FINAL TECHNICAL REPORT

THE DEVELOPMENT AND ASSESSMENT OF A YEAST BIOASSAY FOR THE DETECTION OF MYCOTOXINS

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Executive Summary

Mycotoxins are highly toxic fungal metabolites which occur both in foods and feeds. The occurrence of these toxins has a deleterious effect on the health and productivity of both animals and humans and, if not controlled, can seriously compromise valued export markets.

The aim of the project was the development of a novel yeast bioassay which provides a non-mammalian system for the detection of the presence of toxins, as a precursor to the determination of specific toxins using traditional analytical procedures. It was also envisaged that the bioassay would provide a vehicle for an evaluation of the structure/activity relationship of mycotoxins.

The research activities resulted in the development of a novel colorimetric, microtitre plate-based bioassay for the determination of the trichothecene mycotoxins using the yeast *Kluyveromyces marxianus*. The assay uses the inhibition of β-galactosidase as a sensitive indicator of toxicity, the cultures remaining yellow rather than turning deep green-blue in the presence of X-gal, a chromogenic substrate. Factors which were likely to effect the efficacy of the bioassay were evaluated and optimised including the carbon source, solvent type, inoculum cell density and membrane-modulating agents (MMAs). Polymyxin B nonapeptide, for example, was the most effective toxicity-enhancing MMA tested, enabling the trichothecene mycotoxin, verrucarin A, to be detected at a concentration of about 1ng/ml.

The bioassay was used to study the relative toxicity of thirteen differing trichothecenes, and also to analyse the important structural features which determined the toxicity of individual toxins. The macrocyclic trichothecenes, verrucarin A and roridin A, were the most potent toxins, whereas T-2 toxin was the most potent of the nonmacrocyclic trichothecenes. A high level of potency was imparted by the presence of acetoxy groups at the C-4 and C-15 positions, in the epoxysesquiterpene ring system, together with a hydroxy group at the C-3 position.

The project outputs contribute towards the development of strategies which improve the food security of poor households. Specifically, bioassays have an important role to play in the control of food safety, providing simple, cost-effective means of monitoring toxicity, and a vehicle for evaluating the toxic mechanism. The better understanding of the behaviour of toxins which results will facilitate the establishment of preventative, food safety management systems which can be applied in rural areas in developing countries.

DEVELOPMENT AND ASSESSMENT OF A YEAST BIOASSAY FOR THE DETECTION OF MYCOTOXINS

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ABSTRACT

The development and evaluation of a yeast bioassay for the detection of mycotoxins is described. The assay is based on the inhibition of growth of the yeast *Kluyveromyces marxianus* var. *marxianus* (GK1005), and toxicity can be determined by the spectrophotometric determination of cell density, or by a colorimetric endpoint - the inhibition of β-galactosidase activity, determined by the cleavage of its colorimetric substrate, Xgal. Additionally, the use of Xgal as a colorimetric substrate for β-galactosidase allowed the visualisation of toxicity and permitted a qualitative determination of toxicity to be made in the absence of microtitre plate instrumentation.

The yeast bioassay provided a sensitive method for the detection of the trichothecene mycotoxins, and was used to determine structure-activity relationships between this group of mycotoxin; the order of toxicity was found to be:

verrucarin A> roridin A> T-2 toxin> diacetoxyscirpenol> HT-2 toxin> acetyl T-2 toxin>neosolaniol> fusarenon-X> T-2 triol> scirpentriol> nivalenol> deoxynialenol> T-2 tetraol.

However, although the yeast bioassay could be used for the sensitive detection of the trichothecene mycotoxins, it displayed marked insensitivity to several other important mycotoxins, including aflatoxin B_1. The cause of this insensitivity was shown not to be caused by the poor penetration of the toxin into the yeast cell, and appears to be due to the inability of the cytochrome(s) P450 of *K. maxianus* to activate aflatoxin B_1.

ABBREVIATIONS

ΔA	change in absorbance
a_w	water activity
A_{xxx}	absorbance at a given wavelength (xxxnm)
A	absorbance
AcT-2	acetyl T-2 toxin
AFB_1	aflatoxin B_1
AFB_1-dhd	aflatoxin B_1 2,3-dihydrodiol
AFB_1-FAPyr	aflatoxin B_1 formamdopyrimidine
AFB_1-N^7-gua	aflatoxin B_1 N^7 guanine
AFB_2	aflatoxin B_2
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFM_1	aflatoxin M_1
AFP_1	aflatoxin P_1
AFQ_1	aflatoxin Q_1
AFL	aflatoxicol
ATA	alimentary toxic aleukia
B(*a*)P	benzo(*a*)pyrene
B:A	benzene:acetonitrile
B. megaterium	*Bacillus megaterium*
°C	degrees centigrade
CF	correction factor
cm	centimeter
conc	concentration
CPA	cyclopiazonic acid
CTAB	cetyl trimethyl ammonium bromide
CV	coefficient of variation
cyt P450	cytochromes P450
4-DAN	4-deacetylneosolaniol
15-DAN	15-deacetylneosolaniol
D	volume of B:A used for sample extract dilution
DAS	diacetoxyscirpenol
DDA	disk diffusion assay
DE 3'-OH HT-2	deepoxy 3'-hydroxy HT-2 toxin
DE HT-2	deepoxy HT-2 toxin
DE TET	deepoxy T-2 tetraol
DE TRI	deepoxy T-2 triol
DMF	dimethylformimide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DON	deoxynivalenol
E	enzyme
EC_{50}	dose or concentration required to cause 50% inhbition
ELISA	enzyme limited immunosorbant assay

ES	enzyme-substrate complex
FB_1	fumonisin B_1
fg	fentogrammes
FUS	fusarenon-X
µg	microgrammes
g	grammes
GST	glutathione-S-transferase
HBV	hepatitis B virus
HCC	human hepatocellular carcinoma
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HT-2	HT-2 toxin
IC_{50}	tixun concentration required to cause 50% inhibition of growth
IPTG	isopropyl-1-thio-β-D-galactoside
K	rate dissociation or association constant
$K_2Cr_22O_7$	potassium dichromate
kg	kilogramme
K_m	Michaelis-Menten constant
K_S	spectral dissociation constant
K. marxianus	*Kluyveromyces marxianus* variety *marxianus*
µl	microlitre
l	litres
LC	liquid culture
LD_{50}	dose (or concentration) resulting in 50% death of a popuation.
µM	micromolar
M	molar
MA	molar absorbtivity
MAS	monoacetoxyscirpenol
mg	milligrammes
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimeter
mM	millimolar
MMA	membrane modulating agent
mRNA	messenger RNA
MPA	mycophenolic acid
MTG	methly-1-thio-β-D-galactoside
MTT	3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide
M.wt	Molecular weight
NCIMBL	National Collection of Industrial and Marine Bacteria Limited
NCYC	National Collection of Yeast Cultures

NEL	nil effect level
NEO	neosolaniol
ng	nanogrammes
NIV	nivalenol
nm	nanometers
3'-OH HT-2	3'-hydroxy HT-2 toxin
3'-OH T-2	3'-hydroxy T-2 toxin
3'-OH TRI	3'-hydroxy T-2 triol
ONPG	o-nitrophenyl-β-D-galactopyranoside
OTA	ochratoxin A
%	percent
P	product
P_1	sample peak area (mm^2)
P_2	standard peak area (mm^2)
PBS	phosphate-buffered saline
PBN	polymyxin B nonapeptide
PEPCK	phosphoenolpyruvate carboxykinase
pH	acidity
PMBS	polymyxin B sulphate
ppb	parts per billion
ppm	parts per million
λr	reference wavelength
RNA	ribonucleic acid
ROR	roridin A
rpm	revolutions per minute
S	substrate
scc	single cell colony
S. cerevisiae	*Saccharomyces cerevisiae*
sd	standard deviation
SDS	sodium dodecyl sulphate
S. fragilis	*Saccharomyces fragilis*
STC	sterigmatocystin
λt	test wavelength
ϕTG	phenyl-1-thio-β-D-galactoside
T-2	T-2 toxin
TCA	tricarboxylic acid cycle
TEA	tenuazonic acid
TET	T-2 tetraol
TLC	thin layer chromatography
TRI	T-2 triol
tRNA	transfer RNA
TSB	trypticase soy broth
UV	ultra violet

V_1	volume of standard applied (µl)
V_2	volume of sample extract applied (µl)
v/v	volume for volume
VER	verrucarin A
vol	volume
w/v	weight for volume
$\bar{x}$	mean
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YEPG	yeast extract, bacteriological peptone and glucose
YEPL	yeast extract, bacteriological peptone and lactose
ZEN	zearalenone

CHAPTER 1

INTRODUCTION

1.1 MYCOTOXINS

Mycotoxins are a range of toxic fungal secondary metabolites found to contaminate a variety of foods and feedstuffs. The word mycotoxin is derived from the Greek word "mykes", meaning fungus, and the Latin word "toxicum", meaning poison of biological origin. Over 300 mycotoxins are known to be produced by a taxonomically wide range of filamentous fungi (Cole & Cox, 1981) and are diverse in both their structure and toxic effects. Fortunately only a small number of these toxins are found as natural contaminants in foods and feeds. Toxigenic fungi are ubiquitous and as such, when conditions are favourable for their growth, they proliferate on a range of agricultural commodities, producing mycotoxins and thereby contaminating the foodstuff. The occurrence of mycotoxins in agricultural foods and feeds is dependent on several factors including region, season and the conditions under which a particular crop is grown, harvested and stored. Ingestion of foods contaminated with mycotoxins may cause a variety of pathological conditions, in both humans and animals, termed mycotoxicoses which have a severe impact on health and productivity, worldwide.

1.1.1 MYCOTOXIN PRODUCTION AND FORMATION

The production of mycotoxins in the environment is dependent on the physical presence of a toxigenic mould, the presence of a suitable substrate on which the mould may grow, and a suitable environment for toxin production.

1.1.1.1 Toxigenic fungi

Mycotoxins are produced by filamentous fungi from a variety of genera, the most significant toxigenic moulds being *Aspergillus, Penicillium* and *Fusarium*; the subject has been reviewed by Hockling (1991), Pitt (1991), Pitt & Leistner (1991) and Marasas *et al.*, (1984). Some of the more important toxigenic moulds and the mycotoxins they produce are summarised in Tables 1-4.

Tables 1-4 illustrate several important points regarding toxigenic moulds:- a mycotoxin may be produced by several strains of any one genus or by other close or

distant genera, (e.g. production of trichothecene mycotoxins); not all strains of a known toxigenic species necessarily produce mycotoxins, (e.g. less than 50% of *A. flavus* isolates are toxigenic (Klich & Pitt, 1988)); and some toxigenic strains are capable of producing more then one structural type of mycotoxin, (e.g. *A. flavus* may produce cyclopiazonic acid, sterigmatocystin and aflatrem in addition to aflatoxins). This co-production of mycotoxins causes a multiple toxigenic threat.

Species	Mycotoxin	Species	Mycotoxin
A. clavatus	Cytochalasin E Patulin	*A. flavus*	Aflatoxin B_1 & B_2 Aflatrem Cyclopiazonic Acid Sterigmatocystin
A. niger	Malformins Oxalic acid	*A. ochraceus*	Ochratoxin A Penicillic acid
A. parasiticus	Aflatoxin B_1, B_2, G_1 & G_2	*A. versicolor*	Sterigmatocystin

Table 1 Some toxigenic *Aspergillus* species and the mycotoxins they produce.

Species	Mycotoxin	Species	Mycotoxin
F. equiseti	Trichothecenes including Diacetoxyscirpenol Fusarenon-X Nivalenol T-2 toxin. Zearalenone	*F. graminearum*	Trichothecenes including Diacetoxyscirpenol Deoxynivalenol Fusarenon-X HT-2 toxin Nivalenol T-2 toxin. Zearalenone
F. moniliforme	Fumonisins	*F. sporotrichioides*	Trichothecenes including Deoxynivalenol Diacetoxyscirpenol Fusarenon-X Neosolaniol Nivalenol T-2 toxin. Zearalenone

Table 2 Some toxigenic *Fusarium* species and the mycotoxins they produce.

Species	Mycotoxin	Species	Mycotoxin
P. aurantiogriseum	Penicillic acid	*P. canescens*	Penitrem A
P. citreonigum	Citreoviridin	*P. citirinum*	Citrinin
P. commune	Cyclopiazonic acid	*P. crustosum*	Cyclopiazonic acid Penitrem A Roquefortine
P. expansum	Citrinin Patulin	*P. purpurescens*	Ochratoxin A
P. simplicissimum	Penicillic acid Verruculogen	*P. roquefortii*	Patulin PR toxin Roquefortine
P. verrucosum	Citrinin Ochratoxin A	*P. viridicatum*	Cyclopiazonic acid

Table 3 Some toxigenic *Penicillium* species and the mycotoxins they produce.

Species	Toxin	Species	Toxin
Alternaria	Alternariol methyl ether Alternariol Tenuazonic acid	*Clavicep*	Paspaline Paspalicine Secalonic Acids
Chatomium	Sterigmatocystin Chaetoglobosins	*Diplodia*	Diplodiol
Myrothecium	Verrucarins Roridins	*Phoma*	Cytochalasins Secalonic acids
Stachybotrys	Verrucarins Satratoxin Roridins	*Trichoderma*	Gliotoxin Trichothecenes

Table 4 Other toxigenic fungal genera and the mycotoxins they produce.

Tables 1-4 collated from Pitt, 1991; Hockling, 1991; Pitt & Leistner, 1991 & Marasas *et al.*, 1984.

1.1.1.2. Biosynthesis of mycotoxins

The biosynthesis of mycotoxins has been comprehensively reviewed by Steyn, (1980), mycotoxins being defined as typical fungal secondary metabolites that are produced by a consecutive series of enzyme-mediated reactions from a few intermediates of primary metabolism. Only a brief outline of the various biosynthetic pathways for the production of mycotoxins will be given here, with the emphasis on those toxins most relevant to this study.

The biosynthesis of secondary metabolites is distinct from primary metabolism. Secondary metabolite production is restricted to lower forms of life and is not found in animals, whereas primary metabolism is identical in all organisms (Betina, 1989). Secondary metabolites are synthesised by organisms using either enzymes of primary metabolism or special synthetases. The latter are formed in a manner similar to those of primary metabolism, but are usually of a low specificity, and result in the production of chemical families of structurally related metabolites, e.g. aflatoxins, trichothecenes. Secondary metabolism occurs optimally after a phase of balanced growth, and production of a particular secondary metabolite is usually restricted to a small number of species and may even be strain-specific; furthermore, it has not been possible to confidentally rationalise the biological function of secondary metabolites. Although secondary metabolites have a wide range of chemical structures, they are formed by only a few biosynthetic pathways, which are linked to primary metabolism via common intermediates: acetyl Co A, mevalonic acid and amino acids (Figure 1).

Primary metabolism		**Intermediate**		**Secondary metabolism**
Fatty acids	←	**Acetyl Co A**	→	Polyketides
Sterols	←	**Mevalonic acid**	→	Terpenes
Proteins	←	**Amino Acids**	→	Cyclic polypeptides

Figure 1 Intermediates linking primary and secondary metabolism.

The most important pathways in the biosynthesis of mycotoxins are the polyketide and mevalonate (terpenoid) pathways, and those that utilise essential amino acids; however several mycotoxins are synthesised from precursors of more than one route and for a few mycotoxins intermediates of the tricarboxylic acid (TCA) cycle act as precursors.

1.1.1.2.1 Polyketide pathway

Most fungal secondary metabolites are produced by the polyketide pathway. Polyketides and fatty acids (of primary metabolism) are both derived by the condensation of acetate and a small number of malonate molecules. Fatty acids are synthesised using acetate and 7-12 molecules of malonate whereas polyketides are produced using one acetate and a variable number of malonate molecules. During the biosynthesis of fatty acids a ketone group is formed as each successive 2C malonate molecule is added; this ketone group is continuously reduced producing the paraffin chain characteristic of fatty acids. However, if the ketone group is not reduced, the reactive intermediate formed can undergo a series condensation reaction, leading to the formation of a ring compound known as a polyketide (Smith & Moss, 1985). Polyketides have been classified as tri-, tetra-, pentaketides, etc., according to the number of acetyl groups incorporated. Mycotoxins derived from various polyketides are shown in Table 5.

Group	Mycotoxin
Tetraketides	Patulin, Penicillic acid
Pentaketides	Citrinin, Ochratoxin
Heptaketides	Viomellein, Alternariol. Altenuene
Octaketides	Secalonic acid
Nonaketides	Citreoviridin, Zearalenone
Decaketides	Aflatoxin, Sterigmatocystin

Smith and Moss, 1985.

Table 5 Polyketide-derived mycotoxins.

The folding and condensation of the initial polyketide chain is mediated by specific enzymes and is strain-specific. It is mediated by divalent metal ions such as Ca^{2+}, Mg^{2+} and Zn^{2+}. The close relationship between the initial stages of polyketide biosynthesis and fatty acid synthesis suggests the polyketide synthetase enzyme complex is evolved from the fatty acid synthetase complex (Smith & Moss, 1985).

Most mycotoxins are produced by the modification of the initial polyketide condensation product; e.g. patulin is synthesised by the condensation, decarboxylation, oxidisation and recyclisation of a tetraketide. However, a single polyketide chain may

also be used to produce a diverse range a mycotoxins by the addition of substituents at different positions on the original ketide skeleton; e.g. a pentaketide skeleton may produce ochratoxin or citrinin depending on the position of the methyl substituent (Betina, 1989). The biosynthesis of most polyketide-derived mycotoxins involves oxidative processes, with the exception of zearalenone, for which a nonaketide skeleton is partially reduced. The biosynthesis of aflatoxin from a decaketide skeleton is the most well characterised biosynthetic pathway of the mycotoxins.

1.1.1.2.2 Mevalonate (terpenoid) pathway

Mevalonic acid (derived from three molecules of acetyl CoA) is an important intermediate in both primary and secondary metabolism. In primary metabolism it undergoes pyrophosphorylation, decarboxylation and dehydration to form isopentyl pyrophosphate, a precursor in the formation of sterols, steroids and caretenoids (Stryer, 1981); in secondary metabolism, mevalonate is the starting point for the biosynthesis of trichothecenes.

Trichothecenes are part of the terpene family, which may be categorised as mono-, sesqui-, di- and tri-terpenes based on their C_{10}, C_{15}, C_{20}, and C_{30} skeletons, respectively; the trichothecenes being sesquiterpenes. The trichothecenes are derived from mevalonate via the intermediate farnesyl pyrophosphate, by a cyclisation sequence initiated by enzyme attack at C_{10} of farnesyl pyrophosphate and subsequent methyl group migrations (Tamm & Breitenstein, 1980). This ultimately produces the characteristic epoxy-trichothecene nucleus, which may be substituted at five positions with an oxygen function which may or may not be acylated, thereby producing the large number of trichothecene derivatives.

In the production of the macrocyclic trichothecenes (verrucarins and roridins) two of the hydroxyl groups on the trichothecene nucleus are linked by a long di- or tri-ester chain to form a large ring; these toxins are produced by genera other than *Fusarium* (Tamm & Breitenstein, 1980).

PR toxin is a non-trichothecene mycotoxin synthesised via the mevalonate pathway (Smith & Moss, 1985).

