explained in detail in next page). However, at 3.67 nM 17α-estradiol and estrone levels the responses were found to be 26% and 33% of the signal from the same concentration of 17β-estradiol. These values are close to those established in the equimolar competitive binding assays system (Burshell et al., 1984).

The significant difference in response to estrone between hER *S. cerevisiae* and wild type *S. cerevisiae* is the result of the former strain containing the human estrogen receptor α in addition to the native estrogen receptor. Hence, the responses seen are due to the dual receptor systems compared to the wild type. The sensitivity of the yeast assays is generally approximately an order of magnitude lower compared to *in vitro* bioassays based on mammalian cells (Anderson et al., 1999; Legler et al., 2002). However, the relative potencies of estrogens determined with yeast assays seem to be comparable to those determined using in vitro bioassays (Leskimen et al., 2005).

The possible reasons for the drop of electrochemical responses above 11 nM 17β-estradiol in *S. cerevisiae*

The dramatic decrease of electrochemical responses observed in *S. cerevisiae* above 11 nM 17β-estradiol could be related to the expression of estrogen transporter genes (*SNQ2* and *PDR5*). *SNQ2* is involved in cell growth, PDR5 is involved in toxicity of the cell when over expressed, and at 11 nM 17β-estradiol concentration, PDR5 might be over expressing and exerting toxic effects (Decottignies et al., 1995). Single and double mutations in these two genes have been created and the effects of growth on yeast strains harbouring single mutants either *snq2*or *pdr5* and a strain harbouring double mutants *snq2* and *pdr5* have been studied. The mutant with the disruption in the PDR5 gene reaches a higher cell density than the cells over expressing the gene, suggesting that the protein could be toxic to cells. In contrast, the disruption in SNQ2 gene results in an increase in the lag phase without changing the exponential growth rate, suggesting the role of this protein is in the transition from stationary phase to exponential growth. The double mutant deleted in both genes alters both in logarithmic and the lag phases during growth and slows the growth rate although neither of them are essential genes (Decottignies et al., 1995). Hirata et al. (1994) suggested that these transporters might extrude intracellular cytotoxic metabolites accumulated during growth.

The idea that estradiol has a direct effect on yeast growth is not new (White & Larsen, 1997). Their studies have shown that higher estradiol concentration (1 μM) has on *C. albicans* colony size and germ tube formation. It has been reported that estradiol has a direct effect on *S. cerevisiae* cell growth by regulating the cell cycle via increased levels of adenylate cyclase (cyclic AMP) mRNA in the early G1 phase (Tanaka et al., 1989). The correlation between intracellular cAMP levels and the nutritional status of cells is well established; stress and nutritional starvation result in higher intracellular levels of cAMP, which results in cells that are unable to grow on non-fermentable carbon sources and unable to sporulate (diploids). The increased intracellular cAMP signal is transmitted through the distinct signalling cAMP- PKA cascade. cAMP- PKA cascade is one of the key signalling cascades in central yeast metabolism and might be involved in the drop of signal above 11 nM estradiol. It is known that cAMP- PKA cascade involved in a variety of mammalian cancers and tumours (Gangiano, Bauer & Pretorius, 2002)

4.5.3 Proposed mechanism of estrogen binding in wild type *S. cerevisiae*

Estrogen sensitivity in 3 yeast species was demonstrated in the early 1980s. A protein designated estrogen binding protein (EBP) was isolated from *C. albicans* and its gene was also sequenced. The estrogen binding protein gene in *C. albicans* encodes an oxidoreductase enzyme activity which is inhibited > 69% by the addition of 17β-estradiol *in vitro* (Madani et al., 1994). These findings demonstrate that the absence of 17β-estradiol allows the cellular NADH oxidation to proceed resulting in a decreased level of reduced redox molecules in the cellular pool. Similar responses to 17β-estradiol have been demonstrated in *S. cerevisiae* and *C. albicans,* suggesting that an EBP is also involved in estrogen binding (Baronian & Gurazada, 2007). A model hypothesising the role of EBP in estrogen binding protein in wild type *S. cerevisiae* has been proposed by me and depicted in the next page (Figures 4.6a, 4.6b).

Proposed mechanism of estrogen binding: A possible role of EBP

Key:

EBP
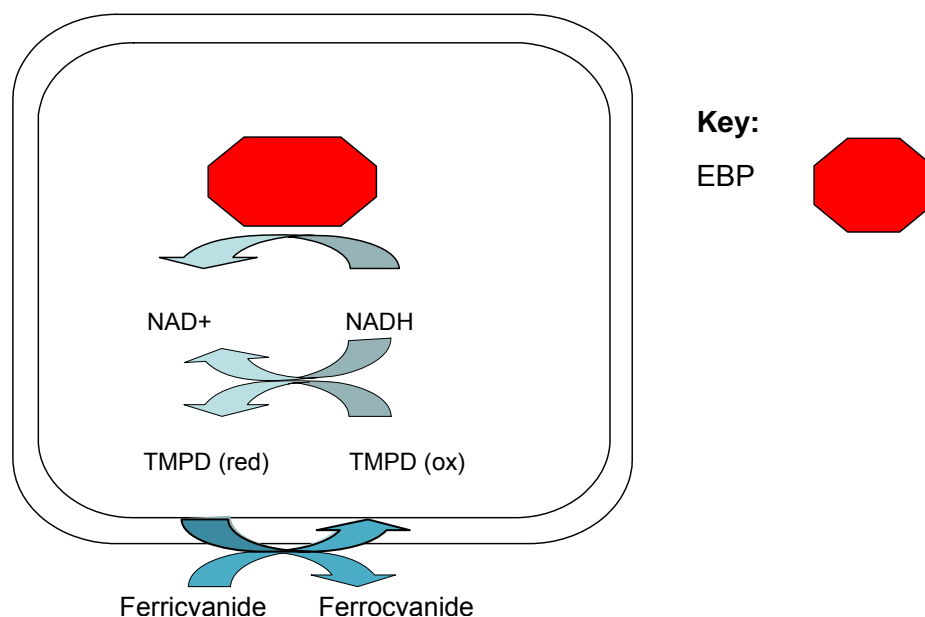
Figure 4.6a Schematic representation of EBP oxidoreductase enzyme activity and electrochemical signal production by double mediators.

Estrogen transport (Import) by SNQ2

Key

Estrogen transporter - SNQ2

Estrogen -

Steps of estrogen binding:

1 Estrogen binding to SNQ2

2 Import of Estrogen inside the cell

3 Estrogen binding to EBP
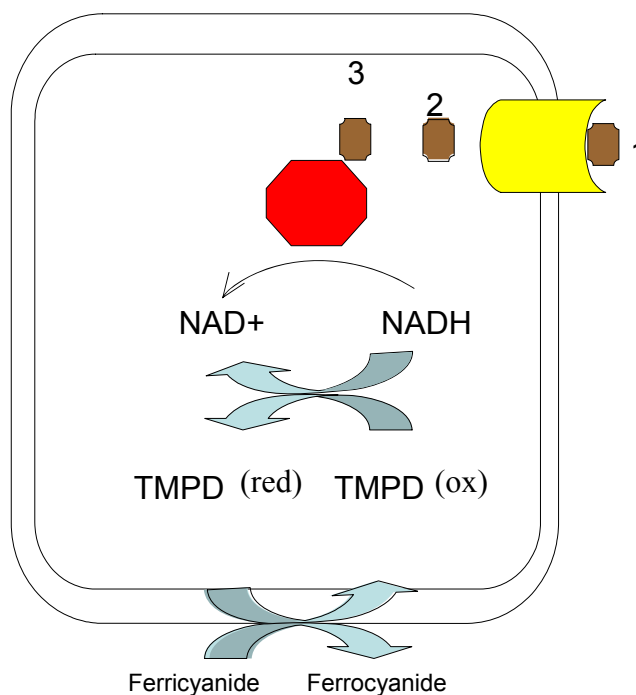
Figure 4.6b Schematic representation of 17β-estradiol import by SNQ2 into the cell, binding to EBP and inhibiting the oxidoreductase activity of EBP, resulting in more NADH. This NADH is available to reduce TMPD resulting in a larger electrochemical signal.

4.5.4 A possible role of EBP in estrogen sensing and generating an electrochemical signal

The results obtained in this study suggest that EBP possesses an NADH oxidoreductase activity and allows cellular oxidation of NADH/NADPH to proceed, perhaps decreasing the cellular pool of reduced redox molecules. The responses which were obtained in this study were large and these could not just be arise from the metabolism of estrogens, because such low concentrations of estrogens were used which, if metabolised, could not generate the large electrochemical responses that were obtained. These electrochemical responses are generated because of estrogen binding protein responses to estrogens. According to our model depicted in Figures 4.6 a and b, estrogens that are present in the sample are imported into the cell by SNQ2 which is a member of ATP dependent ABC transport cassette protein. EBP has a high affinity and specificity for estrogens, especially 17β-estradiol has a unique binding site on EBP. Once the binding site on EBP is occupied by estrogen (17β-estradiol), the EBP oxidoreductase enzyme is inhibited, resulting in increased level of cellular NADH. Increased intracellular NADH levels result in increased reduction of TMPD and subsequent generation of larger electrochemical responses. Hence, our double mediated electrochemical detection technique can be used as a tool to investigate the behaviour of EBP and its ligands (Baronian & Gurazada, 2007).

However, to confirm the role of EBP in estrogen binding and NADH oxidoreductase activity an EBP null mutant needs to be created to show that the wild type *S. cerevisiae* EBP is able to detect the estrogens whereas the mutant fails to do so. A future direction could be to isolate the EBP gene from haploid wild type *S. cerevisiae* cells and create mutants. These mutant studies would confirm without any ambiguity that EBP binds specifically to estrogen and generates the electrochemical signal.

4.5.5 Significance of specificity of EBP to estrogens and estrogen analogues develop an estrogen bioassay using wild type *S. cerevisiae*

All the reported *in vitro* and *in vivo* bioassays used in estrogen screens are based on genetically modified cells that have receptor and reporter system that guarantee specificity for a target molecule. Thus obtaining specificity to estrogens in genetically modified system is not difficult. Whereas achieving specificity in wild type yeast is difficult. The electrochemical detection system used in this study to quantify estrogens also responds to NADH/NADPH, which is produced in catabolism, and any exogenous

catabolite substrate that is present in the sample will provide a signal that is not controlled. For an estrogen screen to have an application in environmental bioassays, the system must be able to achieve the specificity for estrogens in the presence of other catabolisable substrates that are likely to be present in the environmental samples. There are methods to address this problem e.g. prepare samples in a manner that removes catabolisable molecules and concentrate the analyte. For example, the sample could be extracted with lipid solvents to exclude catabolisable molecules and to concentrate the estrogenic molecules in the samples for analysis (Schwirzerb et al., 1998; Colucci, Bork & Topp, 2001). A solid phase extraction method was reported to remove all catabolites (Komer et al., 1999).

An approach in using non-extracted samples is to exploit the glucose catabolite repression property and use glucose in the analysing sample. A large number of catabolic enzymes are repressed by the presence of glucose either by transcriptional repression or by inhibition of the uptake of an inducer molecule, which is known as glucose catabolite repression (Rose, Albig & Entain, 1991). This has been explained in detail earlier (4.4.7).

In these present studies, the glucose catabolite repression property has been exploited to block the catabolism of the other catabolisable molecules and achieve specificity for estrogens. Results in Figure 3.53 demonstrate that glucose 0.1 mM (this concentration was determined in experiments with hER *S. cerevisiae*, please refer to Figure 3.40) with concentrations up to 3.67 nM 17β-estradiol. The optimum response observed was at 0.367 nM above 0.367 nM 17β-estradiol the response dropped. Hence, it was decided to use 0.1 mM glucose and 0.367 nM of 17β-estradiol in the blocking experiments, which were used for hER *S. cerevisiae* blocking experiments. The blocking experiments used simple monosaccharide fructose and complex mixtures of molecules such as peptone. Results in Figure 3.54 and Table 3.17 demonstrate that 0.1 mM glucose and 0.367 nM 17β-estradiol responses are significantly different statistically from 0.1 mM glucose response p-value < 0.05 indicating that 0.367 nM 17β-estradiol responses were detectable and were not masked by 0.1 mM glucose. 17β-Estradiol and 0.1 mM glucose responses were comparable and were not significantly different statistically to those of glucose, fructose and 17β-estradiol p-value > 0.05 indicating that 0.1 mM glucose could efficiently block the catabolism of simple monosaccharide fructose. Results in Figure 3.55 and Table 3.19 show that the 0.1 mM glucose, 0.367 nM 17β-estradiol responses

are not significantly different statistically to glucose, 0.018% peptone and 0.367 nM 17β-estradiol (p-value > 0.05). All these results demonstrate that 0.1 mM glucose can efficiently block the catabolism of a simple monosaccharide such as fructose and complex mixtures of molecules such as peptone and provide specificity of the assay to estrogens.

Although all experiments discussed above were performed with 8 hours incubation to maintain identical experimental conditions with hER *S. cerevisiae,* the minimum incubation time that is required to obtain the maximum estrogen response was explored in this study. Results in Figure 3.56 and Table 3.18 show that the difference between the control and the sample started at 3 hours p-value < 0.05 and continued to diverge for up to 5 hours p-value < 0.05. This period provides a sufficient dynamic range for measurements and it was decided to use 5 hours incubation for estrogen analysis in environmental samples.

In this study lower detection limits were achieved than those reported for the estrogen bioassays using genetically modified organisms. The incubation time was also reduced from several days to five hours. Using wild type yeast as the biocomponent also overcomes the difficulties encountered when using genetically modified organisms as the sensor element because the need for obtaining approval to work with genetically modified organisms can be eliminated. Additionally compared with tissue and organism based assays where special equipment such as tissue-culture equipment and animal house facilities are required, preparation of wild type yeast cells is simple and requires only a basic microbiology laboratory.

4.5.6 Fungal BLAST search using *Candia albicans EBP1* gene sequence to identify EBP1 homologues in other fungal species

It is important to know the sequence similarities of *C. albicans EBP1* gene to other yeast genes to identify the related genes. A computer based fungal BLAST search was performed using *C. albicans EBP1* gene (nucleotide) and amino acid sequences to identify *EBP1* related genes such as other flavoproteins with oxidoreductse enzyme activity in *C. albicans* and other fungal species.

Table 4.2 Nucleotide sequence homology of *C. albicans EBP*1gene with other known yeast gene sequences- (Fungal genome and *EMBL/EBI* WU-BLAST 2 search)

Species	Gene/Protein	Sequence - Identity	Probability p-value	Accession Number
C. albicans	EBP1	100%	1.1 E-270	L25759.1/YSAEBP1A/ WU-BLAST-2 (Fungal Genome search).
C. albicans	NADH dependent flavin oxidase	99%	2.2 E-268	XM708963.1/WU-BLAST-2 (Fungal Genome search).
C. albicans	(Chromosome 6) 136,025	74%	1.5 E-126	NW139484.1/WU-BLAST-2 (Fungal Genome search).
C. albicans	OYE22- Potential NADH-flavin dependent oxidoreductse	75%	1.5 E-199	XM711588.1/WU-BLAST-2 (Fungal Genome search).
D.hansenii	Predicted mRNA 1,272	67%	6.9 E-86	XM458484.1/WU-BLAST-2 (Fungal Genome search)
K.lactis	*KYE1*-old yellow enzyme NADPH oxidase	61%	5.9 E-42	L37452.1/YSKKYE1 A/ WU-BLAST-2 (Fungal Genome search).
S. cerevisiae	NADH oxidoreductase	58%	5.3 E-41	L06124.1/YSCNADPH OR/WU-BLAST-2 (Fungal Genome search).
S. cerevisiae	RM-11-1a (28,078)	58%	7 E-39	AAEGO1000053.1/ WU-BLAST-2 (Fungal Genome search)
S. cerevisiae	YHR179W gene	58%	2 E-40	AY558286.1/WU-BLAST-2 (Fungal Genome search).
S. cerevisiae	OYE 3	46%	4.7 E-75	AY693226;AAT93245, 1/ WU-BLAST-2 (EMBL-EBI database)
S. carlsbergensis	*OYE 1*	57%	5.3 E-33	X53597.1/SCOYELE/ WU-BLAST-2 (Fungal Genome search).
S. *castellii*	NRRLY-12630	60%	9.4 E-41	AACF01000002.1/ WU-BLAST-2 (Fungal Genome search).
P. brasiliensis	pbEBP	Very similar	10 E-56	Fungal Molecular Biology Meeting. Nicola et al. (2005)

BLAST search (Table 4.2) resulted in 75% sequence homology to *C. albicans OYE22*, 46% homology with *OYE2 &OYE3* genes of *S. cerevisiae* and 57% sequence homology with *S. carlsbergensis OYE1* (Baronian & Gurazada, 2007). Nicola et al. (2005) used the *EBP1* sequence from *C. albicans* and searched the *P. brasiliensis* transcriptome (database) finding a cDNA, which they designated as *PbEBP*. *PbEBP* has a very high sequence similarity to *EBP1* of *C. albicans* with 1453 base pair contiguity, encoding a putative protein of 43 Kda, which is very similar in size to EBP1 and OYE of 44 and 45 Kda respectively.

4.5.7 Functional and structural similarities and differences between estrogen binding protein (EBP) and old yellow enzyme (OYE)

Buckman and Miller (1998) have reported the similarities and differences between EBP and OYE with respect to 17β-estradiol, phenol and 19-nortestosterone binding properties and oxidoreductase activities with NADPH and other compounds in *C. albicans*. In addition to a 75% sequence identity of EBP to *OYE*22 in *C. albicans* and 46% sequence homology to *OYE2* & 3 of *S. cerevisiae*, there are two functional similarities of these proteins that are interesting (Madani et al., 1994). Firstly, like EBP, OYE has been shown to bind competitively to a number of phenolic compounds, including phenol and 17β-estradiol. The second feature is that OYE also shows FMN (flavin monooxigenase) dependent oxidoreductase activity. However, in contrast to EBP, phenol binds to OYE with very high affinity and to 17β-estradiol with very much lower affinity (Buckman & Miller, 1994). In their titration studies with EBP in *C. albicans* at pH 7.3 with 17β-estradiol, that the flavin absorbance changes are quite different to phenol absorbance changes at the same pH. The K_D (dissociation constant) of EBP for 17β-estradiol at pH 7.3 is < 0.01 μM and at the same pH, the Kd of EBP for phenol is 80 μM. The K_D values are a strong measure of the ligand binding affinity, the smaller the Kd, the stronger is the binding (De Dutta et al., 2005). Hence, the dissociation constant of phenol is 8,000 times greater than that of 17β-estradiol. This suggests that 17β-estradiol has much higher binding affinity to EBP than phenol. The same group have shown that the EBP prefers NADPH to NADH with a 40-fold higher turnover rate using NADPH as the electron donor. The OYE family of proteins is growing rapidly with new members being discovered through the genome sequencing projects. Some members of the OYE family are quite distantly related to OYE, including EBP1 of *C. albicans*, the bile acid inducible flavoenzymes BaiH and BaiC (Franklund, Baron & Hylemon, 1993) and trimethylamine dehydrogenase (Boyd, 1992).

4.5.8 Phenol interference

Phenol contamination in surface water containing industrial waste is quite common (Nistor et al., 2002) owing to the release of by-products from the petrochemical industry (Batley, 1987), pulp industry and production of plastics and dyes (Allard, Renberg & Nielson., 1987). 4-Nonyl- phenol and other alkyl phenols have been shown to have estrogenic properties (Soto, Justica & Wray, 1991). Due to the toxic properties of phenol, the efficient removal of phenol and phenolic compounds from industrial aqueous effluents is of great practical significance for environmental protection (Lajtner, Erben & Klobucar, 1996).

Similar oxidoreductase enzymes such as OYE, could behave in a similar manner to EBP on binding to their ligand and effect the concentrations of NAD(P)H in the cell and this was tested using phenol, a strong ligand for OYE and a possible source of interference in environmental samples.