1.1.1.2.3 Other routes

A number of mycotoxins are derived from amino acids, often with part of their skeletal carbon atoms being derived from polyketide- or terpenoid-derived precursors. For example, the tremorgens are derived from an amino acid, usually tryptophan, linked to a number of mevalonate-derived isoprene units (Smith & Moss, 1985), roquefortine is derived from tryptophan and histidine, substituted in the indole ring with an isoprene unit (Yamazaki, 1980) and the cytochalasins are also derived from amino acid (tryptophan) and polyketide precursors (Tamm, 1980). Additionally, the tetramic acids, cyclopiazonic acid and tenuazonic acid are derived from the condensation of an amino acid (L-tryptophan and L-isoleucine, respectively) with a polyketide (Holzapfel, 1980).

The rubratoxin group of mycotoxins are produced from precursors of the TCA cycle, by the cyclisation of an acetate-derivative decanoic acid and oxaloacetate. This reaction is analogous to the citric acid synthetase reaction of the Krebs' cycle (Vleggaar & Steyn, 1980).

1.1.1.3 Environmental factors affecting mycotoxin production

The production of mycotoxins is influenced by both the genotype of the fungus and the physiochemical micro-environment in which it is growing. Fungal growth is a prerequisite for mycotoxin production, but optimum conditions for growth may differ from those for mycotoxin production; and mycotoxins are usually produced in the late exponential or early stationary phase of growth, when nutrient availability is low.

General environmental factors affecting mycotoxin production by fungi, include strain variation in the fungus, the presence of other microorganisms, moisture, temperature, pH, the gaseous environment, the presence of agricultural biocides and preservatives and, for growing plants, varietal resistance and plant stress. These factors have been reviewed by Moss, (1991), Scott, (1991), Bullerman *et al.*, (1984), and Hesseltine, (1976), and will be discussed only briefly here.

Different strains of a toxigenic mould may differ in respect of which mycotoxins they produce, the extent of mycotoxin production and their response to changes in

conditions affecting mycotoxin production . In the field, fungi usually occur as mixed populations of species (and strains) and cultural competition can reduce mycotoxin yields (Scott, 1991).

Moisture and temperature also have a critical effect on both mould growth and mycotoxin production; the two factors being closely linked. Moisture may be expressed in terms of relative humidity or as an index of water available for fungal growth, water activity (a_w); and mycotoxin production is generally favoured by high water activity.

Fungi that invade cereal grains have been categorised into three groups which have different moisture requirements, as shown in Table 6. Foods with a moisture content above 13% are susceptible to growth of toxigenic fungi and mycotoxin contamination, although commodities with a high oil content (e.g. tree-nuts and cotton seed) may be susceptible to mould growth at lower moisture contents.

Group	Organism	Occurrence and moisture requirement.
Field fungi-invade grain in the field prior to harvest	*Alternaria, Fusarium,*	Field; 20-25% moisture
Storage fungi-invade grain during post-harvest storage	*Aspergillus, Penicillium*	Storage; 13-18% moisture.
Advanced decay fungi-invade grain after considerable deterioration has occurred	*Fusarium, Chaetomium.*	Storage; 20-25% moisture.
Bullerman *et al.*, 1984		

Table 6 Moisture requirements of fungi invading cereals.

The optimum temperature and water activity for growth are often different to those for toxin production. Northolt *et al.* (1977, 1978, 1979a,b) examined the effects of water activity in combination with temperature on fungal growth and the production of several mycotoxins including aflatoxins, patulin, penicillic acid and ochratoxin A. Generally, the minimum water activity for the production of mycotoxins was greater than the minimum water activity required for growth, the minimum water activities required for the growth of *Penicillium, Aspergilli* and *Fusarium* species being 0.8-0.9, 0.7-0.8, and 0.8-0.92, respectively. Mould strain and substrate were also shown to affect the minimum water activity required for toxin production.

Growth and toxin production at a given temperature has also been shown to be related to water activity (Bullerman *et al.*, 1984). Generally, the higher the water activity the lower the temperature at which growth and toxin production may occur. The optimum temperatures for growth and aflatoxin production by *A. parasiticus* are 35°C and 25°C, respectively (Faraj *et al.*, 1991); however, species of *Penicillium* and *Fusarium* are capable of growth and toxin production at temperatures as low as 5°C.

Toxigenic fungi also require oxygen for growth (Hesseltine, 1976). Atmospheres with high carbon dioxide (20-40%) and low oxygen concentrations have been shown to prevent mould growth and toxin production in a number of stored commodities, including peanuts (Bullerman *et al.*, 1984), and may offer some promise in preventing mould growth and mycotoxin contamination of such products. The use of chemicals to control mycotoxin formation has also been studied; organic acids (e.g. sorbic acid, benzoic acid and propionic acid) have been shown to inhibit mould growth (Scott, 1991), and pesticides such as fonofos, carbaryl and maneb have been shown to inhibit mycotoxin production by *F. roseum* growing on corn (Draunton & Churchville, 1985). Damage and plant stress can also result in an increased incidence of contamination of plant products with mycotoxins. Insect or mechanical damage, moisture, and drought stress can all increase the invasion of oilseeds by *A. flavus* or other fungi (Scott, 1991). However, varieties of groundnuts and maize have been developed that are more resistant to *A. flavus* and hybrids of maize have been produced that have some resistance to zearalenone formation by *F. graminearum* (Dalvi & Salunkhe, 1990).

The effects of the physiochemical environment on toxin production have been best characterised in the production of the aflatoxins. The presence of glutamic and aspartic acids enhance aflatoxin production, and zinc is essential for maximum biosynthesis. It is thought that zinc may stimulate glycolysis during stationary phase ensuring there is sufficient acetyl coenzyme A available for polyketide biosynthesis (Failla & Niehaus, 1986).

1.1.2 NATURAL OCCURRENCE OF MYCOTOXINS

Although over 300 mycotoxins are now known, only a small number of these have been found as natural contaminants in food materials and are considered to pose a risk to human health as food contaminants. These include aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2, sterigmatocystin, ochratoxin A, citrinin, patulin, penicillic acid, zearalenone, fumonisin B_1 and the trichothecenes: T-2 toxin, diacetoxyscirpenol, deoxynivalenol, HT-2 toxin, neosolaniol, nivalenol and fusarenon-X (Bullerman, 1986) and the occurrence of toxigenic fungi in the food chain is a potential hazard to both human and animal health. Toxigenic fungi can grow and produce toxins on a wide range of substrates, including growing crops, leaves and stems, grains and seeds, fruits and vegetables and plant and animal products. Many important agricultural commodities are therefore susceptible to mould growth and possible mycotoxin contamination at some stage in their production, processing, transport and storage (Table 7).

Toxin	Fungus	Commodities damaged
Aflatoxins	*A. flavus, A. parasiticus*	Groundnut, tree nuts cereals, cottonseed soybean, spices, fruits, groundnut meal
Ochratoxins	*A. ochraceus, P. verrucosum*	Legumes, cereals, coffee beans
Zearalenone	*Fusarium* spp.	Fungal infested corn, wheat, barley etc.
Trichothecenes	*Fusarium* spp.	Fungal infested corn, wheat
Rubratoxin	*P. rubrum, P. purpurogenum*	Fungal infested corn
Citrinin	*P. verrucosum, P. citrinum, P. expansum*	Cereals, including barley, wheat, oats, rye and rice
Tremorgens	*P. cyclopium, P. patulins Aspergillus* spp.	Fungal infested feeds, peanuts and rice
Patulin	*Penicillium* spp. *Aspergillus* spp.	Fungal infested fruits, including apples, plums, peaches, pears, apricots
Ergotoxins	*C. purpurea, C. paspali*	Fungal infested grains and grasses.
Dalvi & Salunkhe, 1990; Salunkhe *et al.*, (1980).		

Table 7 Occurrence of mycotoxins in some important agricultural commodities.

1.1.3 MYCOTOXICOSES

Evidence of human and animal disease caused by mycotoxins was initially established following retrospective investigations of diseases of unknown causes, apparently associated with the ingestion of mouldy foods. Mycotoxins thought to be involved in mycotoxicoses are summarised in Table 9, along with the producing fungi, susceptible hosts and biological effects.

Animals demonstrate variable susceptibilities to mycotoxins, depending on a number of factors, including sex, genetic variation, physiological factors - age, nutritional status, presence of other diseases, and environmental factors - climate, husbandry and management (Smith & Moss, 1985).

Mycotoxicoses in animals assume one of three forms: acute primary mycotoxicoses, chronic primary mycotoxicoses and secondary mycotoxicoses (Pier *et al.,* 1980); their symptoms are summarised in Table 8. Natural contamination levels of mycotoxins are not usually high enough to cause acute primary mycotoxicoses and data have generally been obtained from laboratory-based LD_{50} studies; and most feed- or field-contamination with mycotoxins causes chronic mycotoxicosis symptoms or secondary mycotoxin disease (Smith & Moss, 1985).

Mycotoxicoses	Symptoms	Organ or tissue affected
Acute primary mycotoxicoses: produced when high to moderate concentrations of mycotoxins are consumed causing a specific acute disease syndrome or death	Hepatitis	Liver
	Oestrogenism	Reproductive system
	Enteritis	Small intestine
	Nephropathy	Kidney
	Ataxia	Nervous system and muscle
	Dermal necrosis	Skin
	Carcinoma	Various
	Death	
Chronic primary mycotoxicoses: occur following the intake of moderate to low levels of toxins and cause reduced productivity	Slower growth rates Reduced reproductive efficiency Inferior market quality Reduced milk yields Reduced egg production	
Secondary mycotoxin diseases: produced form the intake of very low concentrations of specific mycotoxins:	Suppression of the immune response	

Smith and Moss, 1985; Pier *et al.*, 1980.

Table 8 Symptoms of mycotoxicoses in animals.

Mycotoxin	Mycotoxicoses	Producing Fungi	Susceptible host	Biological Effect
Ergot alkaloids	Ergotism	*C. purpurea* *C. paspalli*	Human, cattle, sheep, horse	Nervous or gangrenous forms (Ataxia)
Aflatoxins	Aflatoxicoses	*A. flavus* *A. parasiticus*	Mammals, birds, fishes	Acute hepatotoxicity and caridotoxicity Chronic liver toxicity Indian childhood cirrhosis Reye's syndrome Primary liver cancer Esophagal cancer Death
Ochratoxin A	Ochratoxicoses Balkan nephropathy Renal porcine nephropathy	*A. ochraceus* *P. verrucosum*	Humans, swine, turkey, chicken, horse	Nephropathy Urinary tract tumours Balkan nephropathy
Citrinin	Nephropathy	*P. citrium* *P. expansum* *P. verrucosum*	Swine	Nephropathy
Zearalenone	Oestrogenic mycotoxicoses	*F. graminearum* *F. sportrichioides* *F. equiseti*	Swine	Oestrogenic syndrome Vulvovaginitis Vaginal & rectal prolapse
Trichothecenes: Deoxynivalenol Nivalenol Fusarenon-X	Fusariotoxicoses (Akakabi-byo)	*F. graminearum* *F. sportrichioides* *F. tricinctum* *F. poae*	Human, livestock, birds	Feed refusal, nausea, vomiting, diarrhoea, leuopenia, haemorrhage, reproductive disorder, immune suppression
T-2 toxin Diacetoxyscirpenol	Alimentary toxic aleukia (ATA)	*F. sportrichioides* *F. poae*	Humans, livestock	Leukopenia, haemorrhage, necrotic angina, death
Satratoxin H Verrucarins Roridins	Stachbotrio-toxicoses	*S. atra* *Fusarium* spp.	Horse, cattle, sheep, swine, human	Leukopenia, gastrointestinal and pulmonary haemorrhage, death
Sterigmatocystin	Slobber photosensitising disease	*A. versicolor*	Rat, chicken	Hepatocarcinoma, acute oral toxicity
Penitrem A	Tremorgen intoxication	*P. canescens* *P. crustosum*	Cattle, sheep	Tremorgenic
Fumonisins	Leukoencephal-macia	*F. moniliforme*	Swine Horses Humans	Pulmonary oedema Leukoencephalomalacia Oesophageal cancer
Citreoviridin	Yellow rice toxicoses	*P. citreonigum*	Human	Neurotoxicity

Angsubhakorn, 1991; Kurata, 1990; Smith and Moss, 1985.

Table 9 Mycotoxins involved in mycotoxicoses.

1.2 PHYSIOCHEMICAL AND BIOLOGICAL PROPERTIES OF SOME MYCOTOXINS

A vast amount of literature has been published on mycotoxins since the 1960s, and this discussion will therefore be limited to the mycotoxins relevant to this study, the emphasis being on the aflatoxins and trichothecenes, although other mycotoxins studied will be mentioned briefly.

1.2.1 AFLATOXINS

The discovery of the aflatoxins emanated from the outbreak of "Turkey X" disease in 1960, which caused the deaths of thousands of young turkeys, pheasants and ducklings in England. Turkey X disease was shown not to be infectious, but of a dietary nature: a common ingredient in the turkey feed was shown to be Brazilian groundnut meal, contaminated by the moulds *A. flavus* and *A. parasiticus* (Blount, 1961). A chloroform extract of this meal yielded a toxic component, which produced disease in ducklings (Allcroft *et al.* 1961) and gave a blue fluorescence under UV light (Sargeant *et al.*, 1961). Subsequent investigations showed the single blue-fluorescent spot of the toxin-containing extract could be split into four components using thin-layer chromatography (TLC). Two of these components fluoresced blue under UV light and were designated aflatoxin B_1 and B_2, (1 & 2 designating their position on a TLC plate) and two fluoresced green and were designated aflatoxin G_1 and G_2 (Nesbitt *et al.*, 1962; Hartley *et al.*, 1963); their structures are shown in Figure 2. Following the isolation of these four major aflatoxins, numerous other analogues were discovered that are derivatives of the four major aflatoxins, produced by metabolism or spontaneously in response to the chemical environment and are summarised in Table 10.

Aflatoxin	Molecular Weight	Molecular Formula	Source
B_1	312	$C_{17}H_{12}O_6$	Secondary metabolite of A*spergillus* spp.
B_2	314	$C_{17}H_{14}O_6$	Secondary metabolite of A*spergillus* spp.
G_1	328	$C_{17}H_{12}O_7$	Secondary metabolite of A*spergillus* spp.
G_2	330	$C_{17}H_{14}O_7$	Secondary metabolite of A*spergillus* spp.
M_1	328	$C_{17}H_{12}O_6$	Metabolic product of B_1, found in milk and urine of animals
M_2	330	$C_{17}H_{12}O_6$	Metabolic product of B_2, found in milk and urine of animals
GM_1	344	$C_{17}H_{12}O_8$	Metabolic product of G_1, found in milk and urine of animals
GM_2	346	$C_{17}H_{14}O_8$	Metabolic product of G_2, found in milk and urine of animals
M_{2a}	362	$C_{17}H_{14}O_8$	Metabolic product of M_1
GM_{2a}	346	$C_{17}H_{14}O_9$	Metabolic product of GM_1
B_{2a}	330	$C_{17}H_{14}O_7$	Fungal metabolite of *A. flavus* produced in acidic conditions (biotransformation product of B_1)
G_{2a}	346	$C_{17}H_{14}O_8$	Fungal metabolite of *A. flavus* produced in acidic conditions (biotransformation product of G_1)
Parasiticol (B_3)	302	$C_{16}H_{14}O_6$	Metabolic degradation of G_1
Aflatoxicol	314	$C_{17}H_{14}O_6$	Metabolic product of B_1 in animals and micro-organisms. Two stereoisometric forms.
P_1	298	$C_{16}H_{10}O_6$	Metabolic product of B_1 in animals.
Q_1	328	$C_{17}H_{12}O_7$	Metabolic product of B_1 in animals.
Cole and Cox, 1981; Betina, 1989.			

Table 10 The aflatoxins and some of their properties.

1.2.1.1 Production of aflatoxins

The production of aflatoxins is mainly associated with certain strains of *Aspergillus flavus* and *A. parasiticus*, which are ubiquitous, and capable of growth over a wide temperature range on substrates of high carbohydrate content (Pitt, 1991). Many commodities are therefore vulnerable to aflatoxin contamination including peanuts, Brazil nuts, pistachio nuts, cottonseed meal, corn, rice, wheat and sorghum, during growth, harvesting, processing, storage and shipment; and aflatoxin contamination causes a serious economic and health problem. The most important factors regulating fungal growth and aflatoxin production are the moisture content of the substrate and

temperature. For maximum toxin production the optimum moisture content is 18% for starch cereal grains and 9-10% for oil-rich nuts and seeds (WHO, 1979), the minimum, optimum and maximum temperatures for toxin production are 12, 27 and 42°C respectively (Dalvi & Salunkhe, 1990). Insect damage is also often associated with aflatoxin contamination (Scott, 1991).

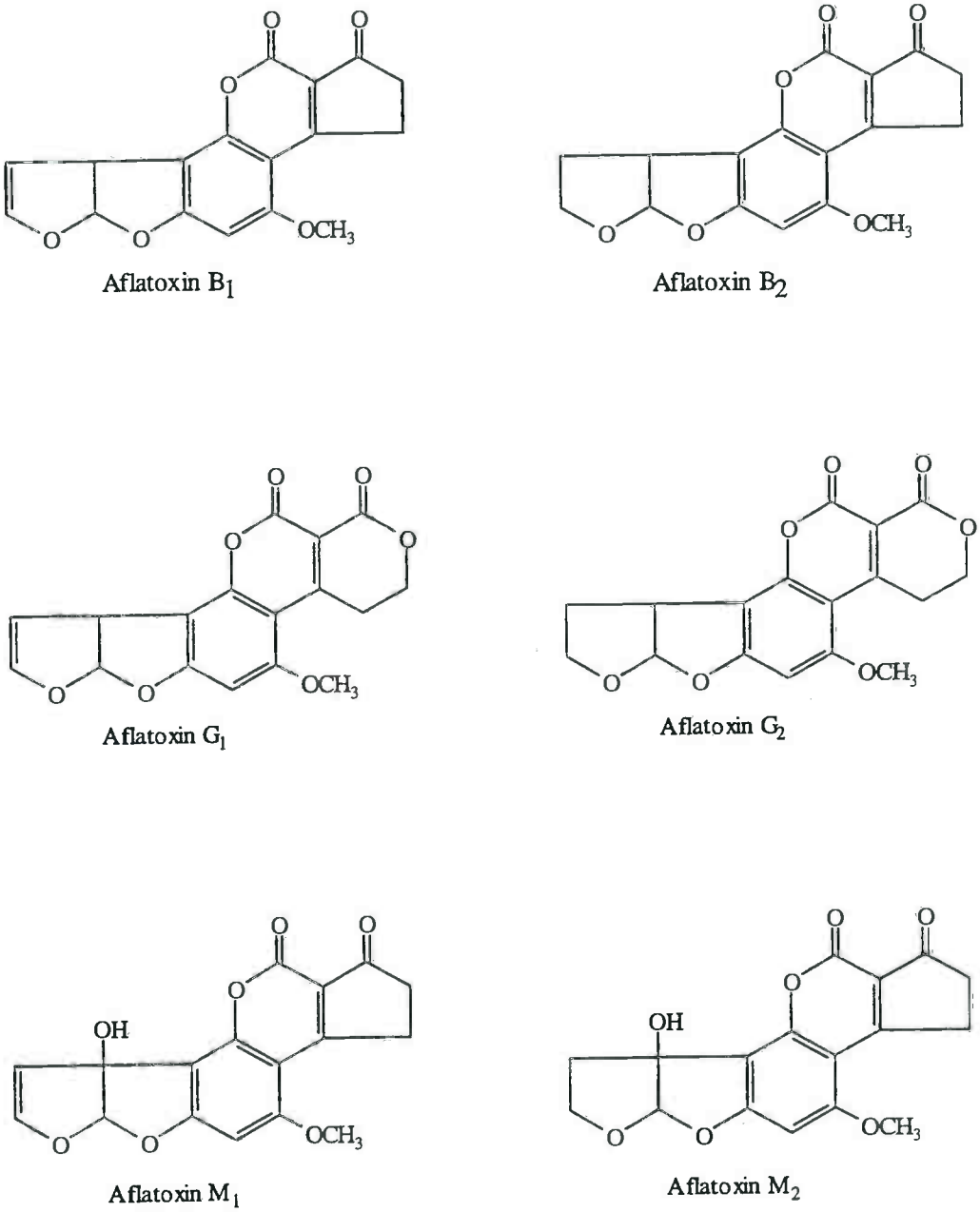


Figure 2 Structures of some of the major aflatoxins.

1.2.1.2 Physiochemical properties of aflatoxins

The aflatoxins are a group of polycyclic unsaturated compounds that differ considerably in their biological effects. All these toxins, however, contain a coumarin ring fused to a bisfuran moiety and, additionally, either a cyclopentenone ring (characteristic of the B series) or a six membered lactone (characteristic of the G series). Although over 20 aflatoxins have been isolated (Cole & Cox, 1981), only 4 are well known and studied extensively. These are aflatoxin B_1, B_2, G_1 and G_2 (Figure 2) and they are normally the major components of crude extracts of aflatoxins. Aflatoxins M_1 and M_2 (Figure 2) are metabolites of B_1 and B_2 which are found in the milk and urine of animals which have ingested aflatoxins B_1 and B_2.

1.2.1.3 Biological effects of aflatoxins

The aflatoxins are both acutely and chronically toxic to a range of animals, including man. They produce four distinct effects: acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Pitt, 1991).

Acute toxicity to animals varies between species (Table 11) and is dependent on age, strain, gender and the route of admission. Common symptoms following acute exposure to AFB_1 include malaise, loss of appetite and decreased growth rates.

Animal species	LD_{50} (mg/kg body weight)	Animal species	LD_{50} (mg/kg body weight)
Rabbit	0.3	Baboon	2.0
Duckling (1 day old)	0.3	Monkey	2.2
Cat	0.6	Rat	5.5-17.9
Pig	0.6	Chicken	6.3
Trout	0.8	Rat (male)	5.5-7.2
Dog	1.0	Rat (female)	17.9
Guinea pig	1.4-2.0.	Mouse	9.0
Sheep	2.0	Hamster	10.2

Betina, (1989); Smith & Moss, (1985).

Table 11 Variation in species susceptibility to aflatoxin B_1.

Among the four aflatoxins, AFB_1 has the greatest acute toxicity, followed by G_1, B_2 and G_2. The LD_{50} (dose or concentration of toxin resulting in 50% death of the population) of these toxins in one day old ducklings is shown in Table 12, alongside comparable values for aflatoxins M_1 and M_2.

Aflatoxin	LD_{50} (µg 50g body mass)	Aflatoxin	LD_{50} (mg kg^{-1} body weight)
B_1	18.2	B_1	23.0
G_1	39.2	M_1	16.6
B_2	84.8	M_2	62.0
G_2	172.5		
Carnaghan *et al.*, (1963)		Holzapfel *et al.*, (1966)	

Table 12 Toxicity of aflatoxins in one-day old ducklings.

AFB_1 is carcinogenic in a variety of animal species, the liver being the primary target organ for carcinogenesis, although tumours in other organs may result from long-term exposure. Primary lesions in the liver include hemarrhagic necrosis, fatty infiltration and bile duct proliferation. AFB_1 is a designated human carcinogen and has been implicated in the high incidence of liver cancer in Africa and Asia. Considerable epidemiological data support the hypothesis that dietry AFB is an important risk factor for human hepatocellular carcinoma (HHC); in many geographical areas where the incidence of HHC is high, (e.g. sub-Saharan Africa and South-east Asia), there is a linear relationship between AFB_1 contaimination of food and the incidence of HHC (Hall & Wild, 1994; Ross *et al.*, 1992; Yeh *et al.*, 1989; VanRensberg, *et al.*, 1985). However, the precise role of AFB_1 in aetiology of the disease is not, at present clear, as there is also a coincidence of hepatitis B virus (HBV), which is another reputed factor for HHC in these regions Additionally, diets containing appreciable quantities of aflatoxins may also contain other contaminants such as other mycotoxins (e.g. sterigmatocystin, ochratoxin A, cyclopiazonic acid, etc.), insecticides or other plant toxins (e.g. alkaloids) which may also have a synergistic effect in liver tumour induction (Angsubhakorn 1991).

Aflatoxins have also been implicated as one of the causative factors in Reye's syndrome in Thailand, New Zealand, USA, Germany and other developing countries (Nelson *et al.*, 1980; Angsubhakorn, 1991), in Kwashiorkor in Sudan (Hendrickse, 1984), and outbreaks of hepatitis in India (Krishnamachari *et al.*, 1975); the inhalation of aflatoxin-contaminated dust has also been linked with the increased incidence of liver and lung cancer in employees at a Dutch peanut processing factory (Baxter *et al.*,, 1981).

AFB_1 is mutagenic to a number of microorganisms including *Salmonella typhimurium* (Ishii *et al.*, 1986; Ueno *et al.*, 1978), *Escherichia coli*, (Auffray & Boutibonnes, 1987), *Photobacterium phosphoreum* (Yates, 1985), *Bacillus subtilis* (Ueno & Kubota, 1976) and *Saccharomyces cerevisiae* (Kuczuk *et al.*, 1978) and possesses insecticidal activity to *Drosophila melanogaster* (Reiss, 1975c).

1.2.1.4 Biochemical mode of action

A requisite step in the toxic and carcinogenic action of AFB_1 is its biotransformation to metabolites which may then bind covalently to cellular macromoleclules such as DNA, RNA and proteins (Figure 3). Biotransformation is mediated primarily by hepatic and extrahepatic cytochromes P450 (cyt P450), and may result in the detoxification as well as the activation of aflatoxin.

The cyt P450-dependent oxidation of AFB_1 produces AFQ_1, AFM_1, AFP_1 and AFB_{2a} (Figure 3), which are all considered to be detoxification products.

Hydroxylation of AFB_1 produces AFQ_1 and AFM_1. AFQ_1 is a major metabolite of AFB_1 produced *in vitro*, (Eaton *et al.*, 1994), and has a lower acute toxicity (Hsieh *et al.*, 1984), mutagenicity (Coulombe *et al*, 1982; Gurtoo *et al.*, 1978) and carcinogenicity (Hendricks *et al.*, 1980) than AFB_1.

AFM_1 is a metabolite of AFB_1 found in the milk and urine of many species following exposure to AFB_1 (Ramsdell & Eaton, 1990b), and although very much less mutagenic than AFB_1 (Coulombe *et al*, 1982; Gurtoo *et al.*, 1978) is only slightly less

acutely toxic (Sinnhuber *et al.*, 1974), and is a relatively potent carcinogen (Eaton *et al.*, 1994).

O-demethylation of AFB_1 produces AFP_1 (Wong & Hsieh, 1980) which is much less toxic and mutagenic than AFB_1 (Coulombe *et al.*, 1982; Gurtoo *et al.*, 1978; Stolloff *et al.*, 1972). It can however be converted to a reactive epoxide (Essigmann *et al.*, 1983) and has been shown to have the highest correlation of all urinary metabolites with the presence of liver cancer (Ross *et al.*, 1992). O-demethylation of AFM_1 is also possible and produces 4,9a-dihydroxyaflatoxin B_1 (Eaton *et al.* 1988).

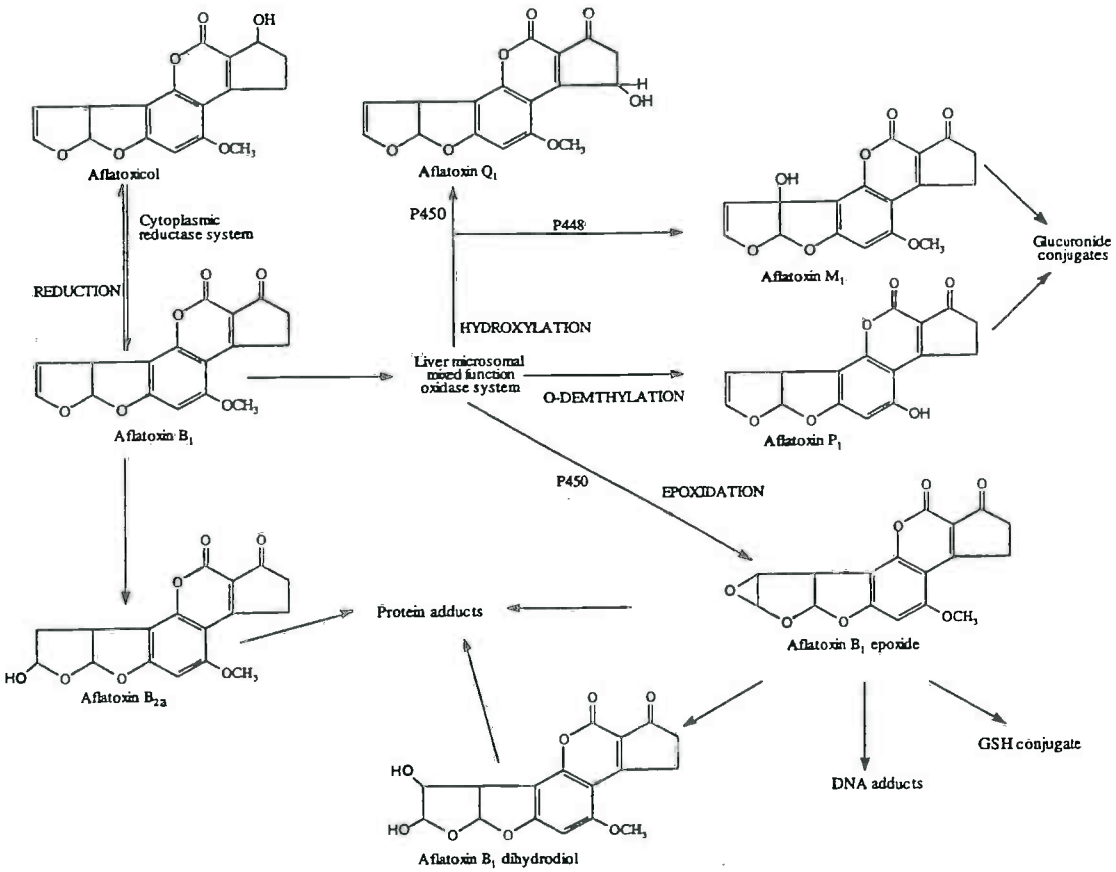


Figure 3 Metabolism of aflatoxin B_1 (adapted from Eaton *et al.*, 1994)

Hydration of the double bond of the terminal furan ring of AFB_1 and AFG_1 produces AFB_{2a} and AFG_{2a}. This reaction can also occur non-enzymatically under acidic conditions (Pohland *et al.*, 1968) and may occur in the stomach following oral

ingestion of AFB_1 (Eaton *et al.*, 1994). Microsomal hydroxylation of AFB_2 to AFB_{2a} has also been reported and may represent an activation mechanism for AFB_2 (Groopman *et al.*, 1981).

Reduction of the 1-keto group of AFB_1 produces aflatoxicol (AFL) (Detroy & Hesseltine, 1970) and this reaction has been shown to be catalysed by a cytosolic NADPH-reductase (Massey *et al.*, 1995; Chen *et al.*, 1981). The reaction is reversible *in vivo* (Loveland *et al.*, 1987) and *in vitro* (Salhab & Edwards, 1977) and AFL may act as a "reservoir" for AFB_1. AFL is as carcinogenic as AFB_1 (Schoenhard *et al.*, 1981) and is only slightly less mutagenic (Coulombe *et al.*, 1982), and as such is not considered to be a detoxification product (Eaton *et al.*, 1994).

Metabolic activation of the aflatoxins occurs via the cytochrome P450-dependent epoxidation of the double bond of the terminal dihydrobisfuran ring of AFB_1 producing the highly reactive aflatoxin B_1-8,9-epoxide, a potent electrophile, capable of alkylating nucleic acids (Essigmann *et al.*, 1977; Swenson *et al.*, 1977). Presumably because of its extreme reactivity, AFB_1-8,9-epoxide has only been isolated from biological systems as an adduct of glutathione or DNA bases (Essigmann *et al.*, 1977; Degen & Neumann, 1978). The epoxide binds covalently to the N-7 atom of guanyl residues in DNA to form DNA adducts; the most prominent adduct is AFB_1-N^7-guanine (AFB_1-N^7-gua) (Figure 4) and of the other adducts isolated, the 'opened-ring' derivative, AFB_1-formamidopyrimidine (AFB_1-FAPyr) (Figure 4) is the most common (Essigmann *et al.*, 1982). The formation of these adducts is thought to be the first step in the development of tumours. Repair of these genetic lesions occurs in living cells either enzymatically or spontaneously and the adducts are excreted in the urine. AFB_1-FAPyr adducts appear to be more resistant to DNA repair enzymes than AFB_1-N^7-gua adducts (Croy & Wogan, 1981).

All aflatoxin derivatives with an unsaturated terminal furan ring are capable of binding to DNA, presumably via epoxidation (Eaton *et al.*, 1994), and DNA adducts of AFP_1 and AFM_1 epoxides have been isolated from rat liver following exposure to AFB_1 (Essigmann *et al.*, 1983); AFQ_1 however, is a poor substrate for epoxidation (Raney *et*

al., 1992). DNA adducts have also been observed following exposure to AFB_2, which is first reduced to AFB_1 which is then epoxidised (Swenson *et al.*, 1977; Roebuck *et al.*, 1978).

Non cyt P450-dependent mechanisms of activation of AFB_1 have also been reported, and include the co-oxygenation of AFB_1 by microsomal prostaglandin H synthetase and cytosolic lipoxygenases (Massey *et al.*, 1995) and the exposure of AFB_1 and AFB_2 to UV light (Eaton *et al.*, 1994).

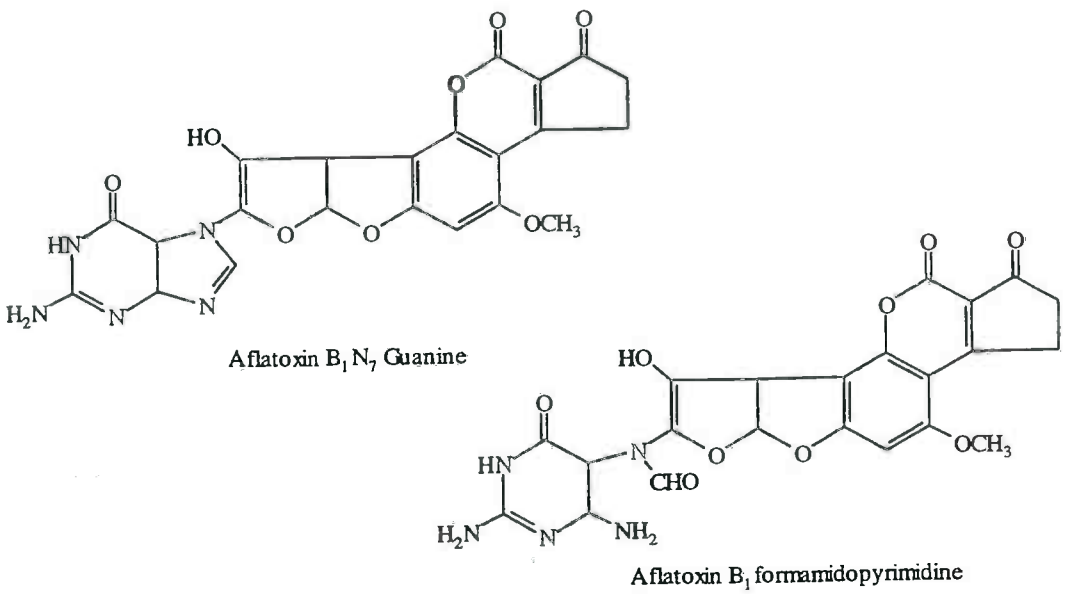


Figure 4 Major aflatoxin-DNA adducts.

Multiple forms of human cyt P450 have been shown to be capable of activating AFB_1 (Massey *et al.*, 1995; Eaton *et al.*, 1994). CYP1A2, CYP2A3, CYP2B7, CYP3A3 and CYP3A4 have all been implicated, by it remains unclear as to which is the most important. However, CYP1A2 appears to be the high-affinity cyt P450, responsible for the activation of AFB_1 at low substrate concentrations, whereas CYP3A4 has a relatively low affinity for AFB_1 and is active at higher substrate concentrations (Massey *et al.*, 1995; Eaton *et al.*, 1994) and CYP1A enzymes produce AFM_1 whereas CYP3A enzymes produce mainly AFQ_1 (Eaton *et al.*, 1994).

AFB_1-8,9-epoxide can be inactivated by conjugation to glutathione in a reaction catalysed by glutathione S-transferase (GST) (Ramsdell & Eaton, 1990a; Neal *et al.*, 1987), this reaction being the first step in the mercaturic acid pathway which leads to. the excretion of a variety of xenobiotics. Inactivation by AFB_1-8,9-epoxide by GST is an alternative to binding to nucleophilic sites in macromolecules and is therefore important in the protection of tissues from AFB_1 toxicity (Neal & Green, 1983) and in determining species susceptibility to AFB_1 (Eaton & Ramsdell, 1992). Other detoxification mechanisms occur via the conjugation of hydroxylated metabolites with sulphates and glucuronic acid (Busby & Wogan, 1984) and AFP_1 and AFL appear to be better substrates for glucuronic conjugation than AFM_1 or AFP_1 (Eaton *et al.*, 1994).

AFB_1-8,9-epoxide may also be inactivated by hydrolysis to AFB_1-dihydrodiol (AFB_1-dhd) (Neal & Colley 1979; Neal *et al.*, 1981), and this reaction may occur spontaneously or be catalysed by epoxide hydrolase. The role of the latter in the inactivation of AFB_1-8,9-epoxide is however unclear and is minimal, at least in hepatic systems (Massey *et al.*, 1995; Eaton *et al.*, 1994). AFB_1-dhd can exist in a resonance form as a phenolate ion that can form Schiff base adducts with protein amino groups, particularly lysine (Eaton *et al.*, 1994), and may be responsible for the acute effects of AFB_1 in various animal species (Neal *et al.*, 1981).

In summary, the fate of AFB_1 is dependent on the relative activity of several biotransformation pathways, in addition to other factors such as DNA repair (Eaton *et al.*, 1994). For carcinogenicity, AFB_1-8,9-epoxide is the key metabolite. The production of hydroxylated metabolites of AFB_1 (AFM_1 AFP_1 and AFQ_1) represents detoxification; and the epoxide may be detoxified via conjugation with glutathione, or hydrolysis to AFB_1-dhd. The latter, although less carcinogenic than AFB_1 is acutely toxic via protein-binding.

1.2.2 TRICHOTHECENES

Although there are now known to be over 100 members of the trichothecene group of mycotoxins, only a few, including T-2, HT-2, DAS, DON, FUS and NIV have been detected as natural contaminants in cereal grains. Due to their widespread natural occurrence and implication in a number of serious outbreaks of human and animal mycotoxicoses they are one of the most hazardous groups of mycotoxins.