4.5.9 Phenol's non-interference with 17β-estradiol's response

Estrogen binding protein shares two characteristic features with Old yellow enzyme (OYE) 1. Both are oxidoreductases enzymes and both have binding sites for 17β-estradiol and phenol (Madani et al., 1994). EBP in *C. albicans* has the same binding site for 17β-estradiol and phenol i.e. competitive binding, although the former has 8000 times greater binding affinity than the latter (Buckman & Miller, 1998). Since *S. cerevisiae* is known to have both estrogen binding protein and OYE, possible interference with estrogen detection by phenol was investigated. Results in Figure 3.57 show that at both phenol concentrations tested, the responses to phenol were the same, and all were less than the control response and they are statistically significantly different (p-value < 0.05). The fact that these responses were less than endogenous (control) response, suggests that phenol may be exerting inhibitory effect to the cells. Responses to the estradiol/phenol mix were also slightly smaller than the response to the corresponding estradiol concentration suggesting that where phenol is known to exist in sample the signal from estrogens may result in an underestimation of their concentration by around ten percent. Thus because EBP has affinity for 17β-estradiol that is several thousands fold higher its affinity to phenol, it is still possible to detect 17β-estradiol in the environmental samples that contain phenol.

Possible mechanism of Phenol inhibitory effect

The phenol inhibitory effect is a general inhibitory effect that was seen even in the endogenous response. Phenol and phenolic substances are known as weak estrogenic substrates and they compete for 17β-estradiol binding sites on EBP. Once these molecules bind to the EBP, they inhibit the oxidoreductase enzyme activity by modifying the enzyme allosterically (Madani et al., 1994). Since the 17β-estradiol and phenol are not directly competing with substrate for the active site of the enzyme, but instead are binding at another site on the enzyme, the inhibition is non-competitive in terms of the actual enzyme inhibition as such. However the inhibition by phenol of 17 β -estradiol binding at the allosteric site of the EBP is competitive between phenol and 17 β -estradiol.

4.5.10 Removal of estrogenic compounds from wastewater treatment plants

Johnson and Sumpter (2001) suggested that the principal mechanism for steroid estrogens removal in activated sludge process could be adsorption and biodegradation. They suggested that 17β-estradiol (E2) is mainly removed by adsorption whereas estrone (E1) is removed by biodegradation (Johnson & Sumpter, 2001). Mastrup et al. (2001) estimated that less than 10% of natural and synthetic estrogens are removed via biodegradation processes, and although a considerable amount is adsorbed to the sludge, the majority of the compounds remain soluble in the effluent. In general for more hydrophobic compounds such as 17α-etheynylestradiol (EE2) adsorption to sludge process is likely to play a significant role in removal of these compounds from solution while for relatively weakly hydrophobic compounds such as estriol (E3) biodegradation would be a preferred method (Danish Environmental Protection Agency, 2004). In a recent Danish study on removal processes in activated sludge shows that 50 –65% of estrone (E1), 17β-estradiol (E2) and 17α-etheynylestradiol (EE2) can be expected to be adsorbed to the sludge. The degradation of these compounds was studied under aerobic and anaerobic conditions in a simulated activated sludge system. It was concluded that under anaerobic conditions, the degradation rates for E1 and EE2 were considerably (10 -20 times) lower than under aerobic conditions while the degradation of E2 was not significantly different (Danish Environmental Protection Agency, 2004). However, there are ambiguities about the mechanisms of various estrogenic compounds removal during treatment process. Filtration processes can give relatively high removal of endocrine disrupting chemicals; however membranes are also expensive and require a significant maintenance to avoid membrane clogging (Auriol et al., 2006). Treatment of

wastewater and sludge contaminated with phenols and estrogens E2 and EE2 with enzymes such as peroxidases and polyphenol oxidases (Suzuki et al., 2003., Ikehata & Nicell, 2000) is a new and interesting strategy.

4.5.11Estrogen bioassay: The double mediated electrochemical detection of total estrogenic compounds in environmental samples

Environmental Sample 1- effluent from Trickling Filters (Christchurch)

Preliminary experiments with environmental samples were performed with Trickling Filters effluent and influent supplied by the Wastewater Treatment Plant (WTP), Christchurch. The experiments with effluent in Figure 3.58a show that no estrogenic compounds were detected by this bioassay. To validate the assay methodology, spiking experiments with 0.367 nM 17β-estradiol were performed with effluent from Trickling Filters. Results shown in Figure 3.58b show that the response to effluent sample (1:7 diluted) spiked with 0.367 nM 17β-estradiol was not statistically significantly different from the response to 0.367 nM 17β-estradiol (p-value > 0.05) suggesting that the assay is working and that the results obtained by this method are authentic.

The total estrogenic compounds in sewage effluents from other parts of world have been reported to be in the range of 1- 38 ng/L (depending on the bioassay method used) whereas New Zealand results are much higher. This data is presented for comparison in (Table 4.3) in the next page.

Table 4.3 Comparison of the total estrogenic compounds concentration (EEQ) in environmental samples from different countries.

Country	Method	EEQ (Influent)	EEQ (Effluent)	Reference
Australia	Estrogen receptor (ER) binding assay	74 - 50 ng/L	1 – 4.2 ng/L	Leusch et al. (2005)
New Zealand		78 –32 ng/ L (Canterbury region)	158 – 128 ng/ L	
New Zealand	Estrogen bioassay Double mediated electrochemical detection	375 ng/L	>1ng/L	Current study (Gurazada, 2007)
Netherlands	ER-binding assay ER-CALUX assay(estrogen receptor-mediated chemically activated luciferase expression)	385 ng/L 1-120 ng/L	30 – 38 ng/L 1-16 ng/ L	Murk et al. (2002)
Japan	YES (yeast estrogen screen)	35 - 72 ng/L	4 - 35 ng/L	Onada et al. (2002)
Germany	E-screen	58 - 70 ng/ L	6 ng/ L	Korner et al. (2001)
United Kingdom	YES	20 - 80 ng/L	<3-13 ng/L	Kirk et al. (2002)

It is known in the literature that there is no consistency in the estrogenicity of the total estrogenic compounds obtained through different bioassays (Table 4.3) and the variations may be large (Murk et al., 2002 & Korner et al., 2001). These variations in bioassay methods are observed not only in the complex environmental samples but also in a defined estrogen mixture (Annexure X, Gutendrof & Westendrof, 2001). They

calculated the EEQ values for a hypothetical mixture of estrogenic compounds including 17β-estradiol, diethylstilbestrol, 4-nonylphenol and β-sistosterol using different *in vitro* assays. The results obtained through the different *in vitro* assays (Table 4.4) varied between 26 pM (yeast assays) and 302 pM (ER- β receptor binding assay) indicating that the value of the estrogenic potency of complex mixtures such as those in environmental samples and defined mixtures vary greatly in different *in vitro* assays and are thus complementary to, but not a substitute for, *in vivo* tests (Guntendrof & Westendorf, 2001).

Table 4.4 Comparison of the EEQ-values of a hypothetical mixture of estrogenic active compound in different test systems

Compound	Concentration (pM)	Luciferase reporter-gene-assay MVLN-cells	Luciferase-reporter-gene-a E-Screen assay HGELN cells		Luciferase-reporter-gene-assay yeast[a]	Competitive binding assay ER α	Competitive binding assay ER β
17- β-Estradiol	10	10	10	10	10	10	10
DES	10	12.5	80	25	6.4	17.5	13
4-Nonyiphenol	30.000	0.35	2.4	6.38	6.00	5.25	69
Bisphenol A	50.000	1.25	*9.5*	1.25	3.33	11.50	130
-Sitostero1	50.000	5.00	36.2	4.80	0.23	43.75	80
Sum of EEQ		29.13	138.10	41.43	25.96	88.00	302

The EEQ values of the different compounds in the mixture were calculated by multiplication of the relative potency of the test compounds (estradiol = 1) with the concentration of the compounds in the mixture (pM).
[a] The data of the luciferase reporter gene assay with yeast are by Gaido et al. (1997).
(From - Gutendrof & Westendrof, 2001)

Leusch et al. (2006) tested the total estrogenic compounds by estrogen receptor binding assay in effluents from municipal sewage treatment plants in Australia (6.0 ± 4.1 ng/L) and New Zealand. In New Zealand, the total estrogenic compounds in effluents from Canterbury (143 ± 15 ng/L) and Taupo/Waikato region (109 ng/ L) varied greatly from the other reported estrogenic contents in the effluents (Table 4.3). Although the wild type yeast detection system could not detect any estrogenic compounds in the effluent (>1 ng/L), the variations in the EEQ values in samples obtained through different

bioassays are known and may account for a part of the difference in the results. The important factor is that the spiking experiment with Trickling Filters effluent worked confirming that the estrogen bioassay is working.

The whole-cell estrogen bioassay developed in this research was tested to detect the total estrogenic compounds in environmental samples: Trickling Filters influent obtained from the WTP (Christchurch), Avon River water obtained from the Antigua boatshed site (Christchurch), Halswell River water from site A (Christchurch) and Tai Tapu River water (Canterbury region).

Environmental Sample 2- Trickling Filters influent from WTP (Christchurch)

Trickling Filters influent from the Wastewater Treatment plant, Christchurch was tested for the total estrogenic compounds by this bioassay method. Results in Figure 3.59a show that the Trickling Filters influent demonstrated estrogenic responses.

Quantitation of glucose present in environmental samples to correct the value of total estrogenic compounds obtained from the estrogen bioassay

It is important to quantify or remove any glucose that may be present in a sample prior to attempting to detect the total estrogenic compounds in the environmental sample (Trickling Filters influent). In this study, glucose was quantified in the samples using before quantitation of the estrogenic compounds. The Trickling Filters influent samples were incubated with glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase) enzyme and the mediator potassium ferricyanide. Glucose oxidase enzyme catalyses the initial oxidation of β-D-glucose to D-glucono-1, 5-lactone that is further hydrolysed to gluconic acid and hydrogen peroxide, in the presence of ferricyanide as an electron acceptor. The resulting reduced ferricyanide, which represents the amount of glucose present in the sample, was quantified by linear sweep voltammetry. Glucose concentration in the samples were calculated using the glucose standards (glucose standard Figure 3.61)

Quantitation of total estrogenic compounds in Trickling Filters influent

The data presented in Figures 3.59a & b to 3.61 was used to calculate the EEQ value (Chapter 3). The total estrogenic compounds in the Trickling Filters influent detected by this method are 375 ng/L. This value is expressed as 17β-estradiol equivalent concentration (EEQ). Despite reported variations among different bioassays, the EEQ value detected in Trickling Filters influent by this bioassay method is of the same

magnitude as the reported value of 385 ng/L (Murk et al., 2002) from Wastewater Treatment Plant influent in the Netherlands by using an ER-binding assay (Table 4.3). They also reported large variations in EEQ values obtained from the wastewater using various assays: ER-CALUX (influent 153 - 123 pmol/L and effluent 9 - 3 pmol/L) and ER-binding (1,463 – 1,330 pmol/L and effluent 142 – 119 pmol/L). Leusch et al. (2005) tested the total estrogenic compounds in STPs from various places in Austalia and in New Zealand using receptor-binding assay. They have tested the raw sewage, primary-treated sewage (influent) and secondary-treated sewage (effluent). Although they only identified the locations of the STP's tested to a Canterbury region, it is obvious through population size that the STP is located in Christchurch. I have compared my results with that result. The EEQ value of Trickling Filters influent from the Christchurch Wastewater Treatment Plant measured in this study, (375 ng/L) is higher than their result in influent from Christchurch Sewage Treatment Plant (72 – 32 ng/L). Their results are quite unexpected and anomalous in that the sewage influent has lower EEQ (72 – 32 ng/L) than the effluent which had undergone treatment (158 -128 ng/L). Although they have explained that due to the late reactivation of the conjugated steroids, their results with sewage influent and effluent samples from Australia contradicts it (Table 4.3) and raises the question of the reliability of their assay method (Leusch et al., 2006). However, it is well known that the concentration of total estrogenic compounds in samples varies and is dependant on the location, population size, number of people using contraceptive pills, patients on hormone therapies, treatment technologies, pH (Leusch et al., 2005) and temperature (Layton et al., 2000). Reliability and accuracy have been tested to validate the preliminary results obtained in this study the Trickling Filters influent has been tested several times using the estrogen bioassay.

Accuracy and precision of the estrogen bioassay developed

It is very important to test the accuracy and precision of any newly developed bioassay method. In this study, the estrogen bioassay that was developed to quantify estrogens has been tested for accuracy and precision by checking the total estrogenic compounds periodically over a period of 3 months during storage at -20ºC. Results presented in Figure 3.62 shows the estrogenic responses dropped from 375 ng/L to 229 ng/L in 33 days (41%). A further drop in estrogen responses was observed in another 13 days from 229 ng/L to 130 ng/L (46 days in total) and then there was little further drop (130 ng/L to 121 ng/L) after another 45 days (90 days in total) suggesting that the loss of estrogenic responses in Trickling Filters influent was rapid in the first 46 days and then

the process slowly diminished. These results are consistent with those of Murk et al. (2002). They tested estrogenic potencies in influent and effluent obtained from a Wastewater Treatment Plant in Netherlands by three different bioassays and reported that estrogen bioassay ER-CALUX show a 27% drop in estrogenic responses in samples stored at room temperature and a 11% drop in estrogenic response in samples stored at 4ºC and no drop in estrogenic responses in samples stored at -20ºC over six week period (Murk et al., 2002). The reason for the higher drop (41%) of the estrogenic activity in 33 days observed in this study compared to a lower percentage drop (27%) of estrogenic activity at room temperatures reported, is probably because they have extracted the samples and preserved them in DMSO, whereas the samples used in this study are non-extracted and unpreserved (Murk et al., 2002).

It is known that temperature has a effect on estrogen degradation as Murk et al. (2002) demonstrated a 27% and 11% loss in estrogenic activity in samples stored at room temperature and at 4ºC over six weeks period. The mechanism for the loss of estrogenic activity even at –20ºC is not well known and could be due to the degradation of estrogens. The degradation of estrogens could not be due to the microbial degradation as the samples were filter sterilised and stored. Probably it could be due to the oxidation of the estrogens due to the presence of oxygen. Nakada et al. (2004) have shown the estrogens and estrogenic compounds removal efficiencies River Water Treatment Plants (RWTP) in Japan are higher with aeration than without aeration. However, to confirm that the loss of estrogenic compounds due to oxidative degradation, a very sensitive chemical assay would be necessary to identify the degraded products with lower estrogenic activity.

However, the results achieved by testing the influent sample 5 times using this bioassay over a period of 90 days suggest the estrogen bioassay developed is reliable. The common observation in this study and Murk et al. (2002) is that the loss of estrogenic responses in environmental samples over time supports the validity of this estrogen bioassay. The robustness of this bioassay has been tested further by testing more environmental samples.

The other environmental samples tested using whole-cell based estrogen bioassay- The Avon river results in Figure 3.63 show that (as expected) .the estrogenic compounds were below the detection limit of this system.

The Halswell River was chosen as a sample site because there are some dairy farms in the area. Dairy herds comprise female animals and it was reasoned that there was a possibility that estrogens excreted in urine could enter the river. Results in Figures 3.64a and b show that this assay system detected total estrogenic compounds (EEQ) is 207 ng/L.

The reasoning for the selection of the Halswell River and the results for that site led to the sampling of a second site located in farmland that also had some dairy farming. The results for the Tai Tapu River sampling site are presented in Figure 3.65a and 3.65b. These show that although there were estrogenic compounds present, they were at a lower level than that detected in the Halswell River. The total estrogenic compounds in Tai Tapu water expressed as 17β-estradiol equivalent concentration is 29 ng/L.

4.5.12 Limitations and validation of estrogen bioassay

The validity of the estrogen bioassay developed through this study in quantifying the total estrogenic compounds in environmental samples has been justified by achieving results consistent with those of Murk et al. (2002). It is known that no two bioassays give the same value even for the hypothetical mixtures (Gutendrof & Westendrof, 2001). Hence, the EEQ values obtained through this estrogen bioassay were not directly compared with other bioassay. However it would have been further justified if the EEQ values obtained in this study by using the estrogen bioassay were compared with an instrumental analysis such as GC-MS analysis or other known bioassay. Nonetheless the GC-MS can only analyse specific individual estrogens whereas the estrogen bioassay developed in this study quantifies the total estrogenic compounds in environmental samples and the individual estrogens in the sample analysed are unknown. In this particular case, GC-MS analysis may not give an accurate value unless the individual estrogens are known. Korner et al. (2001) compared the estradiol equivalent concentrations (EEQ) obtained by E-screen and GC-Ms analysis of effluents from 16 municipal and 2 industrial sewage treatment plants in Germany (Figure 4.7).
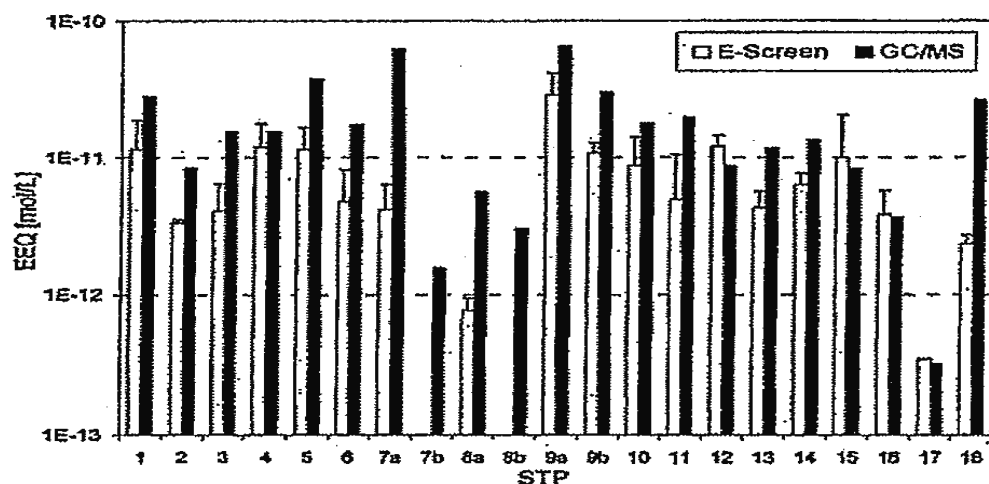
Figure 4.7 Comparison of the estradiol equivalent concentrations (EEQ) determined in the E-screen assay with those calculated from the GC-MS analysis in effluents of 16 municipal and two industrial sewage treatment plants in Baden-Wittenberg, South-western Germany.
(From Korner et al., 2001).

Results in Figure 4.7 show that the GC-MS analysis of total estrogenic compounds in several environmental samples was higher than that derived from bioassays (E-screen) by a factor of two to four, whereas no sample analysed by the bioassay was greater than GC-MS value (Korner et al., 2001).

The cost of developing the GC-MS system for estrogens analysis in a research institute in Cawthron Institute, Nelson (New Zealand) was quoted as $2,500 per sample. Owing to lack of funding, GC-MS analysis of the environmental samples tested in this study was not undertaken.

This estrogen bioassay can be successfully substituted for the estrogen bioassays involving mammalian cell-lines, tissues and genetically modified organisms for routine analysis of environmental samples for estrogenic compounds. The cost of electrochemical equipment is moderate, the system is easy to use and the assay can be completed in one day.

Although the whole-cell estrogen bioassay developed in this study offers several advantages and has the potential for commercial biosensor development, it is at a very early stage. The next step would be to compare analysis of samples tested with this bioassay with other assay systems such as a cell based bioassay and instrumental analysis like GC-MS. However, the chemical assay gives the concentration of a compound or substrate whereas the bioassay gives a measure of the bioeffect or bioresponse to a compound or substance. These two are complimentary but can require considerable interpretation.

Development of a commercial estrogen sensor based on the bioassay using the wild-type *S. cerevisiae* or *C. albicans* developed in this study requires a multi-disciplinary approach. This aspect will be discussed in future work.

4.5.13 The major contributions to the biosensors field that are described in this thesis are:

- **Application of yeast in biosensor systems**

   1. Use of yeast as a biocomponent in biosensor devices.

   2. Versatility of the double mediator system to detect the glucose dependent catabolic responses in four yeast species.

   3. Successful immobilisation of *A. adeninivorans* cells in 'Lentikats'

- **Reduction of the incubation period in the genetically modified hER *Saccharomyces cerevisiae* YES estrogen assays**.

   1. The detection time was reduced by a factor of 10 using the double mediated electrochemical detection system. In addition to the 10-fold reduction in assay time, lower detection limits were also achieved. The limits of detection, determination and the EC_{50} values of this system are of a significant improvement on the other *lac Z* based yeast estrogen assays (Table 4.1). They are equivalent to mammalian cell and tissue based assays.