1.2.2.1 Production of trichothecenes

The trichothecenes are a class of secondary metabolites, closely related in structure, that have been isolated mainly from species of *Fusarium*, and certain other genera of fungi, such as *Myrothecium, Trichoderma, Calconectria, Gibberella, Cylindrocarpon, Verticimonosporium* and *Stachybotrys* (Marasas *et al.*, 1984); some trichothecenes have also been isolated from higher plants (*Baccharis* species) although they are more likely derived from the associated soil fungus (Ueno, 1977). *Fusaria* grow on crops before harvest and grow only at high a_ws; trichothecenes are therefore usually only produced before or immediately after harvest (Hockling, 1991) and toxin production is often triggered by cool temperatures (<15°C) (Moss, 1991). The trichothecenes have been found to contaminate a variety of food- and feed-stuffs including barley, corn, oats, wheat, beans, straw and hay (Marasas *et al.*, 1979).

1.2.2.2 Physiochemical properties of the trichothecenes

Trichothecenes are characterised by a basic, tetracyclic, sesquiterpenoid structure which includes a six-member oxygen-containing ring, an epoxide in the 12,13 position and an olefinic bond in the 9,10 position (Figure 5). Trichothecenes possess oxygen containing substituents at a range of positions, including hydroxyl, ester, keto and epoxide groups (Betina, 1989). Many workers have classified trichothecenes into groups according to chemical structure and properties, and fungal source. In this study the trichothecenes are classified in accordance with Tamm and Tori (1984), (Table 13); and the structures of those toxins relevant to this study are shown in Figures 5 & 6.

Group	Characteristics	Representative toxins	
1	Absence of an oxygen function at the C-8 position	Scirpentriol Diacetoxyscirpenol	(SCR) (DAS)
2	Presence of an oxygen function other than a ketone at the C-8 position	T-2 toxin HT-2 toxin Acetyl T-2 toxin T-2 triol T-2 tetraol Neosolaniol	(T-2) (HT-2) (AcT-2) (TRI) (TET) (NEO)
3	Presence of a ketone group at the C-8 position	Fusarenon-X Nivalenol Deoxynivalenol	(FUS) (NIV) (DON)
4	Macrocyclics	Verrucarin A Roridin A	(VER) (ROR)

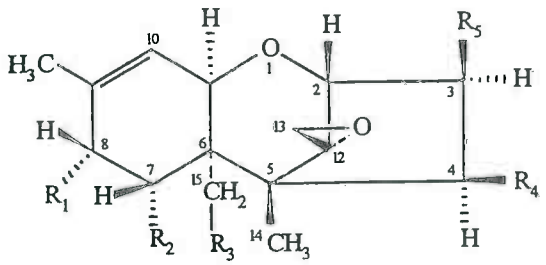
Table 13 Classification of the trichothecene mycotoxins used in this study.

1.2.2.3 Biological effects of the trichothecenes

The trichothecenes produce a wide variety of toxic effects; acute trichothecene poisoning is characterised by gastrointestinal disturbances such as vomiting, diarrhoea and inflammation, dermal irritation, feed refusal, haemorrhage, abortion, haematological changes, necrotic angina, nervous disorders and destruction of the bone marrow (Coulombe, 1993; Ueno, 1983, 1977). Symptoms vary according to the animal and trichothecene used (Ueno *et al.*, 1973) and toxicity varies according to age, but not sex (Ueno, 1977).

Trichothecenes have been implicated in numerous animal diseases, as well as in a number of diseases in man. Two recent outbreaks of trichothecene-related disease in man have been reported: DON was isolated from samples of scabby wheat in China in 1985, following an outbreak of mouldy corn and scabby wheat toxicoses (Luo, 1988) and DON, NIV and T-2 were isolated from wheat flour following an outbreak of disease in India, in 1987 (Bhat *et al.*, 1989).

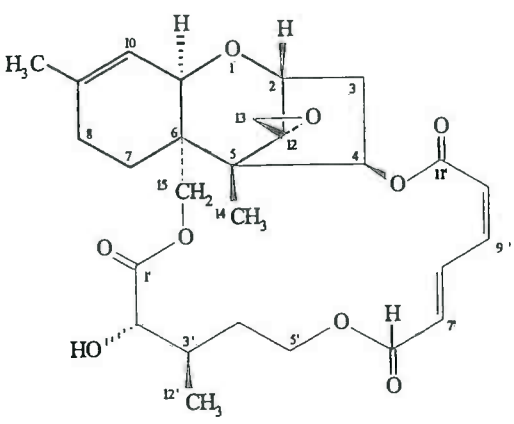
Less recently, T-2 was implicated in the occurrence of alimentary toxic aleukia (ATA) in the U.S.S.R. in the 1940's and this disease is thought to be attributable to the ingestion of overwintered grain contaminated by *Fusarium* species (Mirocha & Pathre, 1973). NIV, DON and FUS have also been implicated in Akakabi disease seen in Japan and Korea between 1946-63 (WHO, 1990).



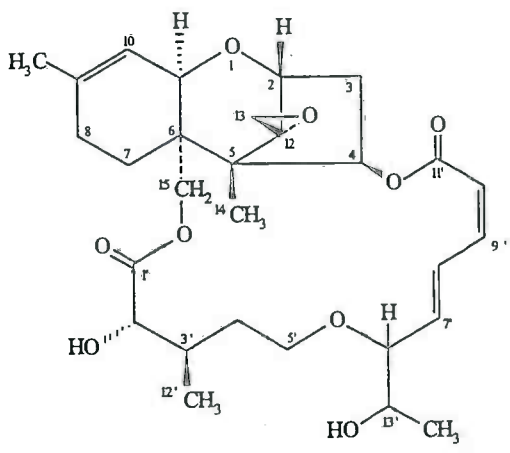
		R_1	R_2	R_3	R_4	R_5
Group 1	**SCR**	H	H	OH	OH	OH
	DAS	H	H	$OCOCH_3$	$OCOCH_3$	OH
Group 2	**AcT-2**	X	H	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
	T-2	X	H	$OCOCH_3$	$OCOCH_3$	OH
	HT-2	X	H	$OCOCH_3$	OH	OH
	TRI	X	H	OH	OH	OH
	TET	OH	H	OH	OH	OH
	NEO	OH	H	$OCOCH_3$	$OCOCH_3$	OH
Group 3	**FUS**	O	OH	OH	$OCOCH_3$	OH
	NIV	O	OH	OH	OH	OH
	DON	O	OH	OH	H	OH

$X=OCOCH_2CH(CH_3)_2$

Figure 5 Structures of the non-macrocyclic trichothecenes (groups 1-3)



Verrucarin A



Roridin A

Figure 6 Structures of the macrocyclic trichothecenes (group 4).

In animals, trichothecene-poisoning has been implicated in stachbotryotoxicosis in horses, caused by the ingestion of satratoxin-contaminated hay and straw (Eppley & Bailey, 1973); equine leukoencephalomalacia in South Africa, caused by T-2 and neosolaniol (Marasas *et al.*, 1979); and bean hull toxicoses in horses, in Japan (WHO, 1990). T-2, neosolaniol, diacetoxyscirpenol, and fusarenon-X have also been associated with emesis in farm animals (WHO, 1990).

The trichothecenes are not carcinogenic (Beasley *et al.*, 1989) and are non-mutagenic to *S. typhimurium* in the Ames test (T-2, DAS & DON) (Kuczuk *et al.*, 1978; Ueno *et al.*, 1978), to *B. subtilis* in the Rec assay (T-2 & FUS) (Ueno & Kubato, 1976) and to *S. cerevisiae* (T-2 & DAS) (Kuczuk *et al.*, 1978).

1.2.2.4 Biochemical mode of action

The trichothecenes are potent inhibitors of eukaryotic protein synthesis in a wide variety of organisms including fungi, plants and animals. All observed toxic effects are thought to be related, either directly or indirectly, to the inhibition of protein synthesis, which has been shown to be the primary mechanism of toxicity to yeasts and mammalian tissue culture (Feinberg & McLaughlin, 1989). Ueno *et al.* (1968) first demonstrated that two trichothecenes, nivalenol and fusarenon-X, inhibited protein synthesis in rabbit reticulocytes and mouse ascites cells. Trichothecenes interfere with protein synthesis by selectively binding to the eukaryotic ribosome, their target being the 60S ribosome. Each ribosome has been shown to have one binding site for trichodermin, (McLaughlin *et al.*, 1977; Barbacid & Vazquez, 1974; Wei *et al.*, 1974) and several studies have shown that trichothecenes compete for the same binding site (Mclaughlin *et al.*, 1977; Cannon, *et al.*, 1976; Jimenez *et al.*, 1975; Wei, *et al.*, 1974; Schindler, 1974) and inhibit protein synthesis via the inhibition of peptidyl transferase activity (Jimenez & Vazquez, 1978; Mizuno, 1975; Wei & Mclaughlin, 1974; Schindler, 1974; Carrasco *et al.* 1973).

Eukaryotic protein synthesis occurs on polysomes, which are composed of several individual ribosomes, that translate a single mRNA molecule encoding for one

polypeptide. During the initiation stage of protein synthesis one 40S and one 60S ribosomal subunit join the mRNA at the initiation codon, (AUG), to form a functional ribosome. This process is complex and involves nine different initiation factors and peptidyl transferase - an integral part of the 60S ribosome, and culminates in the formation of the first peptide bond. The ribosome then undergoes 100-200 elongation events as the amino acids are added, one at a time, to the growing peptide chain. Each elongation requires elongation factors I and II, and peptidyl transferase, and continues until the ribosome reaches a termination or 'stop' codon on the mRNA. Then during the termination step the polypeptide chain is transferred to water, rather than an incoming aminoacyl tRNA, by peptidyl transferase. The peptide is then released from the ribosome which is in turn released from the mRNA, with the aid of a release factor. The free ribosomal subunits then rejoin the mRNA at the 5' end for a new round of initiation. If there is an excess of ribosomal subunits, one 40S and one 60S subunit join without mRNA to form a monosome (80S) for storage.

When protein synthesis is blocked by an initiation inhibitor, ribosomes that have completed elongation and termination cannot rejoin the mRNA and there is a build-up of free subunits which form monosomes; in contrast, inhibition of elongation or termination inhibits protein synthesis without causing the conversion of polysomes to monosomes. The trichothecenes have been divided into two groups, initiation inhibitors and elongation/termination inhibitors, based upon what happens to the polysomes (Table 14).

Initiation	Elongation or termination
Diacetoxyscirpenol	Croticin
Fusarenon-X	Crotocol
HT-2 toxin	Deoxynivalenol
Monoacetoxyscirpenol	Diacetoxyscirpenol
Nivalenol	Fusarenon-X
Scirpentriol	Trichodermin
T-2 toxin	Trichodermol
Verrucarin A	Trichothecin
	Trichothecolone
	Verrucarol
Betina, 1989; Mclaughlin *et al.*, 1977; Mirocha *et al.*, 1979; Ueno, 1991.	

Table 14 Site of action of trichothecenes on protein synthesis.

For a small number of toxins, inhibitors of elongation/termination have been further divided by determining whether polysomes reform after an initiation inhibitor is removed (Mclaughlin *et al.*, 1977). Elongation inhibitors prevent polysome reformation (the first ribosome is unable to move along the mRNA and allow another to join) whereas termination inhibitors allow polysome formation (new ribosomes are able to join the mRNA). Using this assay trichodermin, trichodermol and deoxynivalenol were found to inhibit termination, rather than elongation, in both mammalian cells and yeasts (Cundliffe & Davis, 1977).

As can be seen in Table 14 there are some discrepancies, within the literature, as to in which group a toxin should be placed (e.g. DAS and FUS). This may be due to variations in the concentration at which the toxin was evaluated. Carter and Cannon (1977) showed that the mode of action of some trichothecenes was critically dependent on concentration selected. On the basis of *in vivo* and *in vitro* studies on the effects of 10 trichothecenes on polysomes, they divided the trichothecenes into four groups (I-IV) as shown in Table 15. The stabilisation of polysome profiles is caused by the inhibition of elongation/termination; disaggregation of polysomes into monosomes ('run-off') is caused by the inhibition of initiation or by the binding of the toxin to ribosomes carrying short nascent polypeptides.

Group	Representative toxins	Effect on polysome profile
I	Trichodermin 3,15-didesacetylcalonectrin 15-desacetylcalonectrin Trichothecin	High toxin concentrations stabilise the polysomes Low toxin concentrations cause a limited disaggregation of the polysomes ('run-off').
II	Scirpentriol 3-desacetylcalonectrin 15-acetoxyscirpendiol	High toxin concentrations totally stabilise the polysome. Low toxin-concentrations induce extensive 'run-off'.
III	Diacetoxyscirpenol Calonectrin	High toxin concentrations partially stabilise the polysome. Low concentrations induce total 'run off'.
IV	T2 toxin	All concentrations induce total 'run off'.

Low concentrations = 0.1-20µg/ml; high concentrations = 20-150µg/ml.

Table 15 Concentration dependent effects of trichothecenes on protein synthesis

Trichothecenes are known to be potent inhibitors of peptidyl transferase *in vitro* (Carrasco *et al.* 1973, Mizuno, 1975; Wei & Mclaughlin, 1974; Schindler;1974); however, peptidyl transferase is essential for elongation and termination, but not for initiation. There is evidence that those inhibitors that appear to be initiation inhibitors, do in fact block peptidyl transferase, on small polysomes, when only a small polypeptide has been synthesised, rather then blocking the formation of the initiation complex, which would prevent the ribosome moving along the mRNA and would not allow a second ribosome to attach. Mizuno (1975) showed that fusarenon-X and diacetoxyscirpenol inhibited protein synthesis at initiation, but after the formation of the first peptide bond; Smith *et al* (1975) concluded that T-2 toxin inhibits polypeptide chain initiation by preventing the formation of the first peptide bond, not by preventing the formation of the initiation complex. It appears then that all trichothecenes inhibit peptidyl transferase; those that inhibit peptide bond formation between the first and second amino acid, or when only small nascent peptides are present, result in a build up a single ribosome "polysome" and monosomes and are therefore better-termed initiation-like inhibitors (Feinberg & Mclaughlin, 1991).

Two models have been proposed to explain the mechanism by which trichothecene mycotoxins inhibit peptidyl transferase at the different stages in the translation cycle. In the first the individual trichothecene is able to bind to the polysome only at certain stages of the translation cycle. In the second the toxin binds throughout the translation cycle but only exerts its effect at one stage, possibly due to conformational changes of the ribosome at the different stages (Mclaughlin *et al.*, 1977).

Although all trichothecenes bind to the same ribosomal site they exert different effects on protein synthesis. The 12,13 epoxide group (characteristic of the trichothecenes) is essential for inhibition of protein synthesis, but it is the substituent groups (R_1-R_5) (Figure 5) on the trichothecene nucleus that affect the association between the toxins and the eukaryotic ribosome (Mclaughlin *et al.*, 1977) and thereby produce the different effects on protein synthesis. Substitution at R_2 enhances the toxicity and

trichothecenes with substitution at R_2 only (R_1 & R_3=H) are inhibitors of elongation or termination. Substitution at R_1 and R_3, (which are on the same side of the molecule and opposite to R_2) further enchances toxicity, and produces an initiator-like inhibitor; the more potent trichothecenes are initiator-like inhibitors.

1.2.2.4.1 Mechanisms of resistance to trichothecenes.

Mutants resistant to trichothecenes have been isolated from both yeast and mammalian cells (Feinberg & Mclaughlin, 1989). One group of mutants has an altered 60S ribosomal subunit, that has a lower affinity for trichothecenes (Schindler *et al.*, 1974). These mutants are resistant to all trichothecenes tested, and resistance is due to a single recessive gene in both yeast (Grant *et al.*, 1976) and mammalian cells (Gupta & Simmonovitch, 1978). The gene for resistance has been identified in *Saccharomyces cerevisiae* (Fried & Warner, 1981) and encodes for the 60S ribosomal protein, L3, presumed to be the major structural protein for peptidyl transferase. The mutant ribosomal protein renders the yeast 20-100 times less sensitive to trichodermin. The 60S ribosomal subunit from the trichothecene-producing fungus, *Myrothecium verrucaria*, has also been shown to be resistant to T-2 toxin (Hobden & Cundliffe, 1980).

An additional class of resistant yeast mutants have been isolated which do not have altered ribosomes, but which exhibit lower cellular permeability to the toxin (Stafford & Mclaughlin, 1973).

1.2.2.4.2 Metabolism of the trichothecenes

Metabolism of the trichothecenes produces metabolites that are less toxic than the corresponding parent molecule; oxidation, reduction, hydrolysis and conjugation reactions are all involved. While hydrolysis, oxidation and conjugation occurs in the bodies of animals, reduction is more likely to occur through the action of microorganisms in the gastrointestinal tract (Swanson & Corley, 1989). Both *in vitro* and *in vivo* studies using a variety of organs and animals have been used to study the metabolism of the trichothecnes, and the metabolic profile varies with both organ and

animal used. Most studies have focused on the metabolism of T-2 toxin and 26 metabolites have currently been identified (Swanson & Corley, 1989); a smaller amount of information is available on the metabolism of other naturally occuring trichothecenes including FUS, DAS and DON. Pathways for the metabolism of T-2 toxin are shown in Figure 7.

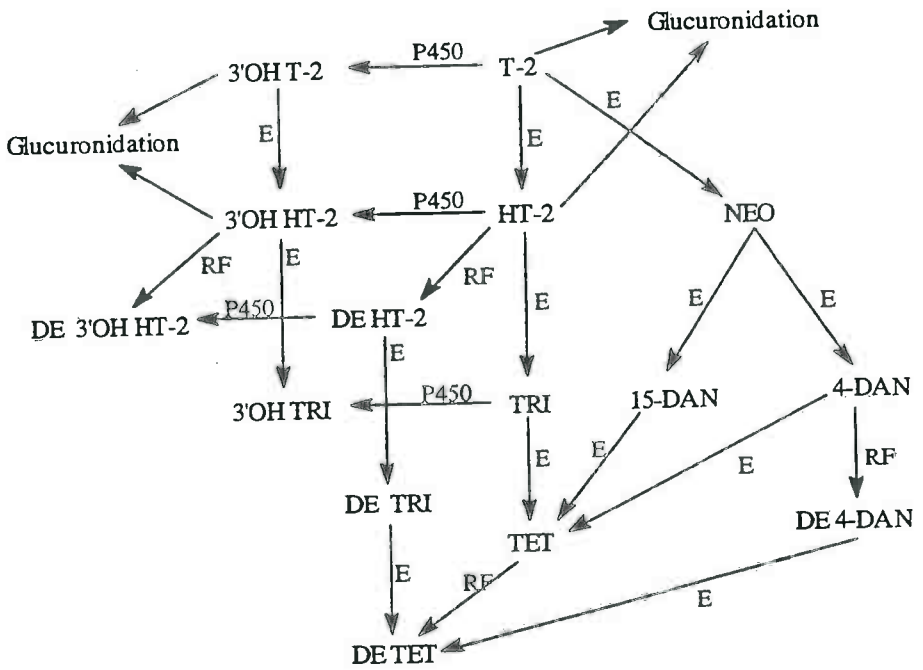


Figure 7 Pathways for the metabolism of T-2 toxin.
E = reactions catalysed by esterases, RF = reactions catalysed by microorganisms found in ruminal fluids and P450 = reactions catalysed by cytochromes P450.