- **Development of an estrogen bioassay using wild type *S. cerevisiae* for environmental application.**

   1. The oxidoreductase property of wild type *S. cerevisiae* estrogen binding protein (EBP) has been exploited in this study to detect estrogens. This is the first report of the development of an estrogen bioassay exploiting the very well characterised wild type *S. cerevisiae*.

2. In this study, glucose catabolite repression in *S. cerevisiae* has been exploited to eliminate interference from the other catabolisable molecules and to achieve specificity to estrogens, eliminating the extraction of environmental samples by means of organic solvents.

3. The preliminary results with environmental samples suggest that the double mediated electrochemical detection system has environmental applications. This system offers a simple, rapid, non-GMO based and cost-effective estrogen bioassay, which has all the advantages of other estrogen screens using cell-lines and genetically modified organisms but none of the major disadvantages.

4. The estrogen bioassay and the sensor technology knowledge are not only applicable to environmental applications but it also has potential in food and meat industry and medical diagnostics.

4.6 Conclusions

This research has been undertaken to explore whether yeast can be used as a biocomponent in whole-cell biosensor devices to detect priority environmental contaminants. Although bacteria have been the most commonly used in the biosensor devices, recent studies have shown that there are several advantages in using yeast as biocomponent. These advantages are:

- Yeast are simple eukaryotic organisms and most of the cellular process are similar to that of higher organisms, hence drug toxicity tests using yeast as the biocomponent have direct relevance to other higher organisms in toxicity screening and evaluations.
- The responses (signal sizes) also are larger (which is an advantage as the background noise is relatively reduced) when compared to bacteria because more redox sites are present and are accessible to mediators in yeast.
- Yeast are generally more robust organisms and have good longevity in storage.

Because of these advantages, yeast has been used as the biological sensing component in the development of biosensors.

It is generally agreed that there is an urgent need for the development of rapid, simple and low cost toxicity screening procedures, which can be used on-line to assess the

impact of the increasing number of toxic chemicals in the aquatic and soil environment. These are the key issues that we have addressed in this research.

4.6.1 Optimisation of the double mediator system in yeast

The double mediator system containing menadione or 2,3,5,6- tetramethyl 1,4 phenylenediamine (TMPD) and ferricyanide was shown to be efficient for probing the intracellular redox activity in living eukaryotic cells. The lipophilic mediator TMPD concentration was optimised to 0.5 mM. Although menadione was used initially, subsequent experiments with four yeast species show that substituting menadione with 0.5 mM TMPD resulted in larger responses, which are preferred because of their ability to reduce the significance of background noise. It is known that even in purified TMPD there is a small proportion of TMPD that is in the reduced form. This is oxidised by ferricyanide prior to functioning as a mediator, which gives rise to a small response, which could then be subtracted from the sample response to give a true measure of mediator reduction. The conversion of this oxidised form by ferricyanide accounts for <4% of the total response. This is constant and was ignored in this work. The generality of the application of the double mediator system was confirmed by using four different yeast species: *S. cerevisiae, A. adeninivorans*, *T. cutaneum* and *R. rubra*. The results confirmed that using the potassium ferricyanide and TMPD double mediator system offered larger electrochemical responses than potassium ferricyanide and menadione double mediator system.

4.6.2 Selection of *A. adeninivorans* as the model organism to test the "IDEA" concept

An important criterion for selecting the model yeast in toxicity tests is its ability to degrade various priority environmental contaminants. In order to monitor the respiratory/catabolic rate of the microorganism (yeast) as the measure of contaminant degradation, the organism must be able to utilise the contaminant as a source of carbon and energy. *A. adeninivorans* has been found to have an extremely broad spectrum of potential substrates. The ability of *A. adeninivorans* to assimilate a wide range of carbon compounds indicates that this organism possesses many degradative enzymes making it very useful as a biocomponent in biosensor devices.

In some organisms, self-splicing introns could be identified in the rDNA, which are characteristic features of the particular organism. 25 S rDNA from *A. adeninivorans* has been cloned (Rosel & Kunze, 1996). It is 3790 bp long and identified as a unique self splicing 410 bp intron. In this study, the authenticity of *A. adeninivorans* LS3 was

confirmed by identifying the unique 410 bp self-splicing intron within the 25 S rDNA by PCR amplification using appropriate primer design based on the DNA sequence data available in a yeast genomic database.

4.6.3 Immobilisation of *A. adeninivorans* on'Lentikats'

A. adeninivorans cells were successfully immobilised in PVAL gels using the 'Lentikats' immobilisation technique. The glucose sensitivity range has been established using immobilised *A. adeninivorans*. The limits of detection and determination of glucose were found to be 0.236 μM and 3.58 μM respectively. In this study the 'Lentikats' prepared were successfully used without significant loss of biological activity for up to 2 months.

4.6.4 Trial model environmental contaminants: gallic acid, naphthalene and di-butyl phthalate: Development of an *A. adeninivorans* based biosensor

Experiments were conducted in an attempt to develop a biosensor using *A. adeninivorans* to detect priority environmental contaminants based on the "IDEA" concept. Gallic acid gave an equivalent response in both pre-conditioned and unconditioned cells i.e. they did not respond as required for "IDEA" concept.

Pre-conditioned (naphthalene grown) *A. adeninivorans* cells show a larger response with naphthalene than with unconditioned cells (glucose grown). Although unconditioned (glucose grown) *A. adeninivorans* cells exhibited a larger response with glucose, the response of the control (without any substrate) was not the same as with naphthalene, which had been expected. These results suggest that this organism probably has a constitutive metabolic pathway to degrade naphthalene and pre-conditioning the *A. adeninivorans* cells by growing with the target molecule, i.e. naphthalene stimulates the degradative pathway, giving a larger response than unconditioned glucose grown cells. Thus, the "IDEA" concept of pre-conditioning the cells with the contaminant requires a differential response and does not work completely with naphthalene. The results show that the naphthalene limits of detection and determination that could be achieved by double mediated electrochemical method are 60 μM and 317 μM respectively. This is very high when compared with the recommended environmentally significant levels of 4 - 40 nM.

Pre-conditioned *A. adeninivorans* cells did however exhibit a full differential response to di-butyl-phthalate (DBP). The DBP limits of detection and determination achieved by the double mediated electrochemical detection method are 25 μM and 50 μM

respectively. The detection limit that could be achieved by this method is not as low as the environmentally significant level of 33 nM. In addition to the higher detection limit, the results also show that glucose depresses the DBP signal, which suggests that the presence of other catabolisable molecules such as glucose in the sample will interfere with the signal of the target molecule. Hence, this biosensor application has relevance only when there are no catabolisable molecules in the sample to interfere with the response in other words the sample should be pure and not a mixture, which is unlikely for the environmental samples.

Because of the high detection limits and interference from other catabolisable molecules in the sample, the original "IDEA" concept to detect priority contaminants has little relevance or application. This finding is in agreement with parallel work with bacteria conducted at Lincoln Ventures Limited.

4.6.5 Using genetically modified hER *S. cerevisiae* as the sensing element to detect estrogenic compounds

Increased sensitivity and specificity for environmental analytes is possible when genetically modified yeast cells are used. Examples include several yeast estrogen screens and bioassays that detect at appropriate concentrations (pM). It was decided to use the double mediated electrochemical detection system to try to reduce the long incubation period of hER *S. cerevisiae* yeast estrogen assay (YES) as the next goal of this work.

Currently a wide variety of different bioassays exist. Because *in vivo* tests are substantially more costly and time-consuming, *in vitro* assays, such as E-Screen, the YES assay or receptor binding assays have gained popularity. The YES assay is often used because of its simplicity of handling. The current YES assay based on optical detection takes 72 hours using hER *S. cerevisiae*. My studies show that the current optical detection system could be successfully substituted with the double mediated electrochemical detection system. The detection time is reduced from 72 hours to 7-8 hours. This system also achieved a limit of detection of 0.041 pM and a limit of determination of 0.42 pM and EC_{50} of 1.48 pM. This limit of detection is several times lower than the reported lowest detection for a genetically modified system. The limit of detection achieved by the mediated electrochemical detection system is a significant improvement over other hER yeast assays (lowest limit of detection is 0.07 pM (Klein et al., 1994). These values are also almost equivalent to *in vivo* mammalian cell and

tissue based assays. The system provides a simple, rapid *in vivo* detection system, which can analyse environmental samples for estrogenic substances. The specificity of the estrogen screen based on the double mediated electrochemical detection method was assessed by the ability of a range of steroids and steroid metabolites to stimulate the expression of β- galactosidase in the yeast.

Achieving specificity of the double mediated detection system to estrogens was the most challenging task in the application of the biosensor. This was achieved by the addition of glucose at 0.1 mM to block the catabolism of all other catabolisable molecules in the sample and primarily allow the organism to respond only to estrogens and the supplied glucose.

During these studies with hER *S. cerevisiae*, a lactose (the reporter molecule) independent response to estrogens was observed, which led to investigation of the native estrogen binding mechanisms in wild type *S. cerevisiae*. The lactose independent response to estrogen strongly suggests that there is a native estrogen receptor molecule that is responding and binding to estrogens and the response that was observed in hER *S. cerevisiae* is probably a result of the dual receptors, i.e. the human estrogen and the native estrogen receptor that the *S. cerevisiae* harbours.

4.6.6 Wild type *S. cerevisiae* responses to estrogens: a possible role of estrogen binding protein (EBP)

Wild type *S. cerevisiae* is of great interest because of its extensive use for industrial and commercial purposes. In addition to its commercial usage, *S. cerevisiae* has been extensively studied at the molecular level and almost the entire genome of this organism has been sequenced. These studies with wild type *S. cerevisiae* clearly demonstrated that the organism responds to estrogens and estrogen analogues. The hypothesis is that estrogen binding protein which has a unique high affinity-binding site and also possesses oxidoreductase activity, plays a role in estrogen binding and generating the electrochemical signal. The binding profiles of estrogen binding protein from *S. cerevisiae* to estrogen and estrogen analogues were tested in this work and are comparable with the estrogen binding protein (EBP) of *C. albicans* (Madani et al., 1994). In this study, the electrochemical response of *C. albicans* to 17β-estradiol has also been demonstrated. The similarity of the responses to 17β-estradiol in *C. albicans* to those in *S. cerevisiae* strengthens the hypothesis that estrogen binding protein plays a key role for these responses.

The limits of detection and determination are 3.6 fM and 21 fM respectively and the EC_{50} value is 51 pM. The limit of detection is less than those reported for most genetically modified yeast estrogen assays and equivalent to mammalian and tissue assays (Baronian & Gurazada, 2007).

Although there are some similarities in the sequences, the estrogen binding properties and oxidoreductase enzyme activity of EBP and old yellow enzyme show some significant differences in ligand binding and catalytic properties (Buckman & Miller, 1998). In addition to 46% sequence identity of EBP1 of *C. albicans* to OYE of *S. cerevisiae,* two functional features are interesting when regarding these two proteins. Like EBP, OYE has been shown to bind a number of phenolic compounds, including 17β-estradiol. However, OYE binds to 17β-estradiol with much lower affinity. In these studies, the 17β-estradiol response at environmentally significant concentrations (3.67 nM) was detectable even in the presence of phenol at very high concentrations of 367 nM. These results show that EBP has a much higher affinity for 17β-estradiol than for phenol. All of the evidence available from literature and the present studies support the notion that EBP and the old yellow enzymes are different proteins with some similarities and differences and that EBP belongs to the OYE super family of proteins.

In conclusion, the hypothesis that arises from my research is that the estrogen binding protein in *S. cerevisiae* has a strong binding affinity for estrogenic compounds and plays a major role in estrogen binding. The results obtained in my research with wild type S. *cerevisiae* are novel and a major breakthrough in the detection of estrogens where all the current yeast estrogen assays are based on genetically modified organisms. The responses that were observed in hER S. *cerevisiae* to estrogenic compounds are actually the result of the dual receptor systems, one is the native estrogen binding protein and the other is the genetically engineered human estrogen receptor.

To confirm the hypothesis that the estrogen binding protein plays a role in estrogen binding in wild type S. *cerevisiae* the following work is necessary:
- The isolation of EBP from wild type S. *cerevisiae*.
- Creation and characterisation of the *ebp* mutants that fail to respond to estrogens and estrogenic compounds.

.

4.6.7 Estrogen whole-cell bioassay - mediated electrochemical detection of estrogenic molecules in environmental samples using wild type *S. cerevisiae*

Unlike hER *S. cerevisiae*, wild type *S. cerevisiae* does not possess estrogen responsive elements and lactose reporter gene to achieve specificity to estrogenic compounds. The mediated electrochemical detection system is based on detection of a signal that can also be generated by catabolism, thus achieving specificity was a major challenge. This problem was successfully overcome and the specificity required to detect estrogenic compounds was achieved by adding glucose to efficiently block the catabolism of all catabolisable molecules that could be present in an environmental sample. Blocking the catabolism of any catabolisable molecules by adding glucose eliminated the need for extraction methods such as ethanol extraction to prepare an environmental sample for an estrogen assay.

This research has lead to the development of a whole-cell estrogen bioassay using wild type *S. cerevisiae* as the biocomponent to detect estrogenic compounds at environmentally significant levels. This bioassay has been tested for the detection of estrogenic molecules in environmental samples obtained from Trickling Filters influent and effluent from the WTP, Christchurch. The Avon River and Halswell River waters were obtained from Christchurch and Tai Tapu River water from Tai Tapu, Canterbury. This system detected total estrogenic compounds (EEQ) at the level of 375 ng/L in the influent. The effluent response was > 1 ng/L, however when the sample was spiked with 0.367 nM 17β-estradiol the response was not significantly different from the 0.367 nM response of 17β-estradiol, supporting the validity of the assay system in the detection of the total estrogenic compounds in environmental samples. The total estrogenic concentration obtained from Trickling Filters influent is higher than reported estrogenic concentrations in influents in Australia (74 – 50 ng/L) and in the Canterbury region, New Zealand (72 -32 ng/L) by Leusch et al. (2005). Although the Canterbury sample used by Leusch et al. (2005) was probably from the same source as the sample used in this study, their results are anomalous in that the EEQ value increases after treatment (from 72 -32 ng/L to 158 -128 ng/L). Considering the fact that there is no consistency in EEQ values obtained within the different bioassays methods used (Murk et al, 2002 ., Kooner et al., 2001) the value obtained in this study (375 ng/L) are close to the values obtained (385 ng/L) in ER-binding assay (Table 4.3, Murk et al, 2002). The observation that there is a 41% drop in estrogenic responses in sewage influent found during storage is consistent with the results of Murk et al. (2002) and supports the validity of this

system. The robustness of the estrogen bioassay has been established by testing influent five times and five other environmental samples.

The values obtained by this estrogen bioassay have not been compared with those of other bioassays methods for validation, because it is reported that the values obtained by these bioassays are highly variable (Murk et al., 2002., Korner et al., 2001., Gutendrof & Westendrof, 2001). Although values from chemical analysis of environmental samples for total estrogenic compounds (EEQ) have been reported to be higher than bioassays (Figure 4.7), GC-MS analysis would provide further validation of the bioassay method developed.

This is the first report of an estrogen bioassay using wild type *S. cerevisiae* to detect estrogenic molecules in environmental samples. This bioassay is simple as it uses non-GMO organism, which is rapid and reliable. Although no systematic work to determine the useful life of cells has been undertaken, cells were regularly used for up to 15 days. Cell preparation was simple and they could be cultured on a regular basis in a laboratory performing this analysis. The mediated electrochemical detection system is cost effective, needs no solvent extractions to prepare the samples for analysis, avoids the specialised laboratory equipment and expertise that is required to handle the mammalian cell assays, and eliminates the requirement to obtain approvals to import and use the genetically modified organisms that are currently used to screen estrogens. Thus, this system offers a simple, rapid cost effective detection system for detecting estrogenic compounds in the environment.

4.7 FUTURE WORK

The whole-cell based estrogen bioassay developed in this study can be improved by:

1. Storing *S. cerevisiae* cells in liquid nitrogen and reviving them whenever required, this saves a lot of time of growing cells.

2. Developing a cartridge that would eliminate glucose from the environmental sample. This would eliminate the estimation of glucose in the sample and could be used for correcting the total estrogenic compounds in the sample

3. Randez-Gal et al. (1995) have shown non-metabolisable glucose analogue 2-deoxygluxose causes catabolite repression in some yeasts. Glucose catabolite repression property was exploited in this study to control the catabolism of other catabolisable

191

molecules catabolism in the sample and achieve specificity to estrogens. This can be modified by substituting glucose with non-metabolisable glucose analogue 2-deoxygluxose. The advantage of using glucose analogue over glucose is non-metabolisable and they would not contribute to the mediator reduction.

However, it is very important to confirm the hypothesis that the estrogen binding protein in wild type *S. cereivisiae* plays a role in estrogen binding. A number of experiments were identified to confirm this. The purification and molecular characterisation of the estrogen binding protein is the essential part to develop an EBP (enzyme) based commercial estrogen sensor. Focus of future work is on the isolation, molecular characterisation, cloning and creation of mutant EBP from *S. cerevisiae.*

4.7.1 Isolation and molecular characterisation of EBP from wild type *S. cerevisiae*
Methodology of preparation of crude cell homogenate and protein purification methods from *S. cerevisiae* cell pellet was described (Zhao et al., 1995). After successful protein isolation and determining its concentration, it is necessary to confirm the authenticity of EBP by performing the ligand-binding assay described by Zhao et al. (1995). The EBP protein that is purified is sent for amino acid sequence analysis to deduce the nucleotide sequence from the sequenced protein to identify the *ScEBP* gene on the *S. cerevisiae* genome. A computer-aided BLAST search is performed using the amino acid sequences obtained to identify the gene, *ScEBP*. The deduced nucleotide sequence from ScEBP is used to design oligonucleotide primers to amplify the *ScEBP* gene in the total genomic DNA of *S. cerevisiae*. Amplified *ScEBP* gene can be cloned into the yeast cloning vector pG5 (Mandani et al., 1994) to produce sufficient quantity of gene product to enable molecular characterisation (EBP).

4.7.2 Molecular characterisation of cloned *ScEBP* gene
To study the structure and function relationship of the cloned *ScEBP* gene, it is necessary to create mutant *Scebp* that is defective in estrogen binding and compare it with wild type *ScEBP* to determine the function of wild type estrogen binding protein. Similar work to that described by Whelan and Miller (1996), Buckman, and Miller (2000) generating estrogen receptor mutants with altered ligand specificity in *C. albicans* can be undertaken in S. *cerevisiae* to refine the understanding of the binding characteristics of EBP.

4.7.3 Checking the inability of the mutant *Scebp* gene to bind to estrogenic compounds

Wild type *ScEBP* and mutant *Scebp* genes are cloned into the pG5 vector. These, and vector alone (control) need to be transformed into an EBP null mutant of *S. cerevisiae.* Then the transformed cells need to be verified for their mediated electrochemical responses to estrogenic compounds. We expect the transformants carrying wild type *ScEBP* produces a bigger responses to estrogenic compounds compared to the transformants carrying mutant *Scebp* and the vector alone (control).

After confirming the role of estrogen binding protein's role in estrogen binding and generating the electrochemical signal the next future direction would be design the enzyme based estrogen biosensor, which would eliminate the problems associated with whole-cell biosensors such as achieving specificity etc.

4.8 Development of an EBP based estrogen biosensor

The isolated EBP from either *C. albicans* or *S. cerevisiae* could be used as a sensing element in estrogen biosensor, preferably from the former as the oxidoreductase properties of the protein is well characterised, more stable and the gene encoding the protein has been cloned, sequenced and characterised at molecular level (Madani et al., 1994). Since the *EBP1* gene has been cloned and expressed in *S. cerevisiae*, the EBP can be isolated from this recombinant strain in large quantities. The essential step during biosensor design and development is the choice of the immobilisation technique for immobilising estrogen binding protein and other factors (redox mediators and surface modifying conducting polymers) on the electrode. After successful immobilization, the next step would be to use appropriate electrochemical techniques such as voltammetry to characterise the modified electrode and then use for analysis of estrogens.

Whichever technique is used for the biosensor design, the biosensor that is developed needs to be tested for its performance. Accuracy, precision and a reasonable shelf life (probably months) are three parameters that need early investigation. The development of a commercial estrogen biosensor based on the laboratory based estrogen bioassay developed in my research to quantify estrogens for environmental application is a complex process, which will need multi-disciplinary skills. At present, the research is at an early stage but the potential for successful biosensor development has been demonstrated.

As well as environmental applications, this biosensor technology may have applications for the detection of estrogens in the food industries and in medical diagnostics.

REFERENCES

Abramovitz, A. S., & Massey, V. (1976). Purification of intact old yellow enzyme using an affinity matrix for the sole chromatographic step. *J. Biol. Chem, 251*, 5321-5326.

Aitchison, J., & Brown, J. A. C. (1957). The lognormal distribution with special reference to its uses in economics. Cambridge: Cambridge University Press

Allard, A. S., Renberg, M., Nielson, A, H. (1987) Bacterial o-methylation of halogen substituted phenol. *Appl. Environ. Microbiol, 53*(4), 839-845.

Anderson, H. R., Anderson, A. M., & Arnold, S. F. et al. (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ. Health. Perspect, 107 (Suppl 1),* 89-108.

Anderson, M., & Adlercreutz, P. (1999). Evaluation of simple enzyme kinetics by response surface modelling. *Biotechnology Techniques, 13,* 903-907.

Anis, N. A., Eldefrawi, M. E., & Wong, R. B. (1993). A fibre optical biosensor for detection of imazethapyr herbicides. *J. Agric. Food Chem, 41,* 843-848.

Anis, N. A., Wright, J., Rogers, K. R., Thompson, R. G., Valdes, J. J., & Eldefrawi, M. E. (1992). A fibre optic immunosensor for detecting parathion. *Anal. Lett, 25*, 627-635.

Antiochia, R., Lavagnini, I., Pastore, P., & Magno, F. (2004). A comparison between the use of a redox mediator in solution and of surface modified electrodes in the electrocatalytic oxidation of nicotine amide adenine dinucleotide. *Bioelectrochemistry, 64*, 157-163.

Arikawa, Y., Ikebukuro, K., & Karube, I. (1998). Microbial biosensors based on respiratory inhibition. . In A. Mulchandani and K. R. Rogers (Eds.), *Enzyme and microbial biosensors: Techniques and protocols* (pp. 225-235). Totowa, NJ: Humanae Press.

Armold, R. G., DiChristina, T. J., & Hoffman, M. R. (1986). Inhibitor studies of dissimilatory Fe (III) reduction by *Pseudomonas* sp stain 200 (*Pseudomonas ferrireductase*). *Appl. Environ. Microbiol. 52*, 281-289.

Atlas, R.M. (1996). Handbook of microbiological media, second edition, CRC Press, New York.

Auret, B. J., Boyd, D. R., Robinson, P. M., Watson, C. G., Daly, J. W., & Jerina, D. M. (1971). The NIH shift during the hydroxylation of aromatic substrates by fungi. *Chem. Commun.* 1585-1587.

Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., Adams, C.D., & Surampalli, R.Y. (2006). Endocrine disrupting compounds removal from wastewater, a new challenge. *Process Biochem, 41*, 525-539.

Avramescu, A., Noguer, T., Magearu, V., & Marty, J. L. (2001). Chronoamperometric determination of D-lactate using screen-printed enzyme electrodes. *Anal. Chim. Acta 433,* 81-88.

Baker, V. A., Hepburn, P. A., Kennedy, S. J., Jones, P. A., Lea, L. J., Sumpter, J. P., & Ashby, J. (1999). Safety evaluation of phytoesterol esters, Part 1: Assessment of estrogenicity using a combination of *in vivo* and *in vitro* assays. *Food Chem. Toxicol, 37,* 13-22.

Banerjee N.B.R., & Vishwanathan L. (1981). Inhibition of glycolysis by furfural in *S. cerevisiae. Eur. J. App Microbial Biotechnol, 11*, 226-228.

Bartlett, M. S. (1947). The use of transformations. Biometrics, 3, 39-52.

Barns, S. M., Lane, D. J., Sogin, M. L., Bibeau, C, & Weisburg, W. G. (1991). Evolutionary relationships among pathogenic Candida species and relatives. *J. Bacteriology, 173*, 2250-2255.

Barrlett, P. N., Tebbutt, P., & Whitaker, R. G. (1991). Kinetic aspects of the use of modified electrodes and mediators in bioelectrochemistry. *Prog. React. Kinet, 16*, 55-155.

Baronian, K.H.R. (2004). The use of yeast and moulds as sensing elements in biosensors. *Biosens. Bioelectron, 19*(9), 953-962.

Baronian, K.H.R. (2006). Interaction of mediators with cellular redox molecules and the application of these interactions in whole-cell electrochemical biosensors and biofuel cells. In K. Riedel, G. Kunze & K. Baronian (Eds.), *Biosensors and bioassays on the basis of microorganisms*, Kerala, India: Research Signpost.

Baronian, K. H., Downard, A. J., Lowen, R. K., & Pasco, N. (2002). Detection of two distinct substrate-dependent catabolic responses in yeast cells using a mediated electrochemical method. *Appl Microbiol Biotechnol, 60*(1-2), 108-113.

Baronian, K.H.R., & Gurazada, S. (2007). Electrochemical detection of wild type *Saccharomyces cerevisiae* responses to estrogens. *Biosens. Bioelectron, 22,* 2493-2499.

Baronian, K. H., Gurazada, S., & Thomas, A. (2005). Electrochemical detection of yeast responses to catbolizable substances. *Aust. J. Chem, 58,* 270-274.

Baronti, C., Curini, R., D'ascenzo, G., Di Corcia, A., Gentili, A., & Sampri, R. (2000). Monitoring natural and synthetic estrogens of activated sludge swage treatment plants and in a receiving river water. *Environ Sci Technol, 34,* 5059-5066.

Batley, G. E. (1987). Applications of liquid chromatography with electrochemical detection to the analysis of oil shale process waters. *J. Chromatograph, 389*, 409-416.

Begley, T. H., & Hollifield, H. C. (1990). The migration of dibenzoate plasticisers and polyethylene terephthlate cyclic oligomers from microwave suspecter packaging into food-simulating liquids and food. *J. Food Prot, 53*, 1062-1066.

Benitez, F. J., Real, J. F., Acero, J. L., Leal, A. I., & Garcia, C. (2005). Gallic acid degradation in aqueous solutions by UV/H$_2$O$_2$ treatment, Fenton's reagent and the photo Fenton system. *J. Hazardous Materials, 126*(1-3)*,* 31-39.

Beresford, N., Routledge, E. J., Harris, C. A, & Sumpter, J. P. (2000). Issues arising when interpreting results from an in vitro assay for estrogenic activity. *Toxicol. Appl. Pharmacol, 162*, 22-33.

Bickerstaff, G. F. (Ed.). (1997). Immobilization of enzymes and cells, *Humanae Press,* Totowa, NJ:

Bitman, J., & Cecil, C. (1970). Estrogenic activity of DDT analogs and polychlorinated biphenyls. *J. Agric. Food. Chem. 18*, pp. 1108-1112.

Billinton, N., Barker, M. G., Michel, C. E., Knight, A. W., Heyer, W. D., Goddard, N. J., Fielden, P. R., & Walmsley, R. M. (1998). Development of a green fluorescent protein reporter for a yeast genotoxicity biosensor. *Biosens. Bioelectron, 13*, 831-838.

Blodgett, K. B., & Langmuir, I. (1937). Build-up films of barium stearate and their optical properties. *Pys. Rev, 51*, 964-982.

Bolger, R., Wiese, T. E., Ervin, K., Nestich, S., & Checovich, W. (1998.). Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ. Health Perspect, 106*, 551-557.

Bollag, J. M., Czaplick, E. J., & Minard, R. D. (1975). Bacterial metabolism of 1-naphthol. *Agric. Food Chem, 23*, 85-90.

Bond, A.M. (1994). Past, present and future contributions of microelectrodes to analytical studies employing voltametric detection. A review. *The Analyst, 119,* 1R-21R.

Bovee, T.F.H., Helsdingen, R.J.R., Koks, P. D., Kuiper, H. A., Hoogenboom, R.L.A.P., & Keijer, J. (2004a). Development of a rapid yeast estrogen bioassay, based on the expression of green fluorescent protein. *Gene, 325*, 187-200

Bovee, T.F.H., Helsdingen, R.J.R., Reitjens, I.M.C.M., Keijer, J., & Hoogenboom, R.L.A.P. (2004b). Rapid yeast estrogen screen bioassays stably expressing human estrogen receptors alpha and beta, and green fluorescent protein: a comparison of different compounds with both receptors types. *J. Steroid Biochem. Mol. Biol., 91*, 99-109.

Boyd, G., Mathews, F. S., Packman, L. C., Scrutton, N. S. (1992).Trimethylamine dehydrogenase of bacterium W3A1. Molecular cloning, sequence determination and overexpression of the gene. *FEBS letters, 308*, 271-276.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the protein-dye binding. *Anal Biochem, 72*, 248-254.

Buckman, J., & Miller, S. M. (2000). Stabilization of a novel enzyme substrate intermediate in the Y206 F Mutant of *Candida albicans* EBP1: evidence for acid catalysis. *Biochemistry, 39*, 10532-10541.

Buckman, J., & Miller, S. M. (1998). Binding and reactivity of *Candida albicans* estrogen binding protein with two steroid and other substrates. *Biochem, 37*, 14326-14336.

Burlage, R. S. (n.d.). Emerging technologies: Bioreporters, biosensors and microporbes. *Publication No. 4557, Environmental Sciences Division, Oak Ridge National Laboratory*.

Burshell, A., Stathis, P. A., Yung Do., Miller, S. C., & Feldman, D. (1984). Characterization of an Estrogen-binding Protein in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem, 259*(6), 3450-3456.

Button, D. K. (1998). Nutrient uptake by microorganisms according to the kinetic parameters from theory as related to cytoarchitecture.*Microbial & Molbiol Reviews, 62*(3), 636-645.

Cadwell, R. C., & .James. G. F. (1992). Randomization of genes by PCR mutagenesis. *PCR Methods and Application, 2*, 28-33.

Cadwell, R. C., & James, G. F. (1994). Mutagenic PCR. *PCR Met Appl, 3*, 136-140.

Cai, C. X., Liu, B., Mirkin, M. V., Frank, H. A., & Rusling, J. F. (2002). Scanning electrochemical microscopy of living cells. 3. *Rhodobacter sphaeroides*. *Anal. Chem., 74*, 114-119.

Cammann, K. (1992). Continuous pollution control by chemical sensors. *Sens. Actuators, B6*, 19-24.

Campanella, L., Favero, G., Mastrofini, D., & Tomassetti, M. (1996). Toxicity order of cholanic acids using an immobilised cell biosensor. *J. Pharm. Biomed. Anal, 14*(8-10), 1007-1013.

Campanella, L., Favero, G., Mastrofini, D., & Tomassetti, M. (1997). Further developments in toxicity cell biosensors. *Sens. Actuators B: Chemical, 44*(1-3), 279-285.

Cass, A.E.G., Davis, G. D., Francis, G. D., Hill, H.A.O., Ashton, W. J., & Higgins, J. (1984). Ferrocene-mediated enzyme electrode for amperometric determination of glucose. *Anal. Chem, 56*, 667-671.

Catterall, F. A., Murray, K., & Williams, P. A. (1971). The configuration of the 1, 2-dihydroxy-1, 2-dihydronapthalene formed in the bacterial metabolism of naphthalene. *Biochim. Biophys. Acta, 237*, 361,364.

Catterall, K. K., Gladman, C., Zhao, H., Pasco, N., & John, R. (2001). The use of microorganisms with broad range substrate utilisation for the ferricyanide-mediated rapid determination of biochemical oxygen demand. *Talanta, 55*, 1187-1194.

Catterall, K. K., Zhao, H., Pasco, N., & John, R. (2003). Development of a rapid ferricyanide mediated assay for biochemical oxygen demand using a mixed microbial consortium. *Anal. Chem, 75*(11), 2584-2590.

Cech, T. R. (1988). Conserved sequences and structures of group-I introns: Building an active site for RNA catalysis: A review. *Gene, 73*, 259-271.

Cerniglia, C. E. (1997). Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. *J Ind Microbiol Biotechnol, 19*(5-6), 324-333.

Cerniglia, C. E. (1981). Aromatic hydrocarbons: metabolism by bacteria, fungi and algae. *In Reviews in Biochemical Toxicology, 3*, 321-361.

Cerniglia, C. E., & Gibson, D.T. (1978). Metabolism of naphthalene by cell extracts of *Cunninghamella elegans*. *Arch. Biochem. Biophys, 186,* 121-127.

Cerniglia, C. E, Hebert, R. L., Szaniszlo, P. J., & Gibson, D. T. (1978). Fungal transformation of naphthalene. *Arch Microbiol, 117*(2), 135-143.

Cerniglia, C. E., & Gibson, D.T. (1977). Metabolism of naphthalene by *Cunninghamella elegans*. *Appl. Environ. Microbiol, 34,* 363-370

Chemla, Y. R., Grossman, H.L., Poon, Y., McDermott, R., Alpert, M.D et al, (2000). Ultrasensitive magnetic biosensors for homogeneous immunoaassay. *Proc. Natl Acad Sci U.S.A, 97*, 14268-14272.

Chan, C., Lehmann, M., Chan, K., Chan, P., Chan, C., Gruendig, B., Kunze, G., & Renneberg, R. (2000). Designing an amperometric thick film microbial BOD sensor. *Biosens. Bioelectron, 15*, 343-353.

Chan, C., Lehmann, M., Tag, K., Lung, M., Gotthard Kunze, Riedel, K., et al. (1999). Measurement of biodegradable substances using the salt-tolerant yeast *Arxula adeninivorans* for a microbial sensor immobilized with poly (carbamyl) sulphonate (PCS) part I: construction and characterization of the microbial sensor. *Biosens. Bioelectron, 14*(2), 131-138.

Clark, W. (1960). *Oxidation-reduction potentials of organic systems*, The Williams & Wilkins Company, Baltimore.

Clark, Jr, L. C., & Lyons, C. (1962). Electrode systems for continuous monitoring in cardiovascular surgery. *Ann, N.Y. Acad. Sci,* 102, 29-45.

Clark, M. G., Partick, E. J., & Patten, G. S. (1981). Evidence for extracellular reduction of ferricyanide by rat liver. *Biochem. J, 200,* 565-572.

Clemons, K. V., Feldman, D., & Stevens, D. A. (1989). Influence of estradiol on protein expression and methionine utilization during morphogenesis of *Paracoccidioides brasiliensis*. *J. Gen. Microbiol, 135*, 1607-1617.

Colborn, T., & Clement, C. (1992). Chemicaly-induced alterations in sexual and functional development: the wildlife/human connection. *Advances in modern environmental toxicology, 21.* Princeton, NJ:

Colborn, T., Vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect, 101*(5), 378-384.

Coldham, N. G., Dave, M., Sivapathasundaram, S., McDonnell, D. P., Connor, C., & Sauer, M. J. (1997). Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect, 105*(7), 734-742.

Colucci, M. S., Bork, H., & Topp, E. (2001). Persistence of estrogenic hormones in agricultural soils: I 17β- estradiol and estrone. *J. Environ. Qual. 30*, 2070-2076.

Crane, F. L., & Low, H. (1976). NADH oxidation in liver and fat cells plasma membranes. *FEBS Letters, 68*, 153-156.

Crane, F. L., Roberts, H., Linnane, A. W., & Low, H. (1982). Trans-plasma ferricyandie reductase in yeast *Saccharomyces cerevisiae. J. Bioenerg. Biomembranes, 14*, 191-205.

Crane, F. L., Sun, I. L., Clark, M. G., Grebing, C., & Low, H. (1985). Trans-plasma membrane redox systems in growth and development. *Biochem. Biophys. Acta, 811*, 233-264.

Cui, Y., Wei, Q., Park, H., & Lieber, C. M. (2001). Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species. *Science, 293*, 1289-1292.

Cullum, B., Griffin, G., Miller, G., & Vo-Dinh, T. (2000). Intracellular measurements in mammary carcinoma cells using fibre-optic nanosensors. *Anal. Biochem, 277*, 25-32.

Cyganski, A. (2004). Podstawy metod elektroanlitycznych (Bais of the electoranalytical methods), WNT, Warsaw (in Polish).

Daniel, H, C. (2003). *Quantitative Chemcial Analysis, 6th Edition.* W.H. Freeman. New York.

Danish Environmental Protection Agency (DEPA).2004. Degradation of estrogens in sewage treatment processes. Environmental Project.no.899. Danish Environmental Protection Agency, Danish Ministry of Environment.

Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and personal care products in the environment: agents of subtle change? Review *Environ. Health Perspect* 107 (supplement 6)), 907-938.

Decottignies, A., Lambert, L., Catty, P, Degand, H., Epping, E. A., Moye-Rowley, W. S., Balzi, E., & Goffeau, A. (1995). Identification and characterization of SNQ2 a new multidrug ATP binding cassette transporter of the yeast plasma membrane. *JBC, 270*(30), 18150-18157.

Dervakos, G. A., & Webb, C. (1991). On the merits of viable cell immobilisation. *Biotech. Adv, 9*, 559-612.

Deshpande, M. S., Rale, V. B., & Lynch, J. M. (1992). *Aureobasidium pullulans* in applied microbiology: A status report. *Enzyme Microb. Technol., 14*(7), 514-527.

De Boever, P., Demare, W., Vanderperren, E., Cooperman, K., Bossier, P., & Verstraete, P. (2001).Optimisation of a yeast estrogen screen and its applicability to study the release of estrogenic isoflavones from a soygerm powder. *Environ. Health Perspect, 109*(7), 691-697.

De, Dutta, D., Kundu, M., Mahato, S., Schiavone, M. T., Chaudhuri, S., Giri, A., Gupta, V., & Bhattacharya, S. K. (2005). Inactive enzymatic mutant proteins (phosphoglycerate mutase and enolase) as sugar binders for ribulose-1,5-bisphoshate regeneration reactors. *Microbial Cell Factories, 4:5*, 1-8.

Dipple, A., Michejda, C. J., & Weisburger, E. K. (1985). Metabolism of chemical carcinogens. *Pharmacol Ther, 27*(3), 265-296.

Di Paolantonio, C. L., & Rechnitz, G. A. (1982). Induced bacterial electrode for the potentiometric measurement of tyrosine. *Anal. Chim. Acta, 141*, 1-13.

Dowd, P. F., Duvick, J. P., & Rood, T (1997). Comparative toxicity of allelochemicals and their enzymatic oxidation products to maize fungal pathogens, emphasizing Fusarium graminearum. *Nat Toxins*, 5, 180-185.

D'Souza, S. F. (2001). Microbial biosensors. *Biosens. Bioelectron, 16*(6), 337-353.

D'Souza, S. F. (1989 a). Immobilized cells : techniques and applications. *Indian J. Microbiol, 29*, 83 - 117.

D'Urso, E. M., and Fortier, G. (1996). Albumin-poly(ethylene glycol) hydrogel as matrix for enzyme immobilization :biochemical characterization of crosslinked acid phophatase. *Enzyme Microb. Technol., 18*, 482-488.

Eaton, A. D., Clesceri, L. S., & Greenberg, A. E. (Eds.). (1995). Standard methods for the examination of water and wastewater. *19th edn. American Public Health Association,* Washington, DC.

Eaton, R. W., & Ribbons, D. W. (1982). Metabolism of Di-butyl phthalate by *Micrococcus* sp. strain 12B. *J. Bacteriol, 151*(1), 48-57.

Eide, D. J. (2000). Metal ion transport in eukaryotic microorganisms: insights from *Saccharomyces cerevisiae. Adv. Microbial. Physio, 4*, 1-38.

Ellem, K.A.O., & Kay, G. F. (1983). Ferricyanide can replace pyruvate to stimulate growth and attachment of serum restricted human melanoma cells. *Biochem. Biophys. Res. Commun., 112*, 183-190.

Elliott, J. M. (1977). Some methods for the statistical analysis of samples of benthic invertebrates. 2nd ed. Freshwater Biological Association scientific publication, no. 25. Ambleside: Freshwater Biological Association.

Eggins, B. (1996). *Biosensors, an introduction*. (np). New York: Wiley.

Emerson, J, D. (1991). Introduction to transformation.In: Fundamentals of explanatory analysis of variance (Hoglin, D. C., Mosteller, F., & Tukey, J. W. Eds.), pp. 365-400. New York: Wiley.