Hydrolysis of esters is a major pathway in the metabolism of trichothecenes containing esterified side chains (e.g. T-2, DAS). Hydrolysis of the C-4 ester appears to the primary site of attack, and liver microsomal non-specific carboxyesterases from a variety of animal species have been shown to metabolise T-2 toxin, DAS, FUS and diacetoxynivalenol to produce HT-2 toxin, MAS, NIV and 15-acetylnivalenol respectively (Ellison & Kotsonis, 1974, Ohta *et al.*, 1977, 1978). C-4 hydrolysis is very common and in most *in vivo* systems HT-2 toxin is the predominant metabolite of T-2 toxin (Yagen & Bailer, 1993); however, C-4 hydrolysis is not considered a

significant detoxification reaction since the C-4 hydrolysis products have similar toxicity to their parent compounds (Swanson & Corley, 1989). Further hydrolysis (by esterases) at C-8 and C-15 to yield scirpentriol (from DAS) and T-2 triol, neosolaniol, 4-deacetylneosolaniol (4-DAN), 15-DAN, and T-2 tetraol (from T-2 toxin) has been reported (Fronnum *et al.*, 1985; Yoshizawa *et al.*, 1980) and does lead to a significant reduction in toxicity.

Cultures of soil and water bacteria have also been shown to deacylate T-2 toxin to produce HT-2 toxin, T-2 triol, neosolaniol, 4-DAN, and T-2 tetraol (Ueno *et al.*, 1983; Beeton & Bull, 1989) using the acetyl group as the sole carbon and energy source; cometabolic interactions between several bacterial species could be a significant factor in the metabolism of naturally occuring T-2 toxin to less toxic derivatives (Beeton & Bull, 1989).

Cyt P450-dependant oxidation of T-2 toxin and HT-2 toxin (the deacylation product of T-2) at the tertiary carbon of the isovaleryl group attached at the C-8 position (C-3' oxidation) yields 3'-hydroxy T-2 toxin (3'-OH T-2) and 3'-OH HT-2 respectively and has been demonstrated in a variety of animals (Kobayashi *et al.*, 1987). C-3' oxidation inhibits the hydrolysis of esters by esterase enzymes with the hydrolysis of 3'-OH HT-2 to NEO, 4-DAN, 15-DAN or T-2 tetraol not occuring (Yoshizawa *et al.*, 1984); and such inhibition most likely accounts for the build up of 3'-OH HT-2 as a major metabolite of T-2 *in vivo* (Swanson & Corley, 1989). As with hydrolysis reactions, oxidation at C-3' does not significantly reduce toxicity but does increase the polarity of the compound which may accelerate detoxification. C-3' oxidation of the C-3' isovaleryl group of T-2 triol and acetyl T-2 toxin to yield their corresponding 3'-OH derivatives has also been reported (Wei & Chu, 1985).

Upon ingestion by humans and animals, mycotoxins are exposed to microorgansims present in ruminal fluids and the gastrointestinal tract; these microorganisms also play a role in the metabolism of trichothecenes, prior to systemic absorbtion and subsequent metabolism in specific tissues. Bacteria in ruminal fluids are able to

deacylate T-2 toxin to HT-2 toxin and to a lesser extent T-2 triol (Swanson *et al.*, 1988) and DAS to MAS (Kiessling *et al.*, 1984); furthermore, under anaerobic conditions they are also capable of the reduction of the 12,13-epoxide to yield a carbon-carbon double bond (deepoxidation). The direct deepoxidation of DON (to a metabolite labelled DOM-1) has been demonstrated (King *et al.*, 1984a, Swanson *et al.*, 1986); however the direct deepoxidation of T-2 and DAS has not been shown (Swanson & Corley, 1989). The deacylation T-2 toxin and DAS, by microbial esterases, occurs prior to deepoxidation, and the major deepoxy metabolites of T-2 are deepoxy HT-2 and deepoxy T-2 triol, and of DAS are deepoxy MAS and deepoxy scirpentriol (Swanson *et al.*, 1986; Yoshizawa *et al.*, 1985; King *et al.*, 1984); enzymatic deepoxidation appears to be sterically inhibited by the presence of an ester functional group at the C-4 postion (Yoshizawa *et al.*, 1985). The epoxide bond present in nearly all the trichothecenes is essential for toxicity, and deepoxidation is a significant detoxification reaction, particlarly in ruminants.

Glucoronide conjugation is also a prominent pathway for the metabolism of trichothecenes and has been reported for DAS, T-2 and DON and their corresponding metabolites. Deacylation and hydroxylation usually precede conjugation and glucuronides of HT-2 toxin, 3-OH HT-2 toxin, 4-DAN and T-2 tetraol were detected following the administration of T-2 toxin to rats (Pace, 1986), dogs (Yagen & Bailer, 1983), and swine (Corley *et al.*, 1985). Glucoronide conjugates are more water soluble than the parent compounds and conjugation leads to biological inactivation and excretion of the toxins. However, following passage into the gastrointestinal tract, via the bile, conjugates may be cleaved by intestinal microflora, thereby liberating the trichothecene and restoring toxicity. Resorbtion can then occur thereby causing increased toxicity (Swanson & Corley, 1989). Gut microflora therefore appear to play a multiple role in the metabolism of the trichothecenes, with reductive epoxidation and ester hydrolysis reducing toxicity and hydrolysis of glucuronide conjugates restoring toxicity.

1.2.3 OTHER MYCOTOXINS

1.2.3.1 Citrinin

Citrinin (Figure 8) is produced by a number of *Aspergillus* and *Penicillium* species, principally *P. citrinum* (Pitt & Leistner, 1991) and is commonly found as a co-contaminant with ochratoxin A in wheat, flour, rye, oats, corn and feed grains (Wilson & Abramson, 1992).

Citrinin is nephrotoxic to a variety of species and has been implicated as a causative factor in renal disease and death in livestock, poultry and humans (Balkan Nephropathy) (Pitt, 1991) and as a causative agent in yellow rice toxicity in Japan (Saito & Tatsuno, 1971). In birds, including chickens, ducklings and turkeys, citrinin causes watery diarrhoea, increased food consumption and reduced weight gain due to kidney degeneration, whereas in animals citrinin is asymptomatic causing a non-specific deterioration in kidney function (Pitt, 1991).

Citrinin is toxic to *S. cerevisiae,* and completely inhibits growth at 25µg/ml, the primary site of action being the mitochondrial electron transport chain (Haraguchi *et al.,* 1987). The carcinogenic potential of citrinin is unclear, it is non-mutagenic to *S. typhimurium* (Ueno *et al.* 1978) and *S. cerevisiae* (Kuczuk *et al.*, 1978), but is mutagenic to *B. subtilis* in the Rec assay (Ueno & Kubota, 1976), furthermore it possess a high polarity due to the carboxylic group, which probably prevents it from crossing the cell membrane and reaching the cell nucleus (Schlatter, 1990).

1.2.3.2 Cyclopiazonic acid

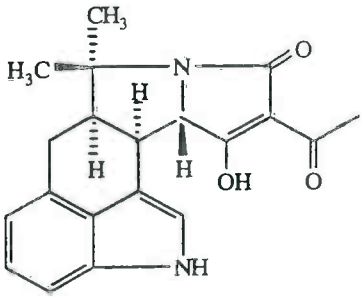
Cyclopiazonic acid (CPA) is an indol tetramic acid (Figure 8), produced by several *Penicillium* and *Aspergillus* species (Pitt, 1991) and has been found as a natural contaminant of several agricultural commodities, including groundnuts, meat, sausages, cheese, and corn (Rao & Hussain, 1985). The co-occurrence of CPA and aflatoxin has been reported as a consequence of the natural contamination of maize by *A. flavus* isolates, although isolates of *A. parasiticus* have not been shown to produce CPA (Lee & Hagler, 1991; Dorner *et al.*, 1984). Recently, CPA was detected in the ground-nut cake implicated in the outbreak of turkey 'X' disease in the 1960's; and its

presence has been suggested to account for some of the clinical symptoms of this mycotoxicosis, not characteristic of aflatoxicosis (catarrhal or haemorrhagic enteritis and opisthotonus) (Bradburn *et al.*, 1994).

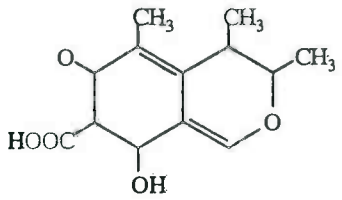
CPA is mutagenic to *Salmonella typhimurium* TA98 both singly and in combination with AFB_1, where activity is additive rather than synergistic (Sorenson *et al.*, 1984). Clinical symptoms of CPA intoxication include weight loss, weakness, vomiting, diarrhoea, dehydration, depression, convulsions and death (Nishie *et al.*, 1985). The target organ varies with species, with the primary target organ in laboratory animals such as the rat (Purchase, 1971) and guinea pig (Pier *et al.*, 1981) being the liver, with lesser effects being seen on the kidney or gastrointestinal system; in the chicken (Dorner *et al.*, 1983), pig (Lomax *et al.*, 1984) and dog (Neuhring *et al.*, 1985) the major target organs are the gastrointestinal system and kidney, and little effect is detected in the liver. Oral administration of CPA to chickens results in substantial accumulation of the toxin in the skeletal muscle, and is a possible route of entry into the human food chain (Norred *et al.*, 1988).

1.2.3.3 Fumonisin B_1

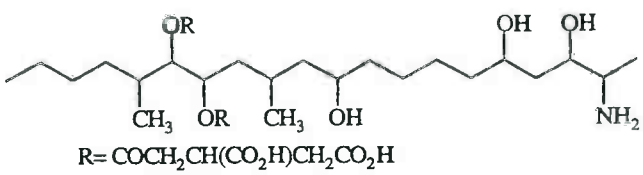
The fumonisins are a group of structurally related mycotoxins that have only recently been identified and characterised. They are produced by a limited number of *Fusarium* species, *F. moniliforme* and *F. proliferatum* being the main producers of high yields (Cawood *et al.*, 1991; Ross *et al.*, 1990; Gelderblom *et al.*, 1988). Of the six known mycotoxins in this group, fumonisin B_1 (FB_1) (Figure 8) is the most abundant in fungal cultures or in naturally contaminated foods and feeds (Ross *et al.*, 1991), and consists of a 20 carbon aliphatic chain with two ester-linked hydrophilic side chains (Bezuidenhout *et al.*, 1988). FB_1 is non-mutagenic to *S. typhimurium* (Gelderblom & Snyman, 1991), but is hepatocarcinogenic in rats (Gelderblom *et al.*, 1991) and has been implicated in leukoencephalomalacia in horses (Kellerman *et al.*, 1990; Marasas *et al.*, 1988), pulmonary oedema in swine (Harrison *et al.*, 1990) and oesophageal cancer in humans (Thiel *et al.*, 1992; Chu & Li, 1994).



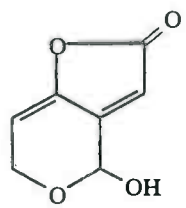
Cyclopiazonic Acid



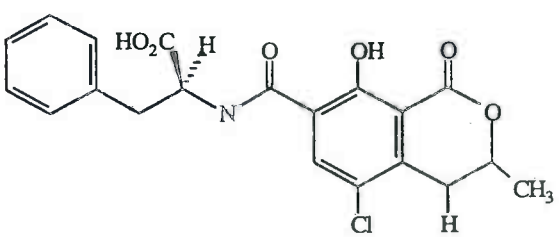
Citrinin



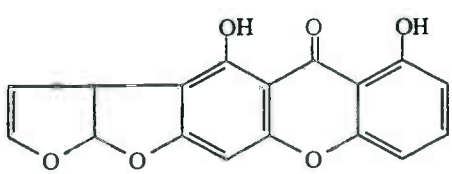
Fumonisin B1



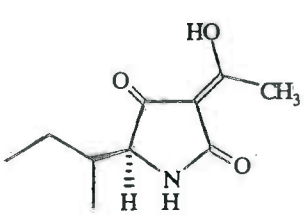
Patulin



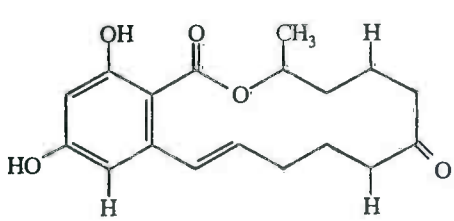
Ochratoxin A



Sterigmatocystin



Tenuazonic acid



Zearalenone

Figure 8 Structures of selected mycotoxins.

1.2.3.4 Ochratoxin A

The ochratoxins are a group of related isocoumarin derivatives that have been isolated as toxic metabolites of several species of *Aspergillus* and *Penicillium* and exhibit various toxicities (Pitt, 1991; Pitt & Leistner, 1991). Of the nine or more ochratoxins that are known to exist, ochratoxin A (OTA) (Figure 8), (the 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methyl isocoumarinamide (ochratoxin α) of L-β-phenylalanine), is the most toxic in embryos, whole animals and cultured cells (Ueno, 1991), is produced in the highest yield (Dalvi & Salunkhe, 1990) and is the only ochratoxin thought to be of significance as a natural contaminant of foods and feeds (Krough, 1977). It is frequently encountered as a contaminant (often in conjunction with citrinin) of grains, legumes and other commodities (Steyn, 1984). Chronically, OTA is predominantly nephrotoxic, and has been implicated in nephrotoxic syndrome in pigs, birds and humans (Krough, 1979, 1974). This syndrome is characterised by retarded growth, gastro-enteritis, polydipsia and polyurea, anaemia and contraction of the kidney (Dalvi & Salunkhe, 1990; Mirocha *et al.*, 1979). Additionally, OTA is fat-soluble and not readily excreted and so accumulates in fatty tissue; consequently it poses a serious health risk to both humans and animals (Pitt, 1991).

Initial toxicological effects in acute toxicity are displayed in the nephron; the proximal tubule being the primary target site in experimental animals (Krough, 1977). OTA is thought to selectively interfere with the anion transport system on the surface of brush-border facing vesicles, leading to a release of membrane-bound enzymes, such as alanine and/or leucine aminopeptidases, into the urine, which is one of the early symptoms of OTA-induced renal toxicity in animals. OTA is also thought to exert its toxicity by inhibiting renal gluconeogenesis, by inhibiting a key enzyme in this process, phosphoenolpyruvate carboxykinase (PEPCK) (Meisner *et al.* 1983). The exact target of the toxin is not known, but is thought to be post-transcriptional due to either the inhibition of the synthesis or the rate of translation of PEPCK mRNA (Ueno, 1991). OTA has also been shown to inhibit protein synthesis in bacteria, yeast and cultured mammalian cells, both *in vitro* and *in vivo* (Creppy *et al.*, 1983, 1984). The L-phenylalanine moiety of OTA is recognised by phenylalanine tRNA synthetase,

and acts as a competitive inhibitor of protein synthesis. The addition of phenylalanine reduces the toxicity and cytotoxicity of OTA (Röschenthaler *et al.*, 1984; Moroi *et al.*, 1985). OTA is not mutagenic to *S. typhimurium* (Ueno *et al.*, 1978), *B. subtilis* (Ueno & Kubota, 1976), *E. coli* (Reiss, 1986) and *S. cerevisiae* (Kuczuk *et al.*, 1978).

1.2.3.5 Patulin

Patulin is a five membered lactone (Figure 8), that is produced by a variety of *Penicillium* and *Aspergillus* species (Pitt, 1991; Pitt & Leistner, 1991). Patulin-producing *Penicillium* species are frequently involved in the spoilage of fruits (apples, bananas, pineapples, grapes, peaches and apricots) and patulin may be found in commercial fruit juices (Mirocha *et al.*, 1979).

Patulin is toxic to a range of biological systems including bacteria, fungi, protozoa, mammalian cell cultures, plants and animals (Yates, 1984, Betina, 1989), is teratogenic in the chick-embryo test (Ciegler *et al.*, 1976) and has been shown to be carcinogenic when injected intradermally into mice at sub-lethal doses, but not when given orally (Becci *et al.*, 1981). Patulin induces single and double strand breaks in HeLa and FM3A cells (Umeda *et al.*, 1972) and is mutagenic to *E. coli* (Lee & Rosenschenthaler, 1986) and *B. subtilis* (Ueno & Kubota, 1976) but non-mutagenic to *S. typhimurium* (Bachmann *et al.*, 1979; Ueno *et al.*, 1978) and to *S. cerevisiae* (Kuczuk *et al.*, 1978).

Patulin has also been shown to inhibit protein synthesis probably via inhibition of translation rather than an effect on the polysomes (Betina, 1989).

The reaction of patulin with SH-containing compounds such as glutathione or cysteine produces products that are less toxic or non-toxic compared to the parent molecule (Betina, 1989).

1.2.3.6 Sterigmatocystin

Sterigmatocystin is characterised by a xanthone moiety fused to a dihydrofuran moiety (Figure 8) and is produced by several *Aspergillus* species, *A. versicolor* being the most important (Pitt, 1991; Pitt & Leistner, 1991). It is formed as an intermediate

in aflatoxin biosynthesis and is similar to aflatoxin in chemical and toxicological characteristics. Sterigmatocystin is carcinogenic, mutagenic and teratogenic, but is less acutely toxic than AFB_1 due to its low solubility in water or gastric juices (Pitt, 1991); and acute toxicity varies with route of administration and species (Ueno, 1991). Sterigmatocystin has the potential to cause human liver cancer; the double bond in the bifuran ring may form an epoxide after incubation with hepatic microsomes, which, like AFB_1, may bind to DNA, and is the probable cause of the mutagenic effects of sterigmatocystin seen in bacterial, yeast, cell and tissue culture systems (Dunn *et al.*, 1982, Ueno & Kubota, 1976, Kuczuk *et al.*, 1978).

Chronic symptoms of sterigmatocystin poisoning include induction of hepatomas in rats, pulmonary tumours in mice, myocardial necrosis of the heart, cellular aberrations, and liver tumours (Betina, 1989), however, due to its low solubility, oral ingestion of sterigmatocystin is often asymptomatic, and detection of a disease syndrome is difficult (Pitt, 1991).

1.2.3.7 Tenuazonic acid

Tenuazonic acid (TEA) (Figure 8) is one of several mycotoxins produced by a variety of *Alternaria* species (Jewers & John, 1990); it is formed by the N-acetoacylation of iso-leucine followed by enzymatic cyclisation (Gatenbeck & Sierankiewicz, 1973) and has been found as a natural contaiminant in blast-diseased rice plants, tomatoes, mandarins, melons, olives and peppers (Logrieco *et al.*, 1988). TEA possesses antibiotic, antiviral and antitumor properties (Miller *et al.*, 1963; Gitterman, 1965), is teratogenic in the chick embryo bioassay (Giambrone, 1978) and is toxic in a variety of animal species (Jewers & John, 1990). Its primary mode of action in mammalian toxicity is via inhibition of protein synthesis and/or interference with the release of polypeptides from the ribosome (Jewers & John, 1990; Shigeura & Gordon, 1963). TEA has been implicated as a cause of onyalai (Steyn & Rabie, 1976; Rabie *et al.*, 1975), a haemotological disorder characterised by blood blisters in the mouth seen in people living in Africa, south of the Sahara (Edginton *et al.*, 1972).