Engelhardt .G, Wallnofer, P. R., & Hultzinger. O. (1975). The microbial metabolism of di-n-butyl phthalate and related dialkyl phthalates. *Bull. Environ. Contam. Toxicol, 13*, 342-347.

Engvall, F., & Perlmann, P. (1972). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglbulin G. *J. Immunol, 109,* 129-135.

Ertl P., & Mikklesen, S. R. (2001). Electrochemical biosensor array for the identification of microorganisms based on lectin-lipopoly-saccharide recognition. *Anal. Chem, 73*, 4241-4248.

Ertl, P., Unterladstaetter, B., Bayer, K., & Mikkelsen, S. R. (2000a). Ferricyanide reduction by *Escherichia coli:* kinetics, mechanism, and application to the optimization of recombinant fermentations. *Anal Chem, 72*(20), 4949-4956.

Ertl, P., Robello, E., Battaglini, F., & Mikkelsen, S. R. (2000b). Rapid antibiotic susceptibility testing via electrochemical measurement of ferricyanide reduction by *Escherichia coli* and *Clostridium* sporogenes. *Anal Chem, 72*(20), 4957-4964.

Fang, H., Tong, W., Perkins, R., Soto, A. M., Prechtl, N. V., & Sheehan, D. M. (2000). Quantitative comparison of in vitor assays for screening estrogenic activities. *Environ Health Perspect, 108*, 723-729.

Faouzi, M. A., Dine, T., Luyckx, M., Gressier, B., Goudalizer, F., Malevais, M. L., Brunet, C., Cazin, M., & Cazin, J. C. (1994). Leaching of diethylhexylphthalate from PVC bags to intravenous teniposide solution. *Int. J. Pharm, 105*, 89-93.

Feldman, D., Do, Y., Burshell, A., Stathis, P., & Loose, D. A. (1982). An estrogen-binding protein and endogenous ligand in *Saccharomyces cerevisiae*: Possible hormone receptor system. *Science, 218*, 297-298.

Feldman, D., & Krishnan, A. (1995). Estrogens in unexpected places: possible implications for researchers and consumers. *Environ Health Perspect, 103*(7), 129-133.

Felix, H. R. (1982). Permeabilized cells. *Anal Biochem, 120*, 710-715.

Ferris, J. P., Fasco, M. J., Stylianopoulou, F. L., Jerina, D. M., Daly, J. W, & Jeffrey, A. M. (1973). Monooxygenase activity in *Cunninghamella bainieri* : Evidence for a fungal system similar to liver microsomes. *Arch Biochem Biophys, 156*, 97-103.

Ferris, J. P., MacDonald, L. H., & Patrie, M. A. (1976). Aryl hydrocarbons hydroxylase activity in fungus *Cunninghamella bainieri*: Evidence for presence of cytochrome P-450. *Arch Biochem Biophys, 175*, 443-452.

Filali-Meknassi, Y., Auriol, N., Adams, C., & Tyagi, R.D. (2004). Natural and synthetic hormones removal by enzymatic degradation. In: Proceedings of 20th Canadian Association on water quality (CAWQ).

Fleschin, S., Bala, C, Bunaciu, A. A., Panait, A., & Aboul-Enein, H. Y. (1998). Enalapril microbial biosensor. *Prep. Biochem. Biotechnol, 28*, 261-269.

Franklund, C. V., Baron, S. F., & Hylemon, P. B. (1993). Characterization of the *baiH* gene encoding a bile-acid-inducible NADH:flavin oxidoreductase from *Eubacterium* sp strain VPI 12708. *Journal of Bacteriology, 175*, 3002-3012.

Fultz, M. L., & Durst, R. A. (1982). Mediator compounds for the electrochemical study of biological redox systems: a compilation. *Anal.Chim.Acta, 40*, 1-18.

Fukumaki, T., Inoue, A., Moriya, K., & Horikoshi, K. (1994). Isolation of a marine yeast that degrades hydrocarbons in the presence of organic solvent. *Biosci. Biotechnol. Biochem., 58*(10), 1784-1788.

Gagiano, M., Bauer, F. F., & Pretorius, I. S. (2002). The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. *FEMS Yeast Research, 2*(4), 433-470.

Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J., & McDonnell, D. P. (1997.). Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol., 143*, 205-212.

Gaisford, W. C., Richardson, N. J., Haggett, B. G., & Rawson, D. (1991). Microbial biosensors for environmental monitoring. *Biochem.Soc.Trnas, 19*, 15-18.

Gancedo, J. M., & Lagunas, R. (1973). Contribution of the pentose phosphate pathway to glucose metabolism in Saccharomyces cerevisiae: a critical analysis of the use of labelled glucose. *Plant Sci. Let., 1*, 193-200.

Gancedo. J. M. (1998). Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev, 2*(2), 334-361.

Gienow, U., Kunze, G., Schauer, F., Bode, R., & Hofemeister, J. (1990). The yeast genus *Trichosporon* spec. LS3: molecular characterization of genomic complexity. *Zbl. Microbial, 145*, 3-12.

Glatz, R.V., Leifert, W.R., Bailey, K., Cooper, T.H., Barton, C.S., Martin, A.S., Alola, A., Bucco, O., Waniganayake, L., Wei, G., Raguse, B., Wieczorek, L., & McMurchie, E.J. (2006). Cell-free receptor based biosensors. *Nanoscience and Nanotechnology International Conference, ICONN,* 06.

Goldenberg, H. (1982). Plasma-membrane redox activities. *Biochem. Biophys. Res. Commun., 694*, 203-223.

Gonzalez-Garcia, M. B., Fernandez-Sanchez, C and Costa-Garcia, A. (2000). Colloidal gold as an electrochemical label of streptavidin-biotin interaction. *Biosens Bioelectron, 15*, 315-321.

Gorton, L., BremLe, G., Csoeregi, E., Joensson-Peterson, G., & Person, B. (1991). Amperometric glucose sensors based on the combination of immobilized glucose-oxidizing enzymes and chemically modified electrodes. *Anal. Chim. Acta, 249*, 43-54.

Grund, A.J., & Gunsalus, A.J. (1983). Cloning of genes for naphthalene metabolism in *Pseudomonas putida, J. Bacteriol, 156* (1), 89-94.

Gutendorf, B., & Westendorf, J. (2001). Comaprison of an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology, 166,* 79-89.

Hadjipetrou, L. P., Gray-Young, T., & Lilly, M. D. (1966). Effect of ferricyanide on energy production by *Escherichia coli. J. Gen. Microbiol, 45*, 479-488.

Hahn, T., Tag, K., Riedel, K., Uhlig, S., Baronian, K., Gellissen, G., & Kunze, G. (2006). A novel biosensor based on recombinant *Arxula adeninivorans* cells. *Bisoens. Biolelectorn , 21*(11)*,* 2078-2085.

Hall, E.A.H. (1992). Overview of Biosensors and Chemical sensors, optimising performance through polymeric materials. In P. G. Edelman & J. Wang (Eds.), *ACS Symposium Series*, *487*, 1-15.

Halling-Sorensen, B., Nielson, S. N., Lanzky, P. F., Ingerslev, F., Holten Lutzhoft, J., & Jorgensen, S. E. (1998). Occurrence, fate and effects of pharmaceutical substances in the environment: A review. *Chenmosphere, 35*, 357-393.

Hampton, R.E., & Havel, J.E. (2006). Introductory biological statistics. 2nd Edition, WAVE LANdD PRESS INC, USA.

Harris, C. A., Henttu, P., Parker, M. G., & Sumpter, J. P. (1997). The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect, 105*(8), 802-811.

Haruyama, T. (2003). Micro-nanobiotechnology for biosensing cellular responses. *Adv.Drug Deliv Rev., 55*, 393-401.

Heiskanen, A., Yakovleva. J., Spegel, C., Taboryski, R., Koudelka-Hep, M., Emneus, J., & Ruzgas, T. (2004). Amperometric monitoring of redox activity in living yeast cells: comparison of menadione and menadione sodium bisulfite as electron tranfer mediators. *Electrochem. Commun, 6*(2), 219-224.

Heitkamp, M. A., & Cerniglia, C. E. (1989). Polycyclic aromatic hydrocarbon degradation by a Mycobacterium sp. in microcosms containing sediment and water from a pristine ecosystem. *Appl Environ Microbiol, 55*(8), 1968-1973.

Heineman, W. R., & Kissinger, P. T. (1996). *Laboratory Techniques in Electroanalytical Chemistry* (2nd ed.). New York: Marcel Dekker, Inc.,1996. Ch. 12 by Adrian Michael and R. Mark Wightman is on microelectrodes.

Hill, H.A.O. (1987). Bioelectrochemicstry. *Pure & Appl. Chem, 59*(6) 743-748.

Hilscherova, K., Kurunthachalam Kanan, M. M., Blankenship, A. L., & Giesy, J. P. (2000). Cell bioassays for detection of aryl hydrocarbons (AhR) and estrogen receptor mediated activity in environmental samples. *ESPR- Environ. Sci. & Pollut. Res., 7*(3), 159-171.

Hikuma, M., Kubo, T., Yasuda, T., Karube, I., & Suzuki, S. (1980). Ammonia electrode with immobilized nitrifying bacteria. *Anal. Chem*, 52, 1020-1024.

Hirata, D., Yano, K., Miyahara, K., & Miyakawa, T. (1994). *Saccharomyces cerevisiae* YDR1, which encodes a member of the ATP-binding cassette (ABC) superfamily, is required for multidrug resistance. *Crr. Genet, 26*(4), 285-294.

Holzer, H. (1984). Mechanisms and function of reversible phosphorylation of fructose-1,6-bisphosphate in yeast. In P. Cohen (Ed.), *Molecular aspects of cellular regulation 3* (pp. 143-144).

Hoyle, M. H. (1973). Transformations—An introduction and a bibliography. International Statistical Review, 41, 203-223.

Ikehata, K., & Nicell, J.A. (2000). Characterization of tyrosinase for the treatment of aqueous phenols. *Bioresour Technol, 74,* 191-199.

Jakel, M., Buhr, A., Willke, T., & Vorlop, K. D. (1998). Immobilization of biocatalysts in Lentikats. *Chem.Eng.Technol, 21*(3), 275-278.

Jain, K. K. (2003). Nanodiagnostics: Application of nanotechnology in molecular diagnostics. *Expert rev. Mol. Diagn, 3*, 153-161.

Jiang, H. (2000). Metabolic responses trigger glucose-induced inactivation of maltose perm ease in *Saccharomyces. J. Bacteriology, 182*, 647-654.

Jianrong, C., Yuqing, M., Nongyue, H., Xiaohua, W., & Sijiao, L. (2004). Nanotechnology and biosensors. *Biotechnol. Advan, 22*(7), 505-518.

Jobling, S., Reynolds, T., White, R., Parker, M. G., & Sumpter, J. P. (1995). A variety of persistent chemicals, including some phthalate plasticisers, are weakly estrogenic. *Enviorn Health Perspect, 103*, 582-587.

Johnson, A.C., & Sumpter, J.P. (2001). Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environ Sci Technol. 35,* 4697-4703.

Jones, C. W. (1981). *Biological energy conservation* (2nd ed.). New York: Champman and Hall.

Joshi, N., T., & D'Souza, S. F. (1999). Immobilization of activated sludge for the degradation of phenol. *J. Environ. Sci. Health Part A Environ. Sci. Engng, 34*, 1689-1700.

Juberg, D. L. (2000). An evaluation of endocrine modulations:implications for human health. *Ecotoxicol. Environ. Saf, 45*, 93-105.

Jung, H.J., Yum, S.H., Lee, J.H., kwon, D.H., & Kang, S.W. (2004). Fiber optic fluoro-immunosensor for detection of *Salmonella* based on evanescent wave absorption. *TENCON 2004 Conference, 21(4),* 668-671.

Kalab, T., & Skaldal, P. (1994). Evaluation of mediators for development of amperometric bioelectrodes. *Electroanalysis, 6*, 1004-1008.

Kang, K. S., Cho, S. D., & Lee, Y. S. (2002). Additive estrogenic activities of the binary mixtures of four estrogenic chemicals in recombinant yeast expressing human estrogen receptor. *J. Vet. Sci, 3*(1), 1-5.

Kano, K., Ohgaru, T., Nakase, H., & Ikeda, T. (1996). Electrochemical evaluation of redox enzyme reaction kinetics based on mediated electrocatalysis in solution. *Chem. Lett*, 439-441.

Karube, I., Matsunga, T., Mitsuda, S., & Suzuki, S. (1977). Microbial electrode BOD sensors. *Biotechnol. Bioeng, 19*, 1535-1547.

Katayama-Hirayama, K., Tobita, S., & Hirayama, K. (1992). Aromatic degradation in yeast *Rhodotorula rubra. Water sci Technol, 26*, 773-781.

Keynes, J. M. (1921). A treatise on probability. London: Macmillan.

Keyser, P., Pujar, B. G., Eaton, R. W., & Ribbons, D. W. (1976). Biodegradation of the phthalates and their esters by bacteria. *Environ Health Perspect, 18*, 159-166.

Kichmer, C, J. (1988). Estimations of detection limits environmental analytical procedures. In L. A. Currie (Ed.), 'Detection in analytical chemistry: importances,therory and practices'. *American Chemical Society,* 79-83. Washington, DC:

Kim, Y. H., Lee, J., Ahn, J. Y., Gu, M. B., & Moon, S. H. (2002). Enhanced degradation of an endocrine-disrupting chemical, butyl benzyl phthalate, by *Fusarium oxysporum f.* sp. pisi cutinase. *Appl Environ Microbiol, 68*(9), 4684-4688.

Kim, B. H., Kim, H. J., Hyun, M. S., & Park, D. H. (1999). Direct electrode reaction of Fe (III) -reducing bacterium. *Shewanella putrefaciens. J.Micorbial. Biotechnol, 9*, 127-131.

Kim, T. S., Kim, H. Y., & Kim, B. H. (1990). Petroleum desulfurization by *Desulfovibrio desulfuricans* M6 using lectrochemically supplied reducing equivalent. *Biotechnol. Lett*, 12, 747-760.

Kinsman, O. S., Pitblado, K., & Coulson, C. J. (1988). Effect of mammalian seteroid hormones and leutinizing hormone on the germination of *Candida albicans* and implications for vaginal conditions. *Mycosis, 31,* 617-626.

Klein, K. O., Baron, J., Colli, M. J., Mcdonnell, D. P., & Cutler, G. B. Jr. (1994). Estrogen levels in childhood determined by an ultrasensitive recombinant cell bioassay. *J. Clin. Invest. Inc., 94,* 2475-2480.

Kodama, T., Ding, L., Yoshida, M., & Yajima, M. (2001). Biodegradation of an s-triazine herbicide, simazine. *J. Mol. Catal.B: Enzymatic, 11,* 1073-1078.

Koenig, A., Zaborosch, C., & Spener, F. (1997 a). Microbial sensors for PAH in aqueous solutions using solubilizers: In J. Gottlieb, H. K. Huck& R. Niessner (Eds.), *Field Screening Europe,* (pp. 203-206). The Netherlands: Kluwer Academic Publishers.

Korner, W., Hanf, V., Schuller, W., Kempter, C., Metzger, J., & Hagenmaier, H. (1999). Development of a sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. *Sci Total Environ, 225,* 33-48.

Korner, W., Spengler, P., Bolz, U., Schuller, W., Hanf, V., & Metzger, J. W. (2001). Sunstances with estrogenic activity in effluents of sewage treatment palnts in Southwestern Germany:2. Biological Analysis. *Environ Toxicol & Chem, 20*(10), 2142-2151.

Kouourtzidou, E., Corgalci, I., Matzavinos, I ., & Marios, T (2005). Anaerobic biodegradability of gallic acid found in olive mill wastewaters. *J. Chemical Toxicol Biotechnol, 81*(9), 1594-1599.

Kozak, R.G., D' Haese, I., Verstraete, W (2001). Pharmaceuticals in the environment focus on 17α-ethinylestradiol. In: Kummerer, K, editor. Pharmaceuticals in the environement. Sources, fate, effects and risk. Heidelberg, Germany: Springer Verlag, 49-65.:

Kulberg, J. M., & Chistensen, H. N. (1979).Electron-transferring enzymes in the plasma membrane of the Ehrlich ascites tumor cell. *Biochemistry, 18,* 1525-1530.

Kumar, V., Ramakrishna, S., Teeri, T. T., Knowles, K. C., & Hartley, B. S. (1992). *Saccharomyces cerevisiae* cells secreting an Aspergillus Niger β-galactosidase grow on whey permeate. *Biotechnol, 10,* 82-85.

Kunze, G., & Kunze, I. (1994). Characterization of*Arxula adeninivorans* strains from different habitats. *Antonie van Leeuwenhock, 65,* 29-34.

Kupier, G. G., Lemmen, J.G., Carlsson, B., Corton, J. C., Safe, S. H., van der Sagg, P. T., et al. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology, 139*(10), 4252-4263.

Lajtner, J., Erben, R & Klobucar, G.I.U.V., Histopathological effects of phenol on the digestive gland of Amphimelania holandri Fér. (gastropoda, prosobranchia), *Bull. Environ. Contam. Toxicol.* **57** (1996), pp. 458–464.

Layton A.C., Gregory B.W., Seward J.R., Schultz T.W., et al. (2000). Mineralization of steroidal hormones by biosolids in wastewater treatment systems in Tennessee *U.S.A. Environ. Science and Technol; 34*,3925-3931.

Legler, J., Dennekamp, M., Vethaak, A. D., Brouwer, A., et al. (2002). Detection of estrogenic activity in sediment-associated compoundsusing *in vitro* reporter gene assays. *Sci. Total. Environ, 293,* 69-83.

Lehmann, M., Chan, C., Lo, A., Lung, M., Tag, K., Kunze, G., et al. (1999). Measurement of biodegradable substances using the salt-tolerant yeast *Arxula adeninivorans* for a microbial sensor immobilized with poly(carbamoyl)sulfonate (PCS): Part II: application of the novel biosensor to real samples from coastal and island regions. *Biosensors and Bioelectronics, 14*(3), 295-302.

Leskinen, P., Michelini, E., Picard, D., Karp, M., & Virta, M. (2005). Bioluminescent yeast assays for detecting estrogenic and androgenic activity in different matrices. *Chemosphere [In Press].*

Leuisse, E., & Labbe, P. (1993). Reductive iron assimilation in *Saccharomyces cerevisiae.* In G. Winkleman & D. R. Winge (Eds.), *Metal ions in fungi* (pp. 149-178). New York: Marcel Dekker.

Leuisse, E., & Labbe, P. (1992). Iron reduction and trans-plasma membrane electron transfer in the yeast *Saccharomyces cerevisiae. Plant Physiol, 100*, 769-777.

Leusch, F.D.L., Chapman, H. F., van den Heuvel, M. R., Tan, B.L.L., Gooneratne, S. R., & Tremblay, L. A. (2006). Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. *Ecotoxicol. Environ. Saf, 65* (3), 403-411.

Limoges, B., & Saveant, J. (2003). Cyclic voltammetry of immobilized redox enzymes. Interference of steady-state and non-steady state Michaelis-Menten kinetics of the enzyme-redox co-substrate system. *J. Electroanal. Chem, 549*, 61-70.

Liu, B., Cheng, W., Rotenberg, S. A., & Mirkin, M. V. (2001). Scanning electrochemical microscopy of living cells- Part-2. Imaging redox and acid basic reactivities. *J. Electroanal. Chem. Interef. electrochemistry, 500*, 590-597.

Liu, J., Bjornsson, L., & Mattiasson, B. (2000). Immobilised activated sludge based biosensor for biochemical oxygen demand measurement. *Biosens Bioelectron, 14*, 883-893.

Liu, R., Zhou, J. L., & Wilding, A. (2004). Simultaneous determination of endocrine disrupting phenolic compounds and steroids in water by solid-phase extraction–gas chromatography–mass spectrometry *J. Chromatogr. A, 1022*, 179-189.

Liu, J & Lu, Y. (2004). Biosensors based on DNAZyme-Assembled gold nanoparticles.

J. Fluorescence, 13, 4, 343-354.

Liu, Z., & Butow, R. A. (1999). A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. *Molecular and Cellular Biology, 19*(10), 6720-6728.

Lloyd-Jones, G. (2002). Biosensors: Bacterial approaches. In *Landcare Research Contract Report: LC0102/122* prepared for Biosensor Consortium, June 2002. Lincoln, New Zealand: Landcare Research New Zealand Ltd.