1.2.3.8 Zearalenone

Zearalenone (ZEN) is a macrocyclic mycotoxin (Figure 8) produced by several species of *Fusarium* and *Gibberella* and is a natural contaminant of corn, wheat, barley, oats, sorghum and hay (Hocklin, 1991). Toxin production is promoted by high humidity and low temperatures (Coulombe, 1993) and ZEN contamination of corn often occurs in conjunction with other trichothecenes (Luo *et al.*, 1990). ZEN possesses potent oestrogenic properties and causes hyperestrogenism in susceptible animals via binding to the endoplasmic oestrogen receptor (Coulombe, 1993); swine are most affected by ZEN, but other animals including cattle, poultry and laboratory animals are also affected to a lesser extent. Symptoms of ZEN poisoning include uterine enlargement, swollen vulva, mammary gland development in immature pigs and testicular hypoplasia (Newberne, 1987; Mirocha *et al.*, 1979). Dairy heifers exposed to ZEN have reduced conception rates (Coulombe, 1993), but there is little carry over of ZEN, or its metabolites, into milk (Prelusky *et al.*, 1990). Following ingestion, zearalenone is absorbed easily from the gastrointestinal tract and is metabolically reduced in the liver by cyt P450 to two stereoisomers, α- and β-zearalenol (Ueno *et al.*, 1983b); α-zearalenol, but not β-zearalenol, is ten times more oestrogenic than the parent molecule and appears to be the active form of zearalenone (Hsieh, 1987). The metabolic capabilities and the proportion of α-zearalenol to β-zearalenol produced by metabolism may account for the variations shown in species susceptibilities.

Zearalenone is non-mutagenic to *S. cerevisiae* (Kuczuk *et al.*, 1978), *E. coli* (Auffray & Boutibonnes, 1987) and *S. typhimurium* (Bartholomew & Ryan, 1980), but is mutagenic to *B. subtillis* (Ueno & Kubota, 1976); α-zearalenol is non-mutagenic to both *S. typhimurium* (Bartholomew & Ryan, 1980) and *B. subtillis* (Ueno & Kubota, 1976) and β-zearalenol is non-mutagenic to *S. typhimurium* (Bartholomew & Ryan, 1980) but is mutagenic to *B. subtillis* (Ueno & Kubota, 1976).

1.3 BIOLOGICAL ASSAYS FOR MYCOTOXINS

There are a wide range of methods available for the detection and quantification of mycotoxins. They are usually detected in foodstuffs by chemical analysis, involving quantification by thin-layer chromatography, gas-liquid chromatography or high-performance liquid chromatography (Smith and Moss, 1985). Recently, immunoassay techniques such as enzyme-linked immunosorbant assays, (ELISAs) have been developed to identify certain mycotoxins. Although sophisticated analytical or immunological methods are often preferred due to their high specificity and reproducibility, they have the disadvantage of only detecting the toxins under examination. They are therefore limited to use with known, well characterised mycotoxins where authentic standards and methodologies are available; immunological methods also require the availability of pure specific antibodies for each toxin. Many potentially dangerous mycotoxins may therefore go undetected by chemical and immunological methods.

Bioassays do not require such characterisation of the toxin, and are therefore attractive as a non-specific screening method in the initial detection of known and unknown toxins in foods and feedstufffs. They are useful in the isolation of new or previously unrecognised toxins, prior to chemical analysis. Bioassays are also essential for working with field cases where the cause of the toxicosis is unclear and evidence is needed to associate a biological effect with the presence of fungally contaminated material. In the area of quality control, bioassays may be used to determine which samples should be subjected to chemical analysis. Unlike many analytical methods, bioassays are simple to perform and do not require expensive equipment to yield semi-quantitative data.

The biological effects of mycotoxins are manifold and include cytotoxicity, carcinogenicity, mutagenicity, teratogenicity, immunosuppresive effects, insecticidal effects and phytotoxicity (Buckle & Sanders, 1990). These effects have all been exploited in attempts to develop bioassays that can be used to detect toxins cheaply, rapidly and yield information about the type of toxicity. Numerous biological methods

have been developed, using a wide variety of test organisms, including vertebrate and invertebrate animals, organ and tissue culture systems, plants and micro-organisms. A general overview and some examples of these methods is given below. The subject has been extensively reviewed by Panigrahi, 1993; Buckle and Sanders, 1990; Yates, 1986 and Watson and Lindsay, 1982.

1.3.1 VERTEBRATE ANIMAL BIOASSAYS

Although vertebrate animal bioassays may be the most dependable type for the detection of vertebrate toxins, a number of considerations must be made when using them. These include species, sex, age or developmental stage, nutritional status and composition of the diet, the frequency and dosage level of the toxin and the presence of more than one toxin (Panigrahi, 1993; Yates, 1986). The lethality of mycotoxins also depends upon the route of administration and decreases in the order intravenous>intraperitoneal> subcutaneous> oral (Ueno & Ueno, 1978).

Vertebrate animal bioassays are also slow, expensive and require specialised laboratory facilities, which, alongside ethical considerations and pressure from animal welfare groups, has promoted the development and use of alternative bioassay methods in which effects are observed at the cellular or subcellular level.

However, some of the more sensitive bioassays that have been developed exploiting the biological effects of mycotoxins on animals include the production of oral lesions in chickens and ducks following the ingestion of trichothecenes (limits of detection for T-2 and DAS being 0.25µg/ml) (Schloesberg *et al.*, 1986), the guinea-pig skin-test for trichothecenes (limits of detect 0.2-0.3ppm T-2 toxin and 0.4-0.6ppm of DAS) (Muller 1987) and bile duct epithelia cell proliferation in the liver of ducklings for the detection of aflatoxins (limits of detection 20µg/kg) (Panigrahi, 1993). The chick embryo bioassay has also been used for the sensitive detection of a wide range of mycotoxins (Vesely *et al.*, 1984); the limits of detection of this assay for selected mycotoxins are shown in Table 16.

Minimum effective dose (μg/embryo)					
0.001	0.01	0.1	1.0	10.0	non-toxic
T-2 toxin	AFB_1	AFB_2	AFG_2	Citrinin	Penitrem A
DAS	PR toxin	AFG_1	Patulin	Penicillic acid	CPA
	Cytochalasin E	MPA	STC	Tenuazonic acid	
		Ochratoxin A	Deoxynivalenol		
		Rubratoxin B	Zearalenone		
Vesley *et al.* 1984					

Table 16 Detection limits of the chick embryo bioassay.

1.3.2 CELL CULTURE BIOASSAYS

Cell cultures originating from a variety of species and organs or tissues have been used as bioassay systems for detecting mycotoxins and have been reviewed, in detail, by Yates, (1986) and Buckle & Saunders, (1990); the same system is frequently used for examining both the cytotoxic and genotoxic effects of mycotoxins.

Cytotoxicity is usually determined by cell death or changes in cell morphology easily recognisable by microscopic examination. Such assays are often tedious, subjective and time-consuming, with assays times varying from 1-10 days (Yates, 1986). Other cell culture assays in which toxicity is determined by measuring protein and DNA synthesis by radio-labelling procedures have also been used, (Robbana-Barnat *et al.*, 1989; Thompson & Wannamacher, 1986), however such techniques require expensive laboratory equipment, radioisotopes and generate radioactive waste. Consequently, a number of cell cultures assays have been developed which incorporate a chromogenic substrate, which can be used to determine cell viability/death. These have included phenol red, which changes from red to yellow, when the pH of the culture medium is lowered, due to the metabolic actvity of viable cells (Sanders, 1984); MTT, which is reduced, by mitochondrial enzymes in viable cells, to a purple formazan dye (Holt *et al.*, 1988; Reubel *et al.*, 1987); and neutral red, which penetrates cell membranes and binds to intracellular sites of the lysosomal matrix, and is retained only by viable cells (Babich & Borenfreund, 1991).

Endpoints used to determined the genotoxic effects of mycotoxins have included chromosomal aberrations, inhibition of mitosis, DNA strand breaks and DNA repair

and assay times vary from 8 hours to 2 weeks (Yates, 1986); often, AFB_1 is the only mycotoxin studied in many of these systems.

An important consideration when using cell culture bioassays is the differences between toxicity sensitivities of different cell lines (Kitabake *et al.*, 1993). Murine fibroblasts have been reported to be particularly sensitive to many of the trichothecenes (Abbas *et al.*, 1984), limits of detection being 0.1ng/ml T-2 toxin, 7.5ng/ml DAS and 500ng/ml DON. Murine lymphocytes have also been used for the detection of the trichothecenes, IC_{50}'s being 1ng/ml T-2 toxin, 3ng/ml HT-2 toxin and 6ng/ml DAS and 60ng/ml DON; and for patulin (37ng/ml), penicillic acid (440ng/ml) and sterigmatocystin (140ng/ml) (Porcer *et al.*, 1986; Robbana-Barnat *et al.*, 1989). For other toxins, rat hepatoma cells were reported to be more sensitive than murine lymphocytes, IC_{50} concentrations, in the former, being 0.5ng/ml VER, 0.6ng/ml ROR, 110ng/ml sterigmatocystin, 320ng/ml CPA and 690ng/ml AFB_1 (Robbana-Barnat *et al.*, 1989). Although cell culture assays are sensitive, they are often slow, relatively expensive and cell lines are difficult to maintain.

1.3.3 INVERTEBRATE BIOASSAYS

Invertebrate bioassays have utilised mainly aquatic organisms, most notably brine shrimp (*Artemia salina*) larvae (Harwig and Scott, 1971; Scott *et al.*, 1980), *Colpidium camopylum* (Dive *et al.*, 1978) and *Tetrahymena pyriforis* (Hayes and Wyatt, 1970). Aflatoxins are less toxic to brine shrimp larvae than trichothecenes, the most potent of which are T-2 toxin, HT-2 toxin and DAS. The brine shrimp bioassay was used in the isolation of five naturally occurring trichothecenes produced by *Stachybotrys atra* (Eppley and Bailey, 1973), and was reported to be sensitive to ten naturally occurring trichothecenes (including T-2 toxin, DAS and the macrocyclics); the limits of detection being in the range of 0.04-0.4µg/ml. Later evaluation of this bioassay showed a high incidence of false positive results in the absence of aflatoxin B_1, ochratoxin A or T-2 toxin (Prior, 1979); it is thought that some naturally occurring fatty acids present in normal feedstuffs possess toxicity toward the larvae comparable to that of several known mycotoxins (Curtis *et al.*, 1974).

Tetrahymena pyriformis has also been shown to be sensitive to the trichothecenes (Nishie *et al.*, 1989a,b), IC_{50}'s (toxin concentration required to cause 50% inhibition of growth) for the most sensitive trichothecenes being 0.014µg/ml DAS, 0.024µg/ml T-2, 0.033µg/ml HT-2 and 0.035µg/ml DON. However, high toxin concentrations (>10µg/ml) of aflatoxins, ochratoxin A and patulin were required to elicit a response in this organism (Bijl *et al.*, 1988).

Colpidium camopylum was reported to be sensitive to patulin and DAS (limits of detection being 0.5µg/ml); however, toxin concentrations of >10µg/ml were required to elicit a response with aflatoxin B_1, ochratoxin B and sterigmatocystin (Dive *et al.* 1978). Zebra fish larvae have been shown to be sensitive to aflatoxin B_1, ochratoxin and patulin, but not penicillic acid at 5ug/ml (Abedi & Scott 1969).

As well as aquatic organisms, insects have been used in bioassays and their use in the detection of mycotoxins has been reviewed by Panigrahi, 1993. The type of effects measured include growth depression and changes in mortality, fertility, egg viability and metamorphosis.

1.3.4 PLANT BIOASSAYS

Many plant bioassays have been developed which exploit the phytotoxic effects of mycotoxins, which include inhibition of seed and pollen germination, reduction of plant growth and development, and chlorosis of the leaves. Although plant bioassays are attractive due to their ease of preparation and relatively low cost, they generally require relatively high concentrations of mycotoxins to elicit an effect, are slow and consequently have had limited application. Pollen germination appears to be more sensitive than seed germination to inhibition by mycotoxins, and has been shown to be particularly sensitive to the verrucarins, though not to aflatoxins, ochratoxins and sterigmatocystin (Panigrahi, 1993). In addition the response by pollen germination may be evaluated in a few hours as opposed to 4-7 days as required for seed germination (Yates, 1986).

30 genera of bacteria, 34 genera of fungi , 4 genera of algae and 1 protozoan. They found *Bacillus megaterium* NRRL B-1368 and *Bacillus brevis* NRRL B-1874 to be the most sensitive, being inhibited at 15 & 10µg/ml, respectively; neither the fungi or protozoan species were inhibited. Boutibonnes *et al.* (1983) assessed the effects of 47 mycotoxins on a single organism, *Bacillus thuringiensis*, using a disk-diffusion bioassay. *B. thuringiensis* was most sensitive to aflatoxin B_1 and patulin, requiring only 3µg/disk for inhibition of growth. However, high concentrations (≥300µg/disk) of T-2 toxin, ochratoxin A, rubratoxin B and sterigmatocystin were required to inhibit growth. A number of bacterial bioassays have been developed, utilising a variety of strains and species, but in particular *Bacillus* strains. These bioassays are often specific to a given mycotoxin or group of mycotoxins, and generally require toxin concentrations ≥1µg to elicit an effect. Toxicity is generally detected as inhibition of growth using either disk diffusion (DDA) or liquid culture (LC) assays. Some bacterial bioassays for mycotoxins are summarised in Table 17.

The mutagenic effects of mycotoxins on bacteria have also been exploited in the development of bacterial bioassay systems (Table 18). There is a strong correlation between the mutagenic and carcinogenic potential of chemicals, creating concern for the long-term health effects of mycotoxins throughout the world (Yates, 1986). One of the most widely used bioassay systems for mutagenicity is the Ames test, which utilises the bacterium, *Salmonella typhimurium*, either with or without a metabolic activating system (cytochrome P450). Five histidine-requiring mutants (TA98, 100, 1535, 1537 and 1538) have been used to study the mutagenic effects of mycotoxins. The media must be supplemented with histidine for these strains to grow. However, in the presence of DNA damaging chemicals these mutants may revert to the wild type condition, thereby allowing growth to occur in the absence of histidine. The *Bacillus subtilis* 'Rec' assay has also been used to determine the mutagenicity of a wide range of mycotoxins (Ueno & Kubota, 1976, D'Aquino *et al.*, 1986). This assay is based the inability of mutant strains (rec^-) to repair DNA damage following cellular recombination.

Bioassay organism	Mycotoxin and limits of detection		Principle of method.	Reference
Bacillus subtilis	Penicillic acid	1µg/disk	Inhibition of growth (DDA)	Olivigni & Bullerman, (1978)
	Aflatoxin B_1 Patulin Rubratoxin DAS	100µg/disk 1µg/disk 100µg/disk 100µg/disk	Inhibition of growth (DDA)	Reiss (1975a)
B. thurigienesis	Aflatoxin B_1 Aflatoxin B_2 Patulin Zearalenone T-2 toxin Ochratoxin A	3µg/mlµg/ml 7.5µg/ml 3.0µg/ml 7.5µg/ml 300µg/ml 600µg/ml	Inhibition of growth (LC)	Boutibonnes *et al.*, (1983)
B. stearothermophillus	Aflatoxin B_1 Patulin Rubratoxin DAS	0.001µg/ml 0.01µg/ml 0.001µg/ml 0.01µg/ml	Inhibition of spore germination (colour change in medium)	Reiss (1975b)
	Roquefortine	25µg/ml	Inhibition of growth (LC)	Kopp (1979)
B. megaterium NRRL 1368	Patulin	1.7µg/disk	Inhibition of growth (DDA)	Stott & Bullerman (1975a)
	Aflatoxin B_1	8µg/ml	Inhibition of growth (LC)	Tiwari *et al.*, (1985)
	Aflatoxin B_1	1µg/disk	Inhibition of growth (DDA)	Clements (1968a,b)
	Aflatoxin B_1 Aflatoxin G_1	1µg/disk 4µg/disk	Inhibition of growth (DDA)	Jayaraman, *et al.*, (1968)
	Aflatoxin B_1	2.5µg/ml	Inhibition of growth (LC)	Lillehoj & Ceigler, (1968)
	Aflatoxin B_1 Aflatoxin B_2 Aflatoxin G_1 Aflatoxin G_2 Ochratoxin A Zearalenone DAS	2µg/disk 2µg/disk 2µg/disk 20µg/disk 2µg/disk 20µg/disk 20µg/disk	Inhibition of spore germination	Buckelew *et al.*, (1972)
B. brevis	Aflatoxin B_1	5µg/ml	Inhibition of growth (LC)	Uwaifo & Bassir (1978)
	Ochratoxin A CPA Aflatoxin B_1 Citrinin Patulin Penicillic acid Zearalenone	0.5µg/disk 0.5µg/disk 1µg/disk 1µg/disk 1µg/disk 1µg/disk 10µg/disk	Inhibition of growth (DDA)	Madhyastha *et al.*, (1994b)
B. cereus mycoides LSU	Ochratoxin A Ochratoxin B	1.5µg/disk 3µg/disk	Inhibition of growth (DDA)	Broce *et al.*, (1970)
Streptococcus fecalis	Ochratoxin A	0.5µg/ml	Inhibition of growth (LC)	Heller *et al.*, (1975)

DDA= disk diffusion assay. LC= Liquid culture.

Table 17 Bacterial bioassays for mycotoxins.

Bioassay organism	Mycotoxin and limits of detection		Principle of method.	Reference
Photobacterium phosphoreum	Patulin	0.89µg/ml	Inhibition of bacterial bioluminescence	Yates & Porter, (1982)
	PR toxin	0.92µg/ml		
	Penicillic acid	3.14µg/ml		
	Citrinin	7.00µg/ml		
	Zearalenone	9.69µg/ml		
	Ochratoxin A	12.56µg/ml		
	Aflatoxin B_1	3.61µg/ml		
	Rubratoxin B	26.36µg/ml		

DDA= disk diffusion assay. LC= Liquid culture.

Table 17 (continued) Bacterial bioassays for mycotoxins.

The *E. coli* SOS chromotest has also been used to evaluate the genotoxic activity of mycotoxins (Auffray & Boutibonnes, 1987; Reiss, 1986; Riesenfield *et al.*, 1985). This assay uses a genetically-engineered strain of *E. coli*, which reacts to damage to its DNA by synthesising β-galactosidase, which produces a measurable colour, with a suitable substrate (Quillardet & Hofnung, 1985). The mutagenic potential of some mycotoxins in these systems in summarised in Table 18.

Mycotoxin	Ames Test	Rec Assay	SOS chromotest
Aflatoxin B_1	+	+	+
Sterigmatocystin	+	+	+
Ochratoxin A	–	–	–
T-2 toxin	–	–	n.d.
Fusarenon X	–	–	n.d.
Cyclopiazonic acid	+	n.d.	n.d
Citrinin	–	+	–
Patulin	–	+	–
Penicillic acid	–	–	–
Zearalenone	–	+	–
Auffray & Boutibonnes, 1987; D'Aquino *et al.*, 1986; Reiss, 1986; Ueno *et al.*, 1978; Ueno & Kubato, 1976;			

– non-mutagenic; + mutagenic; n.d. not determined

Table 18 Mutagenic properties of some mycotoxins in various bacterial systems.

1.3.5.2 Yeast bioassays

Yeasts are eukaryotes and therefore, unlike bacteria, have many features in common with mammalian cells, making them very attractive as bioassay organisms. Numerous genera and strains of yeasts have been screened for their sensitivity to mycotoxins (Table 19) (Baxter *et al*, 1987; Adak *et al.*, 1987a; Sukroongreung *et al.*, 1984; Burmeister & Hesseltine, 1970). Most of these studies have concentrated on sensitivity to the trichothecene mycotoxins, (usually T-2), due to the lack of sensitivity of bacterial bioassays to this group of mycotoxins (Burmeister & Hesseltine, 1970). Disk diffusion assays have generally been used for screening purposes, although liquid culture methods involving impedimetric techniques have also been utilised (Adak *et al.*, 1987a). Bioassays, using sensitive strains identified in these studies, have subsequently been developed.