Loftus, N. J., Laird, W.J.D., Steel, G. T., Wilks, M. F., & Woollen, B. H. (1993). Metabolism and pharmacokinetics of deuterium-labeled di-2-(ehthylhexyl) adipate (DEHA) in humans. *Food Chem Toxicol, 31*, 609-614.

Logan, B. E., & Wagneseller, G. A. (1993). The HBOD test a new method for determining biochemical oxygen demand. *Water. Environ. Res,* 65(7), 862-868.

Loose, D. S., Stover, E. P., Restrepo, A., Stevens, A. D., & Feldman, D. (1983). Estradiol Binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis. Proc. Natl Acad Sci U.S.A, 80*, 7659-7663.

Lutz, W. K., Gaylor, D. W., Conolly, R. B., & Lutz, R. W. (2005). Nonlinearity and threshholds in dose-responsive relationships for carcinogenicity due to sampling variation, logarithmic dose scaling or small differences in individual susceptability. *Toxicol & Appl Pharmacol, 207* (2), 565-569.

Ly, J. D., & Lawen, L. A. (2003). Transplasma membrane electron Transport: enzymes involved and biological function. *Redox Report 8*, 3-21.

Machala, M., & Vondracek, J. (1998.). Estrogenic activity of xenobiotics. *Vet. Med-Czech., 10*, 311-317.

Madani, N. D., Malloy, P. J., Rodriguez-Pombo, P., Krishnan, A. V., & Feldman D. (1994). *Candida albicans* estrogen-binding protein gene encodes an oxidoreductase that is inhibited by estradiol. *Proc. Natl Acad Sci U.S.A, 91*(3), 922-926.

Magee, B. B., D' souza, T. M., & Magee, P. T. (1987). Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species**.** *J. Bacteriology, 169*, 1639-1643.

Mahadevan, A., & Muthukumar, G. (1980). Aquatic Microbiology with reference to Tanin degradation. *Hydrobiologia, 72, 1-2,* 73-79.

Mahe, Y., Lemoine, Y., & Kuchler, K. (1996). The ATP binding cassette transporters Pdr5 and Snq2 of a *Saccharomyces cerevisiae* can mediate transport of steroids in vivo. *J Biol. Chem, 271*(41), 25167-25172.

Malone, K. E. (1993). Diethylstillbestrol (Des) and breast cancer. *Epidemiologic Reviews, 15,* 108-109.

Malloy, J. P., Xi Zhao, Madani, N. D., & Feldman, D. (1993). Cloning and expression of the gene form *Candida albicans* that encodes a high-affinity corticosteroid-binding protein. *Proc. Natl Acad Sci U.S.A, 90*, 1902-1906.

Marty, J. L., Olive, D., & Asano, Y. (1997). Measurement of BOD-Correlation between 5-day BOD and commercial BOD biosensor values. *Environ Technol, 18*, 333-337

Mascini, M., & Memoli, A. (1986). Comparison of microbial sensors based on amperometric and potentiometric electrodes. *Anal. Chem. Acta., 182*, 113-112.

Mastrup, M., Gensen, R.L., Schafer, & A.I., Khan, S (2001). Fate modeling-An important tool for water recycling. In: Schafer A.I., Sherman, P., Waite, T.D, Editors. Recent advances in water recycling technologies. Australia: Brisbane; 103 -112.

Mayer, I., Koch, R., & Puls, J. (2007). Chemical investigations on boiling process waters in face veneer production. *Holtz, 65*(4), 3-10.

Mercure, S., Montplaisir, S., & Lemay, G. (1993(b)). Correlation between the presence of a self-splicing intron in the 25S rDNA of *C. albicans* and stains susceptibility of 5-flurocytosine. *Nucleis Acids Research, 21*, 6020-6027.

Merker, M. P., Bongard, R. D., Kettenhofen, N. J., Okamoto, Y., & Dawson, C. A. (2002). Intracellular redox status affects trans-plasma membrane electron transport in pulmonary endothelial cells. *Am. J. Physiol Lung Cell Mol Physiol, 282*(1), L36-L43.

Meyer, M. T., Bumgarner, J. E., Varns, L. J., Daughtridge, J. V., Thurman, E. M., & Hosteler, K. A. (2000). Use of radioimmunoassay as a screen for antibiotics in confined animal feeding operations and confirmation by liquid chromatography/mass spectrometry.*Total Enviorn., 248*, 181-187.

Michaelis, L & Menton, M. (1913). The kinetics of invertase actiivity. *Biochemische Zeitschrift, 49,*333.

Middlehoven, W. J., de Jong, I. M., & de Winter, M. (1991). *Arxula adeninivorans*, a yeast assimilating many nitrogenous and aromatic compounds. *Antonie Van Leeuwenhoek, 59*(2), 129-137.

Middlehoven, W. J., Hoogkamer-Te Niet, M. C., & Kreger-van Rij, N.J.W. (1984). *Trichosporon adeninovorans* sp.nov., a yeast species utilizing adenine, xanthine, uric acid, putrescene and primary n-alkylamines as sole carbon, nitrogen and energy. *Antonie van Leeuwenhoek, 50*, 369-378.

Miller, E. C., & Miller, J. A. (1974). Biochemical mechanisms of chemical carcinogenisis. *Molecular Biology of Cancer*, 377-402.

Moiroux, J., & Elving, P. J. (1979). Adsorption phenomenon in the NAD+/NADH system at glassy carbon electrode. *J. Electroanal. Chem. Interef. electrochemistry, 102*, 93-108.

Morris, K.C.K., Zhao, H., Pasco, N., & John, R. (2001). Ferricyanide mediated biochemical oxygen demand-development of a rapid biochemcial oxygen demand assay. *Anal Chim Acta, 442*, 129-139.

Murk, A., Legler, J., van Lipzig, M., Meerman, J., Belfroid, A., & Spenkelink, A. (2002). Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. *Environ Toxicol Chem, 21*(16-23).

Murray, R. W., Eving, P. J., & Durst, R. A. (1987). Chemically modified electrodes. Molecular design for electroanalysis. *Anal.Chem, 59*, 379A-390A.

Myers, C. R., & Myers, J. M. (1992). Localization of cytochromes to the outer membranes of anaerobically grown *Shewanella putrefaciens* MR-I. *J. Bacteriology, 174*, 3429-3438.

Nakada, N., Komori, K., Suzuki, Y., Kawano, H., Nagato, Y., Tanaka, H. (2004). Evaluation of the river water treatment facilities for reduction of estrogenic compounds. China -Japan joint symposium on Environmental Chemistry, 2004-10.

Nakagawa, Y., Suzuki, T., & Tayama, S. (2000). Metabolism and toxicity of benzophenone in isolated rat hepatocytes and estrogenic activity of its metabolism in MCF-7 cells. *Toxicology, 156,* 27-36.

Nakamura, H., & Karube, I. (2003). Current research activity in biosensors (Review). *Anal. Bioanal. Chem, 377*, 466-468.

Nelson J. A., Struck, R. F., & James, R. (1978). Estrogenic activities of chlorinated hydrocarbons. *J. Toxicol. Environ. Health., 4*, pp. 325-339.

Nelson, K. H., & Saffarini, D. (1994). Iron and Manganese in anaerobic respiration: environmental significance, physiology and regulation. *Annu. Rev. Micorbiol, 48*, 311-342.

Neter, J., Kutner, M, H., Nachtsheim, C, J., & Wasserman, W. (1996). *Applied linear statistical models* (4th ed.). Illinois, USA: Irwin.

Nicola, A. M., Andrade, R.V., Felipe, M.S.S. (2005). The *Paracoccidioides brasiliensis* estradiol binding protein homolog is differentially expressed in the yeast phase. *Revista do Instituto de Medicina Tropical de Sa'o Paulo- Fungal Molecular Biology Conference, Abstract*(08.042).

Niino, Y. S., Chakraborty, S., Brown, B. J., & Massey, V. (1995). A new old yellow enzyme of *Saccharomyces cerevisiae.JBC, 270*(5), 1983-1991.

Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., &Nishihara, T. (1999). New screening methods for chemcials with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxico & Appl Pharmacol ,154,* 76-83.

Nistor, C., Rose, A., Faree, M., Stoica, L., Wollenberger, U., Ruzgas, T., Pfeiffer, Barcelo, D., Gorton, L., & Emne'us, J. (2002). In-field monitoring of cleaning

efficiency in wastewater treatment plants using two phenol-sensitive biosensors. *Analytica Chimica Acta, 456*, 3-17.

OECD. (2001). Detailed review paper. Appraisal of test methods for sex hormone dirupting chemicals. Environment health and safety publications. Series on testing and assessment no.21. Organisation for Economic Cooperation and Development. Paris.

Olsen, J. O., & Woese, C. R. (1993). Ribosomal RNA a key to phylogeny. *FASEB, J., 7*, 113-123.

Onada, K., Yang, S. Y., Miya, A., & Tanaka, T. (2002). Evaluation of estrogen like activity on sewage treatment processes using recombinant yeast. *Water Sci Technol, 46*(11-12), 367-373.

Ozcan, S., Dover, J., & Johnston, M. (1998). Glucose sensing and signaling by two glucose transporters in yeast *Saccharomyces cerevisiae. The EMBO Journal, 17*(9), 2566-2573.

Ozcan., S., Dover, J., Rosenwald, A. G., Wol, S., & Johnston, M. (1996). Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that genrate a signal for induction of gene expression. *Proc. Natl Acad Sci U.S.A, 93*, 12428-12432.

Pan, D., Chen, J., Yao, S., Tao, W., & Nie, L. (2005). An amperometric glucose biosensor based on glucose oxidase immobilized in electorpolymerized poly(o-aminophenol) and carbon nanotubes composite film on glod electrode. *Anal. Sci, 371*(21), 367-371.

Pandey, P. C. (1998). Enzyme biosensors based on mediator-modified carbon paste electrode. *Meth. Biotechnol, 6*, 81-92.

Pariente, F., Tobalina, F., Moreno, G., Hernandeze, L., Lorenzo, E., & Abruna, H. D. (1997). Mechanistic studies of the electrocatalytic oxidation of NADH and ascorbate at glassy carbon electrode modified with electrodeposited films derived from 3.4-dihydrobenzaldehyde. *Anal. Chem, 69*, 4065-4075

Park, J.W., & Crowley, D.E. (2006). Dynamic changes in *nahAC* gene copy numbers during degradation of naphthalene in PAH contaminated soils. *Applied Microbiol & Biotechnol, 72* (6), 1322-1329.

Park, S. J., Taton, T.A. and Mirkin, C.A. (2002). Array-based electrical detection of DNA with nanoparticle probe. *Science, 295*, 1503-1506.

Parrott, J., Wade, M., Timm, G., & Brown, S. (2001.). An overview of testing procedures and approaches for identifying endocrine disrupting substances. *Water Qual. Res. J. Canada., 36*, 273-291.

Parry, J. M. (1999.). Use of tests in yeast and fungi in the detection and evaluation of carcinogens. In D. B. McGregor, J. M. Rice & S. Venitt (Eds.), *The use of short- and medium-term test for carcinogens and data on genetic effects in*

carcicinogentic hazard evaluation., 146 (pp. 471-485). Lyon: IARC Scientific Publications.

Pasco, N., Baronian, K., Jeffries, C., & Hay, J. (2000). Biochemical mediator demand-- a novel rapid alternative for measuring biochemical oxygen demand. *Appl Microbiol Biotechnol, 53*(5), 613-618.

Pasco, N., Baronian, K., Jeffries, C., Webber, J., & Hay, J. (2004). MICREDOX(R) - Development of a ferricyanide-mediated rapid biochemical oxygen demand method using an immobilised *Proteus vulgaris* biocomponent. *Biosens. Bioelectron, 20*(3), 524-532.

Pasco, N., Hay, J., Scott, A., & Webber, J. (2005). Redox coupling to micorbial respiration: an evaluation of secondary mediators as binary mixtures with ferricyanide. *Aust. J. Chem. 58*, 288-293.

Pasco, N., Hay, J., & Webber, J. (2001). MICREDOX - A new biosensor technique for rapid measurement of BOD and toxicity markers. *Biosensors, 6*(1), 83-89.

Patent Storm. (1996). Mehtod for making electrochemical sensors and biosensors having a polymer modified surface. (United States Patent 5540828).

Paul , A. E., Harford-Cross, C. F., Stevenson, J. A., Rouch, D. A., & Wong, L-L. (1998). The oxidation of naphthalene and pyrene by cytochrome P450cam. *FEBS Letters, 424*, 271-274.

Payne, J., Rajapakse, N., Wilkins, M., & Kortenkamp, A. (2000). Prediction and assessment of the effects of mixtures of four xenestrogens. *Environ. Health Perspect, 108*, 983-987.

Petterson, M., Adolfsson-Erici, M., Parkkonen, J., Forlin, L., & Asplund, L. (2005). Fish bile used to detect estrogenic substances in treated sewage water. *Science of the total Environment (In-press)*

Petterson, M., Schramm, K. W., & Kettrup, A. A. (1999). Applicability of yeast estrogen screen for the detection of estrogen-like activities in environmental samples. *Chemosphere, 38*(14), 3303-3312.

Preininger, C., Kilmant, I., & Wolfbels, O. S. (1994.). Optical fibre sensor for biological oxygen demand. *Anal.Chem. 66*, 1841-1846.

Prez, F. G., Mascini, M., Tothill, I .E., Turner, A. P. F. (1998). Immunomagnatic seperation with mediated flow injection analysis amperometric detection of viable *Escherichia coli* 0157. *Anal. chem, 70*(11), 2380-2386.

Prieto-Simon, B., & Fabregas, E. (2004). Comparative study of electron mediators used in the electrochemical oxidation of NADH. *Biosens. Bioelectron, 19*, 1131-1138.

Prit, S. J. (Ed.). (1994). *Pirtfirm papaers, 6. Stoichiometry and kinetics of microbial growth.7. Bioenergetics of microbial growth, series A*. London: Pritfirm Ltd.

Pons, M., Gangne, D., Nicolas, J. C., & Mehtali, M. (1990). A new cellular model of response to estrgoens a bioluminescent test to characterize (anti)estrogenic moleucles. *Biotechniques, 9,* 450-459.

Qian, J. M., Suo, A. L., Yao, Y., & Zin, Z. H. (2004). Polyelectrolyte-stabilized glucose biosensor based on woodceramics as electrode. *Clin. Biochem, 37,* 155-161.

Quant, P. A. (1993). Experiemental application of top-down control analysis to metabolic systems. *Trends Biochem. Sci, 18*, 26-30.

Quenouille, M. H. (1950). Introductory statistics. London: Pergamon Press.

Quinn, G. P., & Keough, M. J. (2002). Experimental design and data analysis for biologists (chapter 4, pp. 64-65), Cambridge, United Kingdom: Cambridge University Press.

Rabinowitz, J. D., Vacchino, J. F., Beeson, C., & McConnell, H. M. (1998). Potentiometric measurement of intracellular redox activity. *J.Am.Chem.Soc., 120*, 2464-2473.

Racek, J., & Musil, J. (1987). Biosensor for lactate determination in biological fluids. 2. Interference studies. *Clin. Chem. Acta, 167*(1), 59-65.

Randez-Gil, F., Prieto, J.A., & Sanz, P. (1995). The expression of a specific 2-deoxyglucose-6P-phosphatase prevents catabolite repression mediated by 2-deoxyglucose in yeast. *Cuur. Gen, 28(2)*, 101-107.

Rainina, E., Effermenco, S., Varflolomeyev, A. L., Simonian, A. L., & Wild, J. (1996). The development of a new biosensor based on recombinant *E. coli* for the detection of organophophorous neurotoxins. *Biosens. Bioelectron, 11,* 991-1000.

Raloff, J. (1994). EcoCancers. Do environmental factors underlie a breast cancer epidemic? *Science News, 1448*, 10-13.

Ramsay, G., & Turner. A.P.F. (1988). Development of an electrochemical method for the rapid determination of microbial concentration and evidence for the reaction mechanisms. *Anal. Chem. Act, 215*, 61-69.

Ren, S.G., Seliktar, J., Li, X., Braunstein, G.D., & Shlomo, M. (1998). Measurement of leukemia inhibitory factor in biological fluids by radioimmunoassay. *J. Clinical Endocrinology & Metabolism,83(4),* 1275-1283.

Rehmann, K., Schramm, K. W., & Kettrup, A. A. (1999). Applicability of a yeast estrogen screen for the detection of estrogen-like activities in environmental samples. *Chemosphere., 38*, 3303-3312.

Reshetilov, A. N., Efremov, D. A., Iliasov, P. V., Boronin, A. M., Kukushskin, N. I., Greene, R. V., & Leathers, T. D. (1998). Effects of high oxygen concentrations on microbial biosensors responses. Hyper oxygenation by means of perfluorodecalin. *Biosens. Bioelectron*, 13, 795-799.

Richardson, N. J., Gardner, S., & Rawson, D. M. (1991). A chemically mediated amperometric biosensor for monitoring eubacterial respiration. *J.Appl. Bacteriol, 70*, 422-426.

Riedel, K. (1998). Microbial biosensors based on oxygen electrodes. In: A. Mulchandani and K .R. Rogers (Eds.), *Enzyme and Microbial Biosensors: Techniques and Protocols* (pp. 199-223). Totowa, NJ: Humanae Press.

Riedel, K., Renneberg, R., Kuehn, M., & Scheller, F. (1988). A fast estimation of biological oxygen demand using microbial senors. *Appl. Microbiol. Biotechnol, 28*, 316-318.

Rolland, F., Winderickx, J., & Thevelein, J. M. (2001). Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem. Sci, 26*(5), 310-317.

Roller, S. D., Bennetto, H. P., Delaney, G. M., Mason, J. R., Stirling, J. L., & Thuston, T. F. (1984). Electron-transfer coupling in microbial fuel cells. 1. Comparison of redox-mediator reduction rates and respiratory rates of bacteria. *J.Chem. Technol. Biotechnol, 34B*, 3-12.

Rogers, K. R. (1995). Biosensors for environmental application *Biosens. Bioelectron, 10*, 533-541.

Rogers, K. R., Cao, C. J., Valdes, J. J., Eldefrawi, A. T., & Eldefrawi, M. E. (1991). Acetylcholinesterase fibre optic biosensor for detection of anticholinesterases. *Fundam. Appl. Toxicol, 16*, 810-820.

Ronne, H. (1995). Negative control of gene expression by the yeast glucose repression pathway. *Trends in Genetics, 11*, 12-17.

Rose, A. H., & Harrison, J. S. (1987). *Metabolism and physiology of yeast.* London: Academic.

Rose, M., Albig, W., & Entain, K. D. (1991). Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexokinase phophorylation by hexokinase PI and PII. *Eur. J. Biochem, 199*, 511-518.

Rosel, H., & Kunze, G. (1996). Identification of group-I intron within 25S rDNA from the yeast *Arxula adeninivorans. Yeast, 12*, 1201-1208.

Routledge, E. J., & Sumpter, J. P. (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast strain. *Environ. Toxicol. Chem, 15*(3), 241-248.

Routledge, E. J., & Sumpter, J. P. (1997). Structural features of alkylphenolic chemcials associated with estorgenci activity. *J. Biol. Chem, 272* (6) , 3280-3288.

Rusling, J. F., & Ito, K. (1991). Voltammetric determination of electron-transfer rate between an enzyme and a mediator. *Anal. Chim. Acta, 252*, 23-37.

Saito, K., Thiele, D. L., Davio, M., Lockridge, O., & Massay, V. (1991). The clonning and expression of gene encoding old yellow enzyme from *Saccharomyces carlsbergensis, J. Biol. Chem, 266*, 20720-20724.

Sanseverino, J., Gupta, R. K., Layton, A. C., Patterson, S. S., Ripp, S. A., Saidak, L., Simpson, M. L., Schultz, T. W., & Sayler, G. S. (2005). Use of *Saccharomyces cerevisiae* BLYES expressing bacterial bioluminescence for rapid, sensitive detection of estrogenic compounds. *Appl. Environ, Microbiol., 71*(8), 4455-4460.