Genera	Burmeister & Hesseltine (1970)	Sukroongreung *et al.* (1984)	Baxter *et al.* (1987)	Adak, *et al.* (1987a) *
Candida	2 strains	4 strains	11 strains	✓
Crytococcus	1 strain	1 strain		
Debaryamyces		1 strain		
Endomycopsis		1 strain		
Hasenula		11 strains	1 strain	✓
Kluyveromyces		8 strains	1 strain	✓
Picha		15 strains		✓
Rhodoturula	2 strains	1 strain	3 strains	✓
Saccharomyces	2 strains	22 strains	5 strains	✓
Schwanniomyces		1 strain		
Sporobolomyces		2 strains		
Torulopsos		8 strains		
Tremella	1 strain			
Trichosporin			1 strain	✓

*Adak *et al* - Number of strains in each genera was not stated.

Table 19 Yeast genera assessed for sensitivity to T-2 toxin

Rhodotorula rubra (NRRL Y-7222) has been used in a disk diffusion bioassay, the limits of detection for T-2 toxin being 4µg/disk (Burmeister and Hesseltine, 1970) and 0.2µg/disk (Stone *et al.*, 1986). *Hansenula fabianii* (CBS 5640) and *Picha burtonii* (FMBRA) were used in an impedimetric bioassay for T-2, and the limits of detection were 0.012µg/ml and 0.018µg/ml, respectively (Adak *et al.*, 1987b). Two of the screening studies showed strains *Kluyveromyces marxianus* to be the most sensitive,

of the yeasts screened, to T-2 toxin, (Baxter *et al.* 1987; Sukroongreung *et al.*, 1984); the limits of detection for T-2 toxin were 100ng/ml (Baxter *et al.*, 1987) and the concentration required to completely inhibit growth (MIC) was <1μg/ml (Sukroongreung *et al.*, 1984); neither study assessed either *H. fabianii* or *P. burtonii.* However, in a conductimetric assay, *K. marxianus* was shown to be more sensitive than *H. fabianii* to both T-2 toxin and DON (Connolly & Corry, 1990) and a number of bioassays have subsequently been developed using the strain of *K. marxianus* - GK1005 - identified by Sukroongreung *et al.*, (1984).

Schappert & Khachatourians (1984b) used *K. marxianus* (GK1005) in a disk diffusion assay and were able to detect T-2 (0.2μg/disk), verrucarin A (0.01μg/disk), and HT-2 toxin, roridin A and DAS (concentration not stated), but were unable to detect aflatoxin B_1, (50μg/disk) or zearalenone and DON, (20μg/disk). Optimisation of the disk diffusion assay was shown to increase the sensitivity of detection of a range of trichothecenes (Madhyastha *et al.*, 1994a,b); detection limits for VER, ROR, T-2, trichothecin, DAS, HT-2, DON, NEO, FUS and NIV were 0.005, 0.01, 0.02, 0.02, 0.1, 0.5, 10, 10, 10, and 50μg/disk, respectively. The assay was not able to detect aflatoxin B_1, ochratoxin A, citrinin, penicillic acid, penitrem A, and zearalenone at 200μg/disk.

The effect of eight trichothecenes on the inhibition of growth of *K. marxianus* (GK1005) was assessed using a liquid culture assay (Schappert *et al.*, 1986). T-2 triol, T-2 tetraol and verrucarol did not inhibit growth at the highest concentration tested (10μg/ml), but the assay could be used to detect verrucarin A, roridin A, T-2 toxin, diacetoxyscirpenol and HT-2 toxin. MIC and EC_{50} concentrations (μg/ml) were as follow:- verrucarin A- 0.12 & 0.025, roridin A- 0.5 & 0.05, T-2 toxin- 1.0 & 0.1, DAS- 10 & 1 and HT-2 toxin- 19.16 & 8.24.

The interaction of combinations of trichothecene mycotoxins has also been assessed using *K. marxianus* (GK1005) (Koshinsky & Khachatourians, 1992). The effect of combinations of VER, ROR, DAS, H-2 and DON with T-2 on inhibition of growth of *K. marxianus* was determined. HT-2, ROR and VER, in combination with T-2 caused

zero interaction, DAS caused a synergistic effect, and only DON had an antagonistic effect. Of the naturally occurring trichothecenes, DON is the most common; it is less toxic than many of the other trichothecenes, but safety concerns arise due to its frequent occurrence (Scott, 1989). The sensitivity of many bioassays is generally insufficient to allow the detection of DON (Khachatourians *et al.,* 1989) and Koshinsky and Khachatourians, (1992), proposed that the apparently unique antagonist interaction of DON and T-2 could be exploited to develop a sensitive bioassay for the determination of DON-contamination of foods and feeds. The detection of the trichothecenes, T-2 and DON, using *K. marxianus* was also assessed in a conductimetric bioassay (Connolly & Corry, 1990). Detection limits for T-2 were 0.1µg/ml, but DON could not be detected at the highest concentration tested, 10µg/ml. The use of polymyxin B sulphate to enhance sensitivity however reduced the detection limits of T-2 to 0.01µg/ml and DON to 2µg/ml.

Results from the studies summarised above indicated that *K. marxianus* was attractive as a bioassay organism. It is cheap and easy to culture, and being able to grow at 35°C can show responses more rapidly than many other yeasts, which prefer lower growth temperatures. During a previous study, that preceded the work for this thesis, the sensitivity of *K. marxianus* to 62 mycotoxins was determined (Dell, 1993). The bioassay used an automated turbidimeter, the Bioscreen C, (Labsystems), to monitor the growth of up to 200 samples. Toxicity was detected as inhibition of growth in comparison to a control and for each mycotoxin, the percentage inhibition of growth was calculated, after 20 hours. This data was used to construct dose-response curves, from which an EC_{50} estimation (concentration required to cause 50% inhibition of growth) was made for each mycotoxin. Dose-response curves for different toxins varied considerably, were often complex in shape, usually showed plateauing, and rarely showed the simple sigmoidal curve classically expected for growth inhibitors. Many mycotoxins (e.g. aflatoxins) did not strongly inhibit growth even at the highest concentration tested (1µg/ml), but did exhibit distinct partial inhibitory effects at extremely low concentrations (e.g. 1fg/ml). The trichothecenes also showed similar

partial inhibitory effects at the low concentrations tested, but, in contrast to most of the other toxins tested, inhibited growth completely at concentrations of 10-100ng/ml. The plateau effect often made the estimation of EC_{50}'s difficult. A summary of the plateauing effect and EC_{50} estimate for some of the mycotoxins studied using *K. marxianus* in the Bioscreen is shown in Table 20.

Mycotoxin	EC_{50} estimate (µg/ml)	Concentration range of plateaux (µg/ml)	% inhibition seen in plateaux
Aflatoxin B_1	1×10^{-5}	10^{-6}-10^{0}	50-55
Aflatoxin B_2	5×10^{-2}	10^{-9}-10^{-2}	40-45
Aflatoxin G_1	1×10^{-4}	10^{-9}-10^{0}	40-45
Aflatoxin G_2	$>1 \times 10^{0}$	10^{-6}-10^{0}	35-40
Sterigmatocystin	1×10^{-4}	10^{-6}-10^{-1}	60
Cyclopiazonic acid	1×10^{-1}	10^{-7}-10^{-2}	40-50
Ochratoxin A	$>1 \times 10^{0}$	10^{-9}-10^{0}	35-45
Patulin	5×10^{-1}	10^{-4}-10^{-1}	40
Penicillic acid	1×10^{-1}	10^{-8}-10^{0}	45-50
Citrinin	1×10^{-6}	10^{-9}-10^{0}	45-55
Zearalenone	$>1 \times 10^{0}$	10^{-6}-10^{-2}	30-40
Penitrem A	$>1 \times 10^{0}$	10^{-9}-10^{-5}	10-20
Cytochalasin A	5×10^{-1}	10^{-4}-10^{-1}	45
Tenuazonic acid	1.5×10^{-6}	10^{-4}-10^{0}	60
T-2 toxin	1×10^{-2}	10^{-3}-10^{-2}	45
Acetyl T-2 toxin	1×10^{-4}	10^{-9}-10^{-1}	45-50
T-2 triol	$>1 \times 10^{-0}$	-	-
HT-2 toxin	1×10^{-4}	10^{-7}-10^{-2}	45-50
Neosolaniol	1×10^{-4}	10^{-9}-10^{0}	45-50
Diacetoxyscirpenol	5×10^{-1}	10^{-5}-10^{-3}	35
T-2 tetraol	$>1 \times 10^{0}$	10^{-9}-10^{-1}	25-35
Scirpentriol	1×10^{-3}	10^{-9}-10^{0}	45-50
Fusarenon-X	5×10^{-5}	-	-
Nivalenol	1×10^{0}	10^{-9}-10^{-1}	40-45
Deoxynivalenol	1×10^{-3}	10^{-7}-10^{-1}	45-50
Roridin A	1×10^{-3}	-	-
Verrucarin A	1×10^{-8}	10^{-6}-10^{-3}	65

Table 20 EC_{50} estimates of mycotoxins using inhibition of growth of *K. marxianus* in the Bioscreen (Dell, 1993).

The study by Dell, (1993), showed that using *K. marxianus* and the Bioscreen turbidimeter, a wide range of mycotoxins, not only the trichothecenes, could be detected at extremely low concentrations. The Bioscreen however is expensive, (retailing at £30,000), and is therefore unlikely to be used for the routine detection of toxins, especially in developing countries.

A cheaper bioassay for the detection of mycotoxins was therefore necessary and the development of a colorimetric microtitre plate assay was attractive. The use of microtitre plates allows a large number of replicate samples to be analysed simultaneously and using spectophotometric readings (using a conventional microtitre plate reader) the quantitative detection of toxins is possible. However, in the absence of such instrumentation, a colorimetric bioassay would still allow qualitative determinations to be made. *K. marxianus* is known to produce the enzyme, β-galactosidase, for which colorimetric substrates are available and this project aimed to develop a simple rapid bioassay for the detection of mycotoxins, using inhibition of β-galactosidase activity in *K. marxianus* as the colorimetric endpoint.

The enzyme β-galactosidase is therefore of particular interest in the study, and a brief summary of current, relevant knowledge follows.

1.4 β-GALACTOSIDASE

β-Galactosidase (EC 3.2.1.23) has been found in numerous animals, plants and micro-organisms (Gekas & Lopez-Levia 1985), and is responsible for the hydrolysis of β-1,4-D-galactosidic linkages in glycolipids, glycoproteins, polysaccharides and disaccharides such as lactose (Figure 9). The widespread occurrence of β-galactosidase in mammalian organs is probably related to the multiple physiological functions of the enzyme; intestinal β-galactosidase is responsible for the hydrolysis and consequent absorption of dietary lactose and lysosomal β-galactosidase is a key enzyme in the degradation of glycolipids, mucopolysaccharides and glycoproteins (Spiro, 1970). In micro-organisms, β-galactosidase has been found in both prokaryotes and eukaryotes. Of all the β-galactosidases, that from *Escherichia coli* has been the most extensively studied (Beckwith, 1978), and has served as a model in the study of β-galactosidases from other sources. Other micro-organisms known to produce β-galactosidase include *Kluyveromyces marxianus* (Hewitt & Grootwassink, 1984; Mahoney & Whitaker, 1978), *Bacillus subtilis* (Anema, 1964), *Bacillus megaterium* (Rohlfing & Crawford, 1966), *Streptococcus lactis* (Citti *et al.*, 1965), *Neurospora crassa* (Comp & Lester,

1971), *Beauveria bassiana* (MacPherson & Khachatourians, 1991) and *Aspergillus nidulans* (Fantes & Roberts, 1973).

β-galactosidases of bacteria and yeasts are intracellular enzymes induced by the presence of lactose in the growth media (Dickson *et al.*, 1979). Lactose is of low molecular weight and is easily transported across yeast and bacterial membranes by specific permeases (Dickson & Barr, 1983). Once inside the cell it is hydrolysed by β-galactosidase into its constituent monomers, glucose and galactose, (Figure 9), which then enter the glycolytic pathways of the cell.

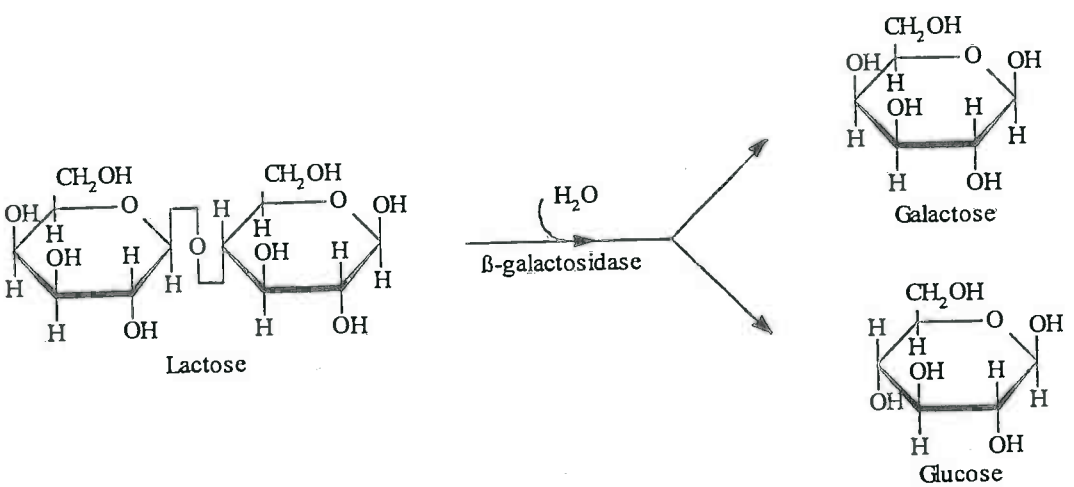


Figure 9 The cleavage of lactose by β-galactosidase.

1.4.1 PHYSIOCHEMICAL PROPERTIES OF β-GALACTOSIDASE FROM *K. MARXIANUS* AND NUTRITIONAL REQUIREMENTS FOR MAXIMAL PRODUCTION

The widespread interest in the reduction of the lactose content of dairy products, for both commercial and nutritional reasons, has led to much of the current knowledge of the physiochemical properties of β-galactosidase from *K. marxianus*, and the nutritional requirements for maximal production.

Utilisation of cheese whey, the major by-product of the dairy industry, is limited by the very high lactose content (Greenberg & Mahoney, 1981) and it is envisaged that

the hydrolysis of lactose in whey will lead to its incorporation into a wider variety of foods and at higher levels than is now possible (Gonzalez *et al.*, 1990). Additionally, many individuals and even whole populations have difficulty in consuming milk and dairy products due to an intestinal insufficiency of β-galactosidase (Shukla, 1975). There has been extensive research into the production of low-lactose milk, especially for those populations where milk is provided - under food-aid programmes - as a major source of dietary protein. The yeast *Kluyveromyces marxianus* var. *marxianus* (*K. marxianus*) is a good source of β-galactosidase and, unlike most bacteria and fungi, is itself approved for food use (Greenberg & Mahoney, 1981). This has led to considerable interest in the characterisation of β-galactosidase from *K. marxianus*. A variety of strains of *K. marxianus* have been used in such studies and the enzyme has been prepared to various degrees of purity. The physiochemical properties of β-galactosidase from *K. marxianus*, in comparison to *E. coli*, are summarised in Table 21.

Property	*K. marxianus*	*E. coli*
Molecular weight (D)	201,000	540,000
Subunits	2 or 10	4
pH optimum	6.6	7.2
pH stability	6.5-7.5	6.0-8.0
Temperature optimum	37°C	40°C
Metal requirements for maximal activity	K^+ Mn^{2+}	Na^+ K^+
Inhibitors	*p*-chloromercuribenzoate Ag^+ Hg^{2+} Cu^{2+}	*p*-chloromercuribenzoate Ag^+ Hg^{2+} Cu^{2+}
Km-lactose Km-ONPG	14-24mM 2.5-4.0mM	2.9mM 0.16mM
Isoelectric point	4.5-5.1	4.61
References	Mahoney & Whittaker, 1977, 1978 Wendorf & Admundson, 1971 Uwajima, 1972	Greenberg & Mahoney, 1981 Wallenfels & Weil, 1972 Wallenfels & Malhorta, 1960

Table 21 Physiochemical properties of β-galactosidase.

Extensive studies have been made to optimise growth conditions and nutritional requirements of *K. marxianus* for the maximal production β-galactosidase (Mahoney

et al., 1974; Wendorff *et al.*, 1970). The primary factors affecting β-galactosidase production are strain selection, lactose concentration, pH and temperature (Mahoney *et al.*,1974, Wendorf *et al.*, 1970) and optimum conditions for β-galactosidase production differ considerably from those for optimum growth (Wendorf *et al.*, 1970). Studies have used both sweet whey media (as lactose source), and conventional liquid media supplemented with lactose. Optimum conditions for β-galactosidase synthesis on sweet whey were found to be 10-15% lactose, with the addition of growth factors in the form of 0.5% corn steep liquor at pH 4.0-4.7 and 28-30°C. (Wendorff *et al.*,, 1970). Under such conditions sweet whey media was shown to produce 80% more β-galactosidase activity/mg yeast than liquid media supplemented with lactose (Mahoney *et al.*, 1974).

1.4.2 ASSAYS FOR β-GALACTOSIDASE

The most common substrates for assaying β-galactosidase are chromogenic galactosides. Lederberg (1950) described the use of o-nitrophenyl β-D-galactoside (ONPG) (Figure 10) as a simple convenient assay substrate for β-galactosidase and assays using ONPG are still widely used today. ONPG is a colourless compound, that in the presence of β-galactosidase is converted to galactose and o-nitrophenol. The o-nitrophenol is yellow and can be measured by its absorption at 420nm. Providing ONPG is present in excess, the assay is linear, and the amount of o-nitrophenol produced is proportional to the amount of β-galactosidase present.

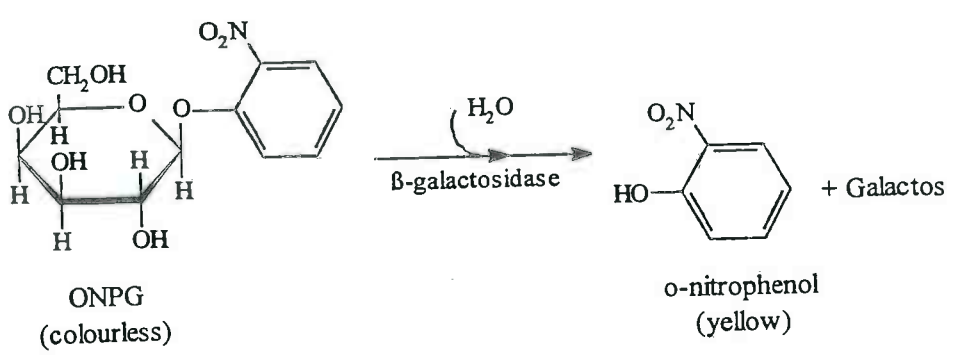


Figure 10 Cleavage of o-nitrophenol-β-D-galactoside (ONPG) by β-galactosidase.

Cohen *et al.* (1958) introduced 6-bromo-2-naphthyl β-D-galactoside, which gives rise to insoluble 6-bromo-2-naphthol upon enzymatic hydrolysis. The low solubility of 6-bromo-2-naphthol made it an attractive substrate for histochemical determinations, and a useful tool for detecting enzymically active zones after electrophoresis of enzyme preparation on polyacrylamide gels (Marchesi, 1969).