Sarmah, A. K., Northcott, G. L., Leusch, F.D.L., & Tremblay, L. A. (2006). A survey of endocrine disrupting chemicals (EDCs) in municipal sewage and animal waste effluents in the Waikato region of New Zealand. *Science of the Total Environment, 355*, 135-144.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry, 30*, 3875-3883

Schwartz-Mittleman A., Baruch, A., Neufeld, T., Buchner, V., & Rishpon, J. (2005). Electrochemical detection of xenestrogenic and antiestrogenic compounds using a yeast two-hybrid-17β-estradiol system. *Bioelectrochem, 65*, 149-156.

Schwirzerb, S.M.G., Hofmaierc, A. M., Kettrup, A. A., Nerdingera, P. E., Schramm, K. W., Thomad, H., Wegenked, M., & Wiebela, F. J. (1998). Establishment of a simple cleanup procedure and bioassay for determining 2,3,7,8-Tetrachlorodibenzo-p-dioxin toxicity equivalents of environmental samples. *Ectotoxicol. Environ. Safety, 41,* 77-82.

Sethi, R. S. (1994). Transducer aspects of biosensors. *Biosens. Bioelectron, 9*, 243-264.

Shultis, T., & Metzger, J. W. (2004). Determination of estrogenic activity by LYES-assay (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase). *Chemosphere, 57*, 1649-1655.

Skoog, D. A., Nieman, H. (2004). Principles of Instrumental Analysis. Standford, Ca: Brools/Cole.

Skowronski, R., & Feldman, D. (1989). Characterization of an estrogen binding protein in the yeast *Candida albicans*. *Endocrinology, 124*(4), 1965-1972.

Slama, M., Zaborosch, C., Wienke, D., & Spener, F. (1996). Simultaneous mixture analysis using a dynamic microbial sensor combined with chemometrics. *Anal Chem, 68*(3845-3850).

Smith, R. V., & Rosazza, J. P. (1974). Microbial models of mammalian metabolism. Aromatic hydroxylation. *Arch Biochem Biophys, 161*, 551-558.

Soboleva, G., & Golubkov, V. (1973). Effect of furfural on the cytochrome systems. *Mikrobiologia, 42,* 441-444.

Sohoni, P., & Soto, J. P. (1998). Several environmental estrogens are also androgens. *J. Endocrinol., 158*, 327-339.

Sokal, R. R., & Rohlf, F. (1995). *Biometry* (3rd ed.). New York: W. H. Freeman.

Soto A. M., Justica, H., Silvia R. M., & Sonnenschein C. (1992). An 'in culture' bioassay to assess the estrogenicity of xenobiotics (E-SCREEN). In T. Colborn & C. Clement (Eds.), Chemically-Induced alterations in sexual and functional development: The wildlife/human Connection. *Advances in Modern Environmental Toxicology* (pp. 295-309). Princeton, NJ:

Soto, A. M., Justica, H., & Wray, J. W. (1991). p-Nonyl-phenol:an estrogenic xenobiotic released from "modified" polystyrene. *Environ. Health Perspect, 92*, 167-173.

Steeter, I., & Compton, G. R. (2007). Steady state voltammetry at non-uniformly accessible electrodes: A study of Tafel plots for micordisc and tubular flow electordes in the reversible and irreversible limits of electron transfer. *Phys. Chem. Phys, 9,* 862-870.

Stredansky, M., Pizzariello, A., Stredanska, S ., & Miertus, S. (2000). Ampermetric pH-sensing biosnesors for urea, pencillin and oxaloacetate. *Analytica Chimica Acta, 415,* 131-157.

Subrahmanyam, S., Kondapani, N., Ahmarshan, J. N., Ranganathan, B., Sahnmugum, K., Jeyakumar, D., & Subramanian, T. V. (2001). Amperometric biochemical characterization of isolated fungal stains. *Electroanalysis. 13*, 1454-1458.

Suzuki, K., Hirai, H., Murata, H., & Nishida, T (2003). Removal of estrogenic activity of 17β-estradiol and ethinylestradiol by legninolytic enzymes from white rot fungi. *Water Res, 37,* 1972-1975.

Suzuki, M., Ozawa, F., Sugimoto, W., & Aso, S. (2002). Miniature surface-plasmon resonance immunosnesors- rapid and repetetive procedure. *Anal & Bioanal Chem, 372(2),* 301-304.

Szczepaniak, W. (2002). *Metody instrumentalne w nalizie chemicznej (Instrumental methods in chemical analysis)*, PWN, Warsaw (in Polish).

Szentrimay, P., Yeh, P., & Kuwana, T. (1977). *Electrochemcial studies of biological systems*, 143, ACS, Washington, DC:

Tabachnick, B., & Fidel, L. (1996). *Using multivariate statistics* (3rd ed.). New York: Harper & Row.

Tag, K., Lehmann, M., Chan, C., Renneberg, R., Riedel, K., & Kunze, G. (2000). Measurement of biodegradable substances with a mycelia-sensor based on the salt tolerant yeast *Arxula adeninivorans* LS3 *Sens. Actuators B, 67*, 142-148.

Tanaka, H., Nakamura, E., Minamiyama, T., & Toyoda, T. (1994). BOD biosensor for secondary effluent from wastewater treatment plants. *Water Sci.Technol. 30*, 215-227.

Tanaka, S., Hasegawa, S., Hishinuma, F., & Kurata, S. (1989). Estrogen can regulate the cell cycle in the early G1 phase of yeast by increasing the amount of adenylate cyclase mRNA. *Cell, 57*, 675-681.

Tan, T. C., Li, F., Neoh, K. G., & Lee, Y. K. (1992). Microbial membrane-modified dissolved oxygen probe for rapid biochemical oxygen demand measurement. *Sens. Actuators, B B8*, 167-172.

Terman, N. G., & McMullan, G. (2002). Iminodiacetate and nitrilotriacetate degradation by *Kluyveromyces marxianus* IMB3. *Biochem. BIophys. Res. Commun., 290*, 802-805.

Terziyska, A., Waltschewa, L., & Venkov, P. (2000). A new sensitive test based on yeast cells for studying environmetal pollution. *Environ. Pollut., 109*, 43-52.

Tescione, L., & Belfort, G. (1993). Construction and evaluation of a metal ion biosensor. *Biotechnol. Bioeng*, 42, 945-952.

Tizzard, A., Webber, J., Gooneratne, R., Hay, J., & Pasco, N. (2004). MICREDOX: application for rapid biotoxicity assessment. *Analytica Chimica Acta, 522,* 197-205.

Towe, B. C., Flechsig, S., & Spaulding, G. (1996). A recirculating-flow fluorescent oxygen sensor. *Biosens. Bioelectron, 11*(8), 799-803 (5).

Tran, D. Q., Jin, L., Chen, J., McLachlan, J. A., & Arnold, S. F. (1997). Evaluation of clinical and environmental anti-estrogens with human estrogen receptor expressed in *Saccharomyces cerevisiae*: a novel role for ABC-cassette transporters in mediating anti-estrogenic activity. *Biochem. Biophys. Res. Commun, 235*, 669-674.

Trosok, S. P., Driscoll, B. T., & Luong, J. H. (2001a). Mediated microbial biosensor using a novel yeast strain for wastewater BOD measurement. *Appl. Microbiol. Biotechnol, 56*(3-4), 550-554.

Trosok, S. P., Driscoll, B. T., & Luong, J. H. (2001b). Mediated microbial biosensor using a novel yeast strain for wastewater BOD measurement. *Appl Microbiol Biotechnol, 56*(3-4), 550-554.

Trosok, S. P., Luong, J. H., Juck, D. F., & Driscoll, B. T. (2002). Characterization of two novel yeast strains used in mediated biosensors for wastewater. *Can. J. Microbiol, 48*(5), 418-426.

Tyagi, R., Batra, R., & Gupta, M. N. (1999). Amorphous enzyme aggregates: stability towards heat and aqueous organic co-solvent mixtures. *Enzyme Microb. Technol., 24*, 348-354.

Uo, M., Numata, M., Suzuki, M., Tamiya, E., Karube, I., & Makishima, A. (1992). Preparation and properties of immobilized mercuric reductase in porous glass carriers. *J. Ceramic Soc. Jpn, 100,* 430-433.

U.S. Patent No. 5540828 (1996). Method for making electrochemical sensors and biosensors having a polymer modified surface. *Patent storm.*

Vallini, G., Frassinetti, S., D'Andrea, F., Catelani, G., & Agnolucci, M. (2001). Biodegradation 4-(1-nonyl)phenol by axenic cultures of the yeast *Candida aquaetextoris*: identification of micorbial breakdown products and proposal of a metabolic pathway. *Int. Biodeterior. Biodegrad, 47*, 133-140.

van der Walt, J. P., Smith, M. T., & Yamada, Y. (1990). *Arxula* gen.nov. (*Candidaceae*), a new anamorphic, arthroconidial yeast genus. *Antonie van Leeuwenhock, 57*, 59-61.

Vo-Dinh, T., Cullum, B. M., & Stokes, D. L. (2001). Nanosensors and biochips: frontiers in biomolecular diagnostics. *Sens. Actuators, B 74*, 2-11.

Vo-Dinh, T., Tromberg, B. J., Griffin, G. D., Ambrose, K. R., Sepaniak, M. J and Gardenhire, E. M (1987). Antibody-based fibre optic biosensor for the carcinogen benzo(a)pyrene. *Appl. Spectrose*, 41, 735-738.

Walker, G. M. (1998). *Yeast physiology and biotechnology*. Chichester, United Kingdom: Wiley.

Walmsley, R. M., Keenan, P. (2000). The eukaryotic alternative: advantages of using yeast in place of bacteria in microbial biosensor development. *Biotechnol. Bioprocess. Eng, 5*, 387-394.

Walmsley, R. M., Keenan, P., Knight, A. W., & Schmuckli, J. (2000.). Rad54-GFP. In: *Proceedings of the BIOSET Technical Workshop on Genotoxicity Testing*. Belgium: TECHNOTOX.

Wang, J., Wu, L. H., & Anges, L. (1991). Organic-phase enzymatic assays with microelectrodes. *Anal. Chem*, 63, 2993-2994.

Wang, H., Peters, G.A., Zeng, X., Tang, M.L., Wallace, I., Khan, S.A. (1995). Yeast two-hybrid system demonstrates that estrogen-receptor dimerization is ligand-dependent *in-vivo*, *J. of Biological Chem, 270,* 23322-23329.

Webb, J. S., Barratt, S. R., Sabev, H., Nixon, M., Eastwood, I. M., Greenhalgh, M., Handley, P. S., & Robson, D. G. (2001). Green fluorescent protein as a novel indicator of antimicrobial susceptibility in *Aureobasidium pullulans. Appl. Environ. Microbiol, 67*(12), 5614-5620.

Werlen, C., Jaspers Marco C. M., & Roelof van der Meer , J. (2004). Measurement of biologically available naphthalene in gas and aqueous phases by use of *Pseudomonas putida* biosensor. *Appl. Environ. Microbiol, 70*(1), 43-51.

Whelan, J., & Miller, N. (1996). Generation of estrogen receptor mutants with altered ligand specificity for use in establishing a regulatable gene expression system. *J. Steroid Biochem. Mol. Biol., 58*(1), 3-12.

White, S., & Larsen, B. (1997). *Candida albicans* morphogenesis is influenced by estrogen. *CMLS, Cell.Mol. Life. Sci. 53*, 744-749.

Wightman, R. M., & Wipf, D.(1996). Voltammetry at Ultramicroelectrodes, D.O. Wipf. *In:Electroanalytical Chemistry, A. J. Bard (Ed.),* New York: Marcel Dekker, v.15, ch. 3, pp. 267-353.

Winde, J.H.D. (1996). Differential requirement of the yeast sugar kinases for sugar sensing in establishing the catabolite-repressed state. *Eur. J. Biochem., 241*, 633-643.

Windhal, S, H., Norgard, M., Kuiper, G, C., Gustafsson, J, A., & Anderson, G. (2000). Cellular distribution of estrogen receptor beta in neonatal rat bone. *Bone, 26* (2), 117-121.

Wise, D, L., & Wingard, L. B. (Ed.). (1991). *Biosensors with Fibreoptics*. Clifton, NJ: Human Press.

Wong, S. A., & Hart, J. P. (1992). Chemically modified carbon-based electrodes and their application as electrochemical sensors for the analysis of biologically important compounds. A review. *Analyst, 117*, 1215-1229.

Wozei, E., Hermanowicz, S. W., & Holman, H.Y.N. (2005). Developing a biosensor for estrogens in water samples: Study of the real-time response of live cells of the estrogen-sensitive yeast strain RMY/ER-ERE using fluorescence microscopy. *Biosens Bioelectron, 21*(8), 1654-1658.

Yadger, J. W., Eastmond, D. A., Robertson, M. L., Paradisin, W. M., & Smith, M. T. (1990). Characterization of micronuclei induced in human lymphocytes by benzene metabolites. *Cancer Research, 50*(15), 393-399.

Yamashoji, S., & Kajimoto, G. (1986). Catalytic action of vitamin K3 on ferricyanide reduction by yeast cells. *Biochem. Biophys. Acta, 849*, 223-228.

Yamashoji, S., Ikeda, T., & Yamashoji, K. (1989). Chemiluminescent assay for determination of viable cell density of yeast, mammalian and plant cells. *Anal Biochem 181*, 149-152.

Yamashoji, S., Ikeda, T., & Yamashoji, K. (1991). Extracellular generation of active oxygen species catalyzed by exogenous menadione in yeast cell suspension. *Biochem. Biophysic. Acta, 1059*, 99-105.

Yamazoe, N & Miura, N. (1998). Gas sensors for oxidic gases. *J of Electroceramics, 2,* 243-255.

Yang-Hoon Kim, J. L., Ji-Young Ahn., Man Bock Gu., & Seung-Hyeon Moon. (2002). Enhanced degradation of an endocrine-disrupting chemical butyl benzyl phthalate, by *Fusarium oxysporum* F.sp.psi cutinase. *Appl. Environ. Microbiol, 68*(9), 4684-4688.

Yashiki, Y., & Yamashoji, S. (1996.). Extracellular reduction of manadione and ferricyanide in yeast cell suspension. *J. Ferment. Bioeng, 82*(3), 319-321.

Yielding, L., & Tomkins, G. M. (1960). Structural alterations in crystalline glutamic dehydrogenase induced by steroid hormones. *Proc. Natl Acad Sci U.S.A, 46*, 1483-1488.

Yin, P., Burns, C. J., Osman, P.D.J., & Cornell, B. A. (2003). A tethered bilayer sensor containing alamethicin channels and its detection of amiloride based inhibitors. *Biosens. Bioelectron, 18*(4), 389-397.

Yin, Z., Hatton, L., & Brown, A.J.P. (2000). Differential post-transcriptional regulation of yeast mRNAs in reponse to high and low glucose concentrations. *Mol. Micorbiol. 35*, 553-565.

Yin, Z., Smith, R. J., & Brown, A.J.P. (1996). Multiple signalling pathways trigger the exquisite sensitivity of yeast glluconeogenic mRNAs to glucose. *Mol. Microbiol, 20,* 751-761.

Yin, Z., Wilson, S., Hauser, N. C., Tourmu, H., Hoheisel, J. D., & Brown, J. P (2003). Glucose triggers different global responses in yeast, depending on the strength of the signal, and transiently stabilizes ribosomal protein mRNAs. *Mol. Microbiol, 48*(3), 713-724.

Zacharewski, T. (1997). *In vitro* bioassays for assessing estrogenic substances. *Environ. Science. Technol. 31*(3), 613-623.

Zeise, R., Wilson, R., & Crouch, E.A.C. (1987). Dose-response relationships for carcinogens: a review, *Environ. Health perspect, 73,* 259-358.

Zhao, J.Y.Z., Gong, Q., Lu, Y., & Yang, Z. (2005). Electrochemical insights into the glucose metabolism pathways within Saccharomyces cerevisiae. *Anal. Lett, 38*, 89-98.

Zhao, J., Wong, M., Yang, Z., Gong, Q., Lu, Y., & Yang, Z. (2005). Mediated electrochemical measurement of the inhibitory effects of furfural and acetic acid on *Saccharomyces cerevisae* and *Candida shehatae. Biotechnol. Lett, 27*, 207-211.

Zhao, J. Wong., M., Yang, Z., & Yang, Z. (2005). Measurement of inhibitory effects of furfural and furfural alcohol using coupled redox mediators. *Enzyme Microb.Technol, 37,* 246-253.

Zhao, X., Malloy, P. J., Ardies, C. M., & Feldman, D. (1995). Ostrogen-binding protein in *Candida albicans*: antibody development and cellular localization by electron immunocytochemistry. *Microbiology, 141*, 2685-2692.

APPENDIX I
YEPD Medium

Glucose = 20 g
110 Peptone = 20 g
Yeast extract = 10 g

The contents were added to 940 mL distilled water and the was stirred with a magnetic bead to dissolve the contents. The volume is made up to 1 L with distilled water. Aliquots of 100 mL were dispensed in 250 ml flasks, sterilised at 121° C for 15 min, and stored at room temperature.

APPENDIX II

Minimal medium (pH 5.5)

Glucose = 20 g
KH_2PO_4 = 1 g
$(NH_4)_2SO_4$ = 5 g
$MgSO_4, 7 H_2O$ = 0.5 g
$CaCl_2, 2 H_2O$ = 0.1 g
Inositol = 2 mg
KI = 1 mg
H_3BO_3 = 0.5 mg
$ZnSO_4, 7 H_2O$ = 0.4 mg
$Mn SO_4, 7 H_2O$ = 0.4 mg
Thiamine, HCl = 0.4 mg
Pyroxidine, HCl = 0.4 mg
Niacin = 0.4 mg
Calcium pantothenate = 0.4 mg
p-Amino benzoic acid = 0.2 mg
Riboflavin = 0.2 mg
$FeCl_3$ = 0.2 mg
$Na_2MoO_4, 4H_2O$ = 0.2 mg
$Cu SO_4, 5 H_2O$ = 0.04 mg
Folic acid = 2 μg
Biotin = 2 μg
Growth supplement solution = 10 mL
Distilled water = 1 L

Aliquots of 90 mL were dispensed in 250 ml flasks, sterilised at 121° C for 15 min, and stored at room temperature.

Growth supplement solution

L- Argininine HCl = 20 mg
L-Histidine, HCl = 20 mg
Uracil = 20 mg

Components were added to 6 mL of distilled water mixed thoroughly and made up the volume to 10 mL. Filter sterilised and stored at 4 °C.

APPENDIX III

Minimal medium (pH 7.1) (Routldege and Sumpter, 1996)

$KH_2 PO_4$ = 13.61g

$(NH_4)_2 SO_4$ = 1.9 g

KOH pellets = 4.2 g,

$MgSO_4$ = 0.02g,

$Fe_2 (SO_4)_3$ solution (40mg/50mlwater) = 1 mL,

L-leucine = 50 mg

L-histidine= 50 mg

 Adenine = 50 mg

L-arginine- HCL = 20 mg

L-metheionine = 20 mg

L-tyrosine = 30 mg

L-isoleucine = 30 mg

L- lysine- Hcl,= 25 mg

L-phenylalanine = 30 mg

L-glutamic acid = 100 mg,

L-valine = 150 mg

L-serine = 375 mg

Distilled water = 1 L

Aliquots of 45 ml were dispensed in 250 ml flasks, sterilised at 121° C for 15 min, and stored at room temperature.

Vitamin solution

Thiamine = 8 mg

Pyridoxine = 8 mg

pantathenic acid = 8 mg

Inositol = 40 mg/ml

Biotin solution (2mg/100ml H_2O) = 20 ml

Distilled water = 180 nmL.

The solution was then filter-sterilised through 0.45 μM pore size Millipore filters disposable, 10- ml aliquots in sterile bottles were stored at 4 °C.

D-(+) glucose

20% w/v solution was sterilised at 121 °C for 15 min and stored at room temperature.

Asparitic acid

Stock solution of 4 mg/mL L-asparitic acid was sterilised at 121 °C for 15 min and stored at room temperature.

L-Thereoinine

A stock solution of 24mg/mL L-thereoinine was sterilised at 121 °C for 10 min and stored at 4 °C.

Copper (II) sulphate

A 20 mM copper (II) sulphate solution was prepared and filter sterised and stored at 4 °C.

APPENDIX IV

Phosphate Buffer (0.05 M PO$_4$ buffer, pH 7)

KH$_2$PO$_4$ = 305. 25 mL of 0.05 M solution
K$_2$H PO$_4$ = 192. 25 mL of 0.05 M solution
Distilled water = 502.20 ml

Added 502.40 mL of distilled water to make up the volume to 1` L. Aliquots of 100 mL were dispensed in 250 mL bottles, sterilised at 121° C for 15 min, and stored at room temperature.

Phosphate buffered saline (PBS, pH.7)

0.1 M PO$_4$ buffer = 500mL
1M KCl = 100 mL

Added 400 mL distilled water to make up the volume to 1 L. Aliquots of 100 mL were dispensed in 250 mL bottles, sterilised at 121° C for 15 min, and stored at room temperature.

APPENDIX V

Mediators

Potassium ferricyanide (0.5 M)

Potassium ferricyanide = 16.42 g was added to 90 mL of distilled water. The content was mixed thoroughly by stirring with a magnetic bead. The volume was made up to 100 mL with distilled water. The stock solution was filter sterilised and stored at 4 °C.

Menadione (20 mM)

344 mg of menadione was dissolved in 100 mL of 96% ethanol. The stock solution was filter sterilised and stored at 4 °C.

2,3,4,6, Tetramethyl 1, 4-phenylenediamine (TMPD, 20 mM)

328 mg of TMPD was dissolved in 100 mL of 96% ethanol. The stock solution was filter sterilised and stored at 4 °C.

APPENDIX VI

Sodium phosphate buffer pH 6.5 (12.5 mL)

100 mM Na_2PO_4 = 4 mL
100 mM NaH_2PO_4 = 8 mL

Solution 1

10 mM Sodium Phosphate buffer
1.2 M Sorbitol
50 mM DTT (Dithiotheitol)

Solution 2

100 mM NaCl
50 mM Tris-HCl pH7.8
100 mM EDTA pH7.8

Tris Ethidium-bromide (TE) Buffer.

Tris-HCl 10 mM, pH 8.0.
0.1 mM ETDA

Tris borate buffer (TBE, 10X)

540g Tris base
275 g boric acid
200 mL 0.5 MEDTA

made up the volume to 1 L with distilled water, sterilised at 121 °C for 15 min and stored at room temperature.

6 X Gel loading buffer

0.25% bromophenol blue
0.25% Xylenecyanol FF1
30% glycerol in water

Store at 4 100 mL of 96% ethanol. The stock solution was filter sterilised and stored at 4 °C.

APPENDIX VII

Table 1.1. Redox potentials (at pH 7) of the selected mediators and their respective biological components.

Mediator	E (V)	Biological component	E (V)
Neutral red	-0.33	NAD+/NADH	-0.32
Menadione	-0.13	FAD/FADH$_2$	-0.22
Phenazine ethosluphate	0.05	Succinate/fumerate	+0.03
Dichloroindophenol	0.22	Flavoproteins	-0.45- 0
2,3,5,6,-Tetramethyl-1,4 phenylenediamine (TMPD)	0.25	Ubiquinone	0.11
N,N-Tetramethyl phenylenediamine	0.26	Cytochrome b	0.07
Benzoquinone	0. 28	Cytochrome c	0.25
Potassium ferricyanide	0..43	Cytochrome a	0.38
		O$_2$/H$_2$0	0.82

(From - Pasco et al, 2005).

APPENDIX VIII

Table 1.2 Priority contaminants- chemical analysis.

Priority ranking	Pollutant	Method	Cost (NZ\$)	Detection limit (mg/m^3)	Aqueous solubility
1	Lead	ICP-MS	9-12	2-0.05	Soluble
2	Cadmium	ICP-MS	9-12	1-0.01	Soluble
3a	Polycyclic Aromatic Hydrocarbons (PAHS)	GC-MS-SIM	90-200	0.01-0.005	-
4	Naphthalene	GC-MS-SIM	90-200	0.5-0.05	0.3 g/L
5	Polychlorinated Biphenyls (PCB)	GC-MS-SIM GC-ECD	90-260	0.1-0.0003	-
6	Bis (2-ethylhexyl) phthalate	GC-MS	200-230	20-2	0.1 g/L
7	Arsenic	ICP-MS	9-12	20-0.5	Soluble
8	Di-n-octyl phthalate	GC-MS	120-180	10-1	0.0039 g/L
4	Mercury	FIMS	35	1-0.08	Soluble
10	Di-butyl phthalate	GC-MS	200-230	10-1	0.4 g/L/l
11	Pentachlorophenol	GC-MS	170	0.5	0.014 g/L
12	Nitrobenzene	GC-MS	200-230	5-0.5	1.9 g/L

From Gareth Lloyd-Jones, Landcare Research Contract Report; LCO102/1222 (2002)

APPENDIX IX

Table 1.3. Examples of endocrine disrupting compounds: natural products (phytoestrogens and mycoestrogens) and synthetic chemicals.

Compound	Mode of Action	Assay	Reference
1 PHYTESTROGENS			
Indole-3-carbinol	ER agonist	RER (MCF-7-luc), YES	23, 24
β-Sitosterol	ER agonist, androgenic after metabolised	YES, *in vivo* fish	24, 25
Coumestrol	ER agonist	RER (MCF-7-luc), YES	23, 24
		in vitro - ER mediated alkaline. induction	26
Enterolactone, Enterodiol	decreased aromatase enzyme activity	*in vitro* - human cell culture system	27
Genistein	ER agonist	RER (ER-CALUX)	28, 26
	estrogenic	*in vitro* and *in vivo* vitellogenin production	29
Biochanin A, Daidzein, Equol	ER agonists, estrogenic	*in vitro* and *in vivo* vitellogenin production	29
		in vitro - ER mediated alkaline induction	26
Quercetin, Naringenin, Luteolin	Estrogenic, antiestrogenic	CB-ER, RER	30, 31
Apigenin, Chrysin, Kaempferol,	ER agonists	RER (MVLN)	32
Hydroxy- and Methoxy-flavons			
2 MYCESTROGENS			
Zearalenone	ER agonist	CB-ER, RER, Vtg-*in vitro*	30, 33
3 PHARMACEUTICALS			
Flutamide	antiandrogenic activity	YES	19
Tamoxifen	antiestrogenic drug binding to ER, ER antagonist or agonist	*in vitro* cell line tests, *in vivo*	34, 35, 36
		E-screen and other effects	37
Hydroxytamoxifen	antiestrogenic and antiandrogenic activity	YES, E-screen and other effects	19, 37
Nafoxidine, Clomiphene	ER agonist	Yes	24
Ethynylestradiol	ER agonist	*in vitro, in vivo*	29, 25

4 ADDITIVES

Parabens	ER agonists	CB-ER, YES, *in vivo* uterotropic response	38
t-Butylhydroxyanisol	estrogenic	E-screen	39

5 PESTICIDES

5.1 Insecticides

o,p'-DDT	ER agonist, antiandrogenic activity	YES, RER (ER-CALUX), Vtg-*in vitro*	24, 28, 19, 40
o,p'-DDD, o,p'-DDE	ER agonists	YES	24
p,p'-DDE	androgen receptor antagonist, weak ER and androgen receptor agonist antiandrogenic and weak antiestrogenic activity	CB-androgen receptor, *in vivo* mice study YES	41 19
p,p'-DDD	ER agonist	YES, CB-ER, RER (MCF-7-luc)	42
p,p'-DDT	ER agonist, estrogenic	E-screen	29
Kepone	ER agonist, estrogenic - after metabolisation	RER (ER-CALUX), E-screen, *in vitro + in vivo*	28, 29, 36
Endosulfan, Dieldrin, Lindane	ER agonist	RER (ER-CALUX)	28
Toxaphene	estrogenic	E-screen	43
Methyl parathion	estrogenic	YES, Vtg-*in vitro* *in vivo* effects on estrus cycle in mice	44 45
Chlordecone	estrogenic	YES, Vtg-*in vitro*	44
Chlordane	ER agonist	RER (ER-CALUX) *in vivo* effects on estrus cycle in mice	44 45
Methoxychlor	ER agonist	RER (ER-CALUX) in vivo effects on endocrine function in mice	28 46
Carbamate insecticides (Aldicarb, Bendiocarb, Carbaryl, Methomyl, Oxamyl)	endocrine modulators, non-ligand binding	*in vitro* modulation of estrogen and progesterone receptor in human breast and endometrial cancer cells	42

232

Pyrethroid insecticides (Sumithrin, Fenvalerate, Allethrin, Permethrin)	estrogenic (different mechanisms)	*in vitro* pS2 gene expression E-screen	47

5.2 Fungicides

Vinclozolin	antiandrogen	*in vitro* androgen receptor binding assay, YES	48 19
Dodemorph, Triadimefon, Biphenyl	estrogenic	YES, Vtg-*in vitro*	43

5.3 Herbicides

Atrazine	estrogen, antiestrogen	RER (MCF-7-luc), *in vivo*	23
Simazine	antiestrogen	*in vivo*	49
Alachlor, Nonachlor	ER agonists	YES, CB-ER, RER (MCF-7-luc)	42
Tributyltins	androgenic	imposex in snails, various *in vivo* effects in gastropods	25, 50

6 INDUSTRIAL CHEMICALS

6.1 Phthalates

Butylbenzylpthalate (BBP)	ER agonist, antiandrogenic activity	*in vitro* + *in vivo*, E-screen, YES	25, 39, 51, 19
Dibutylpthalate (DBP)	ER agonist	*in vitro* + *in vivo*	25, 51

6.2 Alkyl phenols

Nonyl phenol (NP)	ER agonist, estrogenic	RER (MCF-7-luc, ER-CALUX), YES, number of *in vitro* and *in vivo* assays, E-screen, Vtg-in vitro	23, 24, 28, 52, 36, 29, 39
Octylphenol (OP)	ER agonist	RER (MCF-7-luc) number of *in vitro* and *in vivo* assays	52 39
Butyl phenol, Pentylphenol	estrogenic	E-screen	39, 29
Nonyl phenol polyethoxylates and polyethoxycarboxylates	ER agonists	number of *in vitro* and *in vivo* assays	52
Pentachlorophenol (PCP)	decrease in blood testosterone concentration	*in vivo* ewes feeding study	53
BIS phenol A (BPA)	ER agonist	RER (MCF-7-luc, ER-	23, 24

	antiandrogenic activity	CALUX), YES, Vtg-*in vitro* YES	28, 39, 19

7 PERSISTENT ORGANIC POLLUTANTS (POPs)

Polychlorinated dibenzo-p-dioxins (PCDDs)	antiestrogenic - different mechanisms	*in vivo + in vitro* studies	54
Polychlorinated biphenyls (PCBs)	ER agonists or antagonists or other mechanism - depending on the substitution	RER (transient MCF-7-luc), E-screen, *in vivo* - vaginal cell cornification in mice	21, 39
Aroclor 121 (PCBs mix), Aroclor 1260	estrogenic, effect on sexual differentiation, gonadal abnormalities	Vtg-*in vitro* *in vivo* trout study	39 55
Hydroxy-PCBs	ER agonists or antagonists	RER (MCF-7-luc), E-screen, CB-ER, *in vivo* - vaginal cell cornification in mice	53, 56, 21, 39
Polycyclic aromatic hydrocarbons (PAHs)	ER agonists - estrogenic, antiestrogenic - different mechanisms	YES, E-screen RER (MCR-7-luc)	57, 58, 59 60
6-Hydroxy-chrysene	antiestrogenic	YES	57

8 HEAVY METALS

Cations of cadmium, cobalt, copper, mercury, nickel, zinc	depression or increase in testosterone production	*in vitro* substrate stimulated testosterone production by Leydig cells	61
cadmium	decrease in plasma testosterone and cortisol modification of pituitary hormone secretion	*in vivo* juvenile rainbow trout exposure *in vivo* rat feeding exposure	62 63
lead	delayed sexual maturation, suppression of sex steroid biosynthesis	*in vivo* rat feeding study	64

YES = yeast based recombinant estrogen receptor-reporter assay

E-screen = MCF-7 cell proliferation

CB-ER = in vitro competitive receptor binding assay

RER = in vitro recombinant receptor-report cell bioassay

Vtg-*in vitro* =in vitro vitellogenin synthesis in cultured male trout hepatocytes

(From: Hilscherova et al, 2000).

APPENDIX X

Total genomic DNA isolation from *A. adeninivorans*

1. A 10 mL overnight culture of *A. adeninivorans* in YEPD media was set up.

2. The overnight culture was checked microscopically for cell concentration (preferably between $1 \times 10^7 - 5 \times 10^7$) and for contamination.

3. Cells were harvested by centrifugation at 4000 rpm for 5 minutes.

4. Supernatant was discarded and cells were resuspended in 0.5 mL distilled water. The cell suspension was transferred to a microcentrifuge tube.

5. Cells were pelleted in a microcentrifuge (13000 rpm, 1 minute).

6. Steps 4 - 5 were repeated twice to wash cells.

7. The supernatant was discarded and the cells were resuspended in 0.5 mL of solution.

8. They were left at room temperature for 5 minutes.

9. Cells were pelleted in a microcentrifuge.

10. The supernatant was discarded and the cells were resuspended in 0.5 mL of fresh solution 1. A solution of 5mg/mL zymolyase-100T solution was thoroughly mixed and 10μL was added.

11. Cells were incubated at 37°C for 20 minutes.

12. Cells were checked for completion of spheroplasting by withdrawing 1 µL of cell suspension to 10 µL of water on a slide and viewed under a microscope. Cell lysis was observed with the appearance of debris indicating spheroplasting was complete (compared with intact cells).

13. Spheroplasts were pelleted in a microcentrifuge.

14. Spheroplasts were carefully resuspended in 0.45 mL solution 2. 10 µL of 1mg/mL Proteinase K solution and 50 µL of 10% sodium dodecyl sulphate (SDS) was added and mixed gently by inversion.

15. Spheroplasts were incubated at 65°C for 20 minutes.

16. 0.5mL phenol/chloroform was added and emulsified by inversion.

17. Spheroplasts were centrifuged at 13000 rpm for 5 minutes and carefully transferred the aqueous upper phase to a clean microcentrifuge tube avoiding the lower phenol phase and interface .

18. Steps 15-16 were repeated three times until the white interface disappeared completely.

19. DNA was precipitated by adding 0.25 volumes of 10.5 M ammonium acetate and 2 volumes of absolute ethanol. It was mixed well and placed at -20°C overnight.

20. DNA was pelleted by centrifugation for 15 minutes at 13000 rpm in a microcentrifuge.

21. All traces of ethanol were removed and the pellet allowed to air dry.

22. Re-suspended the pellet in 90 µl of sterile distilled water, added 10µL of 10mg/mL RNase A solution and incubated at 37°C for 15 minutes.

23. DNA was reprecipitated by adding 0.25 volumes 10.5 M ammonium acetate and 2 volumes of absolute ethanol. DNA was mixed well and placed at -20°C for three hours.

24. DNA was pelleted in a microcentrifuge at 13000 rpm for 15 minutes.

25. Supernatant was removed and washed pellet with 0.5mL 70% ethanol. The DNA solution was centrifuged at 13,000 rpm for 5 minutes.

26. All traces of ethanol was removed and allowed pellet to air dry.

27. DNA pellet was resuspended in 40 µL of sterile distilled water.

APPENDIX XI

Preparation of 1% agarose gel and gel electrophoresis

1. 1% agarose gel was prepared by weighing 1 g of agarose in 100 mL of 1x TBE buffer prepared by adding 900 mL of distilled water to 100 mL of 10x TBE buffer (see Appendix IV). The agarose was melted in microwave for 3 min, cooled and cast in the gel tray with comb inserted in the agarose.

2. After the gel had solidified it was transferred to the buffered tank and immersed in 500 mL of 1xTBE with 10μg/mL of ethidium bromide (7 μL in 500mL gel running TBE buffer).

3. Loading dye (4μL) was added to 10 μL of PCR products to prepare the samples.

4. Samples were loaded.

5. The gel electrophoresis power pack was set at constant potential of 100 V at the rate of 8V/cm.

6. The gel was run till the blue band of the loading dye in the sample had migrated about three quarters of the way down the gel (approximately one hour).

7. The gel was stopped and transferred to the transilluminator.

8. It was viewed under UV light and a gel photograph taken.

ANNEXURE I

Publications

1. Baronian, K.H.R & Gurazada, S. (2007) - Electrochemical detection of wild type *Saccharomyces cerevisiae* responses to estrogens. *Biosensors and Bioelectronics, 22,* 2493-2499.

2. Baronian, K.H., Gurazada, S and Thomas, A. (2005). Electrochemical detection of yeast responses to catabolizable substances. *Australian Journal of Chemistry, 58,* 270-274.

Conference Presentations

1. Baronian Keith, Gurazada Saroja, Nicholas Haslett, Gotthard Kunze, Manjula Premaratne and Seetha Wanniyike. (2007). Comparison of yeast fuel cell performance: double mediator, single mediator and mediator-less. The 58th Annual Meeting of the International Society of Electrochemistry, Canada, Banff, 9 – 14 September.

2. Gurazada Saroja and Baronian Keith (2006) - Mediated electrochemical detection of wild type *Saccharomyces cerevisiae* responses to estrogens- The Ninth World Congress on Biosensors, Toronto, Canada, 9 - 12 May (Saroja Gurazada, Presenter).

3. Baronian Keith and Gurazada Saroja (2006) - Electrochemical detection of wild type yeast responses to estrogens- International Workshop on Biosensors for environmental analysis, Goa, India, 21 - 23 February.

4. Gurazada Saroja and Baronian Keith Baronian: Double mediated electrochemical detection of redox responses of the yeast hER *Saccharomyces cerevisiae* to 17-β estradiol. - 56th Annual Meeting of the International Society of Electrochemists/ Electrochemistry for the Next Generation. Busan, Korea, 25 - 30 September 2005. (Saroja Gurazada, Presenter)

5. Gurazada Saroja and Baronian Keith: Electrochemical detection of specific catabolic responses to naphthalene and di-butyl-phthalate by the yeast *Arxula adeninivorans*-Interact 2004.Broadbeach, Queensland, Australia, 4 - 8 July 2004. (Saroja Gurazada, Presenter)

6. Baronian Keith, Downard Alison, Gurazada Saroja, Robson David - Electrochemical Detection of Yeast Cellular Responses to External Substrates Interact 2004, Broadbeach, Queensland, Australia, 4 – 8 July 2004.

7. Baronian Keith, Downard Alison, Kunze Gotthard, Tag Kristina, Gurazada Saroja, and Robson David - Use of mediated electrochemical detection of catabolism in yeast for environmental biosensors. Biosensors and BioAnalytical Micro–Techniques in Environmental and Clinical Analysis International Association of Environmental Analytical Chemistry, Italian National Agency for New Technology, Energy and the Environment (ENEA), University of Rome University of Rome and ENEA, 8 – 12 October, 2004.

8. Baronian Keith, Downard Alison, Gurazada Saroja, Robson David - Electrochemical Detection of Yeast Cellular Responses to External Substrates. Chemistry at the Interface, the International Conference of New Zealand, Institute of Chemistry, Nelson, New Zealand. 30 November to 4 December 2003.

Unpublished Research Papers

1. Application of a double mediated electrochemical detection system to hER *Saccharomyce cerevisiae* Yeast Estrogen Screen reduces incubation period and increases sensitivity. Baronian Keith, Saroja Gurazada and Thomas Hahn. *Biosensors and Bioelectronics* (Submitted).

2. *Arxula adeninivorans* based sensor for the estimation of BOD - a comparison of electrochemical measurement of oxygen consumption and mediator reduction by catabolising cells. Tag Kristina, Riedel Klaus, Gurazada Saroja, Baronian Keith, Kunze Gotthard. *Sensors and Actuators* (In-preparation).

Invited Lectures

1. Gurazada Saroja* and Baronian Keith- Double mediated electrochemical detection of redox responses of the yeast hER *Saccharomyces cerevisiae* to 17-β estradiol, Korean Oceanographic Research and Development centre, Busan, Korea. (30.09.05)

2. Gurazada Saroja* and Baronian Keith- Double mediated electrochemical detection of redox responses of the yeast hER *Saccharomyces cerevisiae* to 17-β estradiol. National Environmental Research Institute. Tsukuba, Japan (03.10.05).

3. Gurazada Saroja* and Baronian Keith- Double mediated electrochemical detection of redox responses of the yeast hER *Saccharomyces cerevisiae* to 17-β estradiol. University of Tokyo , Chemistry Department, Tokyo, Japan (04.10.05).

*Presenter