Pearson *et al.* (1963) studied several substituted indolyl β-D-galactosides for the histochemical localisation of β-galactosidase, and found 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) especially suitable for this purpose. Xgal is a colourless compound, which, in the presence of β-galactosidase, is cleaved to a halogenated indoxyl which is rapidly oxidised to form a bisindigo product that is blue-green, insoluble, light-fast and stable (Figure 11). Xgal has also been used for activity staining following electrophoresis. The sensitivity of β-galactosidase assays has been increased further by the use of fluorogenic substrates. These include methlyumbelliferyl β-D-galactoside (McCaman, 1959), 2-naphthyl β-D-galactoside (Asp, 1971) and fluorescein di-β-D-galactoside (Rotman, 1961).

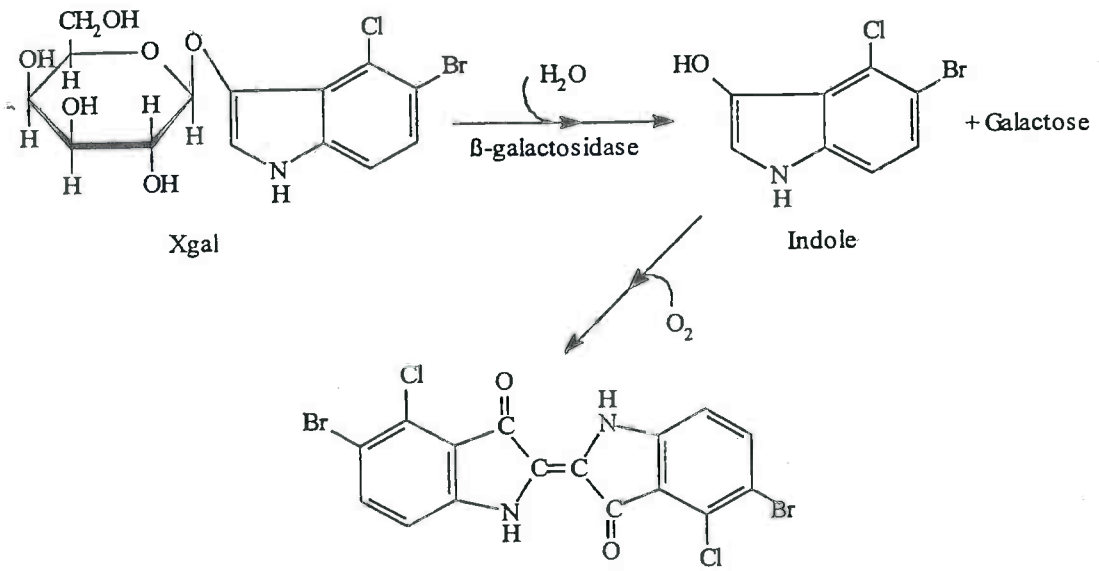


Figure 11 Cleavage of Xgal by β-galactosidase.

1.5 AIMS OF RESEARCH PROGRAMME

The objective of this research programme was to develop a simple colorimetric bioassay for the detection of mycotoxins using the inhibition of induction of a colorimetrically assayed enzyme in *K. marxianus* var. *marxianus* (GK1005). The relevant enzyme is β-galactosidase which is reported to be inducible and toxicity can therefore be detected as interference with any stage of the induction process or the enzyme reaction itself, causing (by comparison with controls) a loss of ability to cleave a chromogenic substrate and hence a reduced rate of - photometrically monitored - colour formation.

Inital objectives were to determine the kinetics of induction of β-galactosidase using a variety of inducers and assay procedures. Conditions permitting maximum speed and efficiency of enzyme induction and greatest sensitivity of detection were also to be established and the system was to be miniaturised so as to minimise the number of cells needed to detect induction, thereby maximising sensitivtiy.

The colorimetric yeast bioassay was then to be used to determine the limits of detection for a range of mycotoxins. Mycotoxins to be assessed included those known to be natural contaminants of foods and feeds and those shown to be particularly toxic to *K. marxianus* in the Bioscreen assay developed by Dell, 1993.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND SPECIALIST ITEMS

Chemicals and specialist items were purchased from suppliers as detailed below.

BDH/Merck LTD., PO Box 15, Freshwater Road, Dagenham, Essex. RM8 1RF:-

Glucose, galactose, glycerol, lactose, sorbitol, Kieselgel 60 silica gel HPTLC plates (5547).

Calbiochem Novabiochem (UK) Ltd., Boulevard Industrial Park, Beeston, Nottingham, NG7 1BR:-

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal).

Dynatech Laboratories Ltd, Daux Rd, Billingshurst, West Sussex, RH14 9SJ.:-

Sterile flat-bottom microtitre plates (MR24A).

ICN Biomedicals Ltd, Wenman Road, Thame, Oxfordshire, OX9 3XA:-

Mylar plate sealers, phosphate buffered saline (Dulbecco's formula), polymyxin B nonapeptide.

Sigma-Aldrich Chemical Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH:-

Aflatoxin B_1, aflatoxin M_1, acetyl T-2 toxin, ascorbic acid, cetyl trimethyl ammonium bromide (CTAB), cyclopiazonic acid, deoxynivalenol, diacetoxyscirpenol, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT), fumonisin B_1, fusarenon-X, HT-2 toxin, isopropyl-1-thio-β-D-galactoside (IPTG), lactose, methyl-1-thio-β-D-galactoside (MTG), neosolaniol, o-nitrophenyl-β-D-galactopyraoside (ONPG), nivalenol, ochratoxin A, patulin, phenyl-1-thio-β-D-galactoside (ϕTG), polymyxin B sulphate, roridin A, scirpentriol, sterigmatocystin, T-2 tetraol, T-2 triol, T-2 toxin, tenuazonic acid, verrucarin A, zearalenone.

Unipath Ltd, Wade road, Basingstoke, Hampshire:-

Yeast extract, bacteriological peptone, agar, trypticase soy broth.

Micro-organisms were purchased or obtained from the following suppliers:-

National Collection of Yeast Cultures (NCYC), AFRC Institute of Food Research, Norwich, NR4 7UA:-

Saccharomyces cerevisiae, NCYC 754.

National Collection of Industrial and Marine Bacteria Limited, 23, St Machar Drive, Aberdeen, AB2 1RY:-

Bacillus megaterium, 10820 (NRRLB-1368).

Ministry of Agriculture Food and Fisheries, Nobel House, 17, Smith Square, London, SW1P 3JR:-

Kluyveromyces marxianus (GK1005).

2.2 DEVELOPMENT AND OPTIMISATION OF THE YEAST BIOASSAY

2.2.1 PHYSIOLOGICAL STUDIES OF THE INDUCTION OF β-GALACTOSIDASE IN *K. MARXIANUS*

An adaptation of the method of Miller, 1972 was used for the determination of β-galactosidase activity in *K. marxianus*. The assay, which uses o-nitrophenyl-β-D-galactopyranoside (ONPG) as the chromogenic substrate for β-galactosidase was optimised (as detailed in section 2.2.1.2) prior to use in this analysis.

2.2.1.1 Experimental procedures

2.2.1.1.1 Media and reagents

Media consisted of 1% (w/v) yeast extract and 1% (w/v) bacteriological peptone supplemented with a carbon source and/or inducer as indicated in each experiment. All media was autoclaved at 121°C for 15 minutes prior to use. Z buffer consisted of 100mM sodium phosphate, 10mM potassium chloride, 1mM magnesium sulphate and 50mM β-mecaptoethanol, pH 7.0. A stock solution of ONPG was prepared, immediately before each assay, at 4mg/ml in water.

2.2.1.1.2 Preparation of yeast inoculation culture

Unless otherwise stated, an inoculation culture was prepared by adding a single cell colony (scc) of *K. marxianus* from an agar plate to 50ml of medium identical to that to be used in the subsequent experiment. The culture was incubated for 16 hours at 35°C and 200 rpm. 1ml of inoculation culture was used to inoculate 50ml of fresh medium prior to each experiment.

2.2.1.1.3 Determination of cell density

Cell densities were measured spectophotometrically at 620nm with a Cecil CE383 spectrophotometer. For such measurement cells were diluted to an A_{620} of less than 0.7 absorbance units, as higher densities did not obey the Beer-Lambert law. The number of cells/ml was determined by counting on a haemocytometer slide. Under these conditions there were 4.1×10^8 log phase cells/A_{620} unit.

2.2.1.1.4 Determination of β-galactosidase activity (ONPG assay)

β-galactosidase activity was assayed spectophotometrically, using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the chromogenic substrate for β-galactosidase, by the modified procedure of Miller, 1972.

Cells were harvested from culture media by centrifugation and resuspended in Z-buffer. The volume of media from which cells were harvested and the volume of Z-buffer used to resuspend the cells was varied depending on the expected level of β-galactosidase activity (section 2.2.1.2.1) and details are given in the relevant experimental sections. Samples were assayed for β-galactosidase activity as follows:- 0.9ml of Z buffer was added to 0.1ml of cells in Z-buffer. Since *K. marxianus* is only slightly permeable to ONPG, cells were made permeable by the addition of two drops of chloroform and one drop of 0.1% sodium dodecyl sulphate (SDS) to each sample, which was subsequently vortexed for 10 seconds. Assay mixtures were equilibrated for 5 minutes at 30°C, and the reaction started by the addition of 0.2ml ONPG (4mg/ml in water). When a suitable yellow colour had developed, reactions were stopped by the addition of 0.5ml of 1M sodium carbonate. The time taken for colour development varied according to the carbon source (section 2.2.1.2.2); generally samples with high levels of enzyme activity (e.g. lactose-grown) were incubated with ONPG for up to 30 minutes and samples with a lower level of activity (e.g. glucose-grown) were incubated with ONPG for up to one hour, and under these conditions the cleavage of ONPG was shown to be linear with time. Cells were removed by centrifugation, and the absorbance of the supernatant at 420nm determined using a Cecil CE383 spectrophotometer. One unit of enzyme activity equals the production of 1nmol of o-nitrophenol/min/ml and the molar extinction coefficient of o-nitrophenol under these conditions is 4.5×10^3 $M^{-1}cm^{-1}$ (Miller, 1972). During an induction experiment where samples are taken over an extended period of time, it was convenient to store and assay them in batches. Samples diluted with Z-buffer could be stored at 4°C for up to 5 hours without loss of enzyme activity (section 2.2.1.2.3).

2.2.1.2 Assessment and optimisation of the ONPG assay

2.2.1.2.1 Effect of cell density of assay samples on the determination of β-galactosidase activity

Medium supplemented with 50mM lactose was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2. Subcultures were incubated for 0, 2, 4 or 6 hours at 35°C and 200 rpm, after which cell density was measured as detailed in section 2.2.1.1.3. Cells were harvested from 5ml of culture media by centrifugation and were resuspended in 5ml Z-buffer. Samples were diluted 2, 5, 10 or 20 fold in Z-buffer to a final volume of 1ml, and β-galactosidase activity was determined as detailed in section 2.2.1.1.4.

2.2.1.2.2 Linearity of the cleavage of ONPG with respect to time

Media supplemented with either 50mM lactose or glucose was inoculated as detailed in section 2.2.1.1.2. The subcultures were incubated for 2, 4 or 6 hours at 35°C and 200 rpm, after which cell density was determined as detailed in section 2.2.1.1.3. Cells were harvested from 5ml of culture media by centrifugation and resuspended in 5ml of Z-buffer and samples originating from lactose cultures were further diluted 10 fold in Z-buffer. All samples were then assayed in duplicate for β-galactosidase activity as detailed in section 2.2.1.1.4, using incubation times with ONPG of 0, 2.5, 5, 7.5, 10, 15, 20, 30, 45 and 60 minutes.

2.2.1.2.3 Effect of storage of samples at 4°C prior to assaying

Medium supplemented with 50mM lactose was inoculated as detailed in section 2.2.1.1.2. The subculture was incubated at 35°C and 200 rpm for 2 hours, after which cell density was measured as detailed in section 2.2.1.1.3. Cells were harvested from 10ml of culture medium by centrifugation, and resuspended in 10ml of Z-buffer. Samples were stored at 4°C for various time intervals between 0 and 5 hours after which β-galactosidase activity was determined as detailed in section 2.2.1.1.4.

2.2.1.3 Effect of carbon source on growth and β-galactosidase activity of *K. marxianus*

Medium supplemented with 50mM of one of the carbon sources was inoculated as detailed in section 2.2.1.1.2; the carbon sources assessed were lactose, galactose, glucose, sorbitol and glycerol. Cultures were incubated at 35°C and 200 rpm and cell density was determined at regular intervals throughout the growth cycle (section 2.2.1.1.3). For the assay of β-galactosidase activity, cells were harvested from 1ml of medium and resuspended in 1ml of Z-buffer, with the exception of samples from lactose or galactose cultures taken after 2 hours of culture growth, which were resuspended in 10ml Z-buffer (Table 22); enzyme activity was then determined as detailed in section 2.2.1.1.4.

Carbon source and/or inducer.	Time (hrs)	Harvest	Resuspend
Lactose or galactose	< 2 hrs	1ml medium	1ml Z-buffer
	> 2 hrs	1ml medium	10ml Z-buffer
All others	All	1ml medium	1ml Z-buffer

Table 22 Volume of media and Z-buffer used to harvest and resuspend cells prior to determination of β-galactosidase using the ONPG assay.

2.2.1.4 Effect of inducers on growth and β-galactosidase activity of *K. marxianus*

An inoculation culture was prepared as detailed in section 2.2.1.1.2, with the following modifications:- medium was supplemented with 20mM sorbitol and the culture was incubated for 24 hours (so that the stationary phase of growth was reached). 1ml of the inoculation culture was used to inoculate 50ml of fresh medium supplemented with 20mM sorbitol and 20mM of the inducer. Inducers assessed included isopropyl-1-thio-β-D-galactoside (IPTG), methy-1-thio-β-D-galactoside (MTG), phenyl-1-thio-β-D-galactoside (ϕTG), lactose and galactose; medium supplemented with 40mM sorbitol was used for control purposes. All cultures were incubated at 35°C and 200 rpm and cell density was determined at regular intervals throughout the growth cycle (section 2.2.1.1.3). β-galactosidase activity was

determined as detailed in section 2.2.1.1.4; cells were harvested from 1ml of medium and resuspended in 1ml of Z-buffer, with the exception of samples from lactose or galactose cultures taken after 2 hours of culture growth, which were resuspended in 10ml Z-buffer (Table 22).

2.2.2 EVALUATION OF ALTERNATIVE CHROMOGENIC SUBSTRATES

The use of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), as a chromogenic substrate for the determination of β-galactosidase activity, and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), as a chromogenic substrate for the determination of cell viability was assessed.

2.2.2.1 Media and reagents

Medium (50mM YEPL) consisted of 1% (w/v) yeast extract and 1% (w/v) bacteriological peptone and 50mM lactose. Xgal was dissolved in dimethylformimide (DMF) at 100mg/ml and stored at -20°C in the dark. MTT was dissolved in phosphate-buffered saline (PBS) (Dulbecco's formula) at 5mg/ml immediately prior to use and the solution was filter sterilised to remove any insoluble residue present.

2.2.2.2 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal)

50mM YEPL medium was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2. 10ml of the subculture was aliquoted into two universal bottles. Twenty drops of chloroform and 10 drops of 0.1% SDS was added to one of the bottles to permeabilise the cells and 20µl of Xgal (100mg/ml in DMF) was added to each bottle. The samples were incubated at 35°C and 200 rpm until the blue precipitate of the indigo product of cleaved Xgal was clearly visible. The absorbance of the solution was measured using a Lambda 3 spectrophotometer (Perkin Elmer).

2.2.2.3 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT)

50mM YEPL medium was inoculated with *K. marxianus*, as detailed in section 2.2.1.1.2. 10ml of the subculture culture was aliquoted into a universal bottle. 2ml of MTT in PBS (5mg/ml) was added, and the bottle was incubated statically at 35°C, for 4 hours, after which the supernatant was removed and 10ml of DMSO was added to dissolve any crystalline material. The absorbance spectra of the formazan product was measured, using a Lambda 3 spectrophotometer (Perkin Elmer).

2.2.3 THE USE OF MICROTITRE PLATE TECHNOLOGY IN THE MINIATURISATION OF THE BIOASSAY

The use of microtitre plate technology for data collection of the bioassay results was assessed, using a Titertek Multiscan Plus Mk II microtitre plate reader (Labsystems), interfaced with an Amstrad microcomputer and Titresoft 1.01 software (Labsystems). Medium supplemented with 50mM lactose was inoculated as detailed in section 2.2.1.1.2 and 150µl was aliquoted into the wells of a mictrotitre plate. Blank wells contained sterile media. The use of single wavelength measurements for the determination of cell density was assessed using a filter at 560nm.

A culture containing the indigo product of cleaved Xgal was prepared as detailed in section 2.2.2.2, and 150µl aliquoted into the wells of a mictrotitre plate. Blank wells contained sterile media and control wells contained cells that had not been exposed to Xgal. The use of dual wavelength measurements for the determination of β-galactosidase activity (cleavage of Xgal) was evaluated using a reference filter at 560nm and a test filter ar 666nm.

A culture contining the formazan product of cleaved MTT was prepared as detailed in section 2.2.2.3, and 150µl aliquoted into the wells of a mictrotitre plate. Blank wells contained sterile media and control wells contained cells that had not been exposed to MTT. The use of dual wavelength measurements for the determination of cell viability (cleavage of MTT) was evaluated using a reference filter at 666nm and a test filter at 560nm.

2.2.4 DETERMINATION OF β-GALACTOSIDASE ACTIVITY USING XGAL

2.2.4.1 Experimental procedures

2.2.4.1.1 Media and reagents

Media and inocula were prepared as detailed in sections 2.2.1.1.1 & 2.2.1.1.2, respectively. Media was supplemented with 50mM carbon source as detailed in each experiment.

2.2.4.1.2 Determination of cell density

Cell density was determined by the measurement of absorbance at 560nm, using a Titertek Multiscan Plus Mk II microtitre plate reader (Labsystems), interfaced with an Amstrad microcomputer and Titresoft 1.01 software (Labsystems). The number of cells/ml was determined by counting on a haemocytometer slide and under these conditions there were 1.1×10^9 log phase cells/A_{560} unit.

2.2.4.1.3 Determination of β-galactosidase activity

β-galactosidase activity was determined spectophotometrically using the Multiscan Plus Mk II microtitre plate reader, using a test wavelength of 666nm and a reference wavelength of 560nm. Unless otherwise stated cells were first permeabilised by the addition of 5µl of 0.1% SDS and 3µl chloroform to each well of the microtitre plate. 1µl of 100mg/ml Xgal in DMF was added to each well and the plate was sealed with a Mylar plate sealer and placed in the shaker-incubator at 35°C for 30 minutes. The cleavage of Xgal was determined at 5 minute intervals throughout the 30 minute incubation by the measurement of A_{666}-A_{560}, and β-galactosidase activity is expressed as the cleavage of Xgal (A_{666}-A_{560}) as a function of time (minutes) and cell density (A_{560}).

2.2.4.2 Toxicity of dimethyl formimide (DMF) to *K. marxianus*

Medium supplemented with 50mM glucose was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2.; 150µl of this subculture was added to the wells of a microtitre plate. DMF was added to triplicate wells at concentrations of 0, 1, 2 and 3% (v/v). The plate was sealed with a Mylar plate sealer and incubated at 35°C in the plate shaker for the duration of the experiment. Cell density was determined at regular intervals throughout the growth cycle as detailed in section 2.2.4.1.2.

2.2.4.3 *In vivo* and *in vitro* approaches to using Xgal as a chromogenic substrate for β-galactosidase

The use of Xgal as a chromogenic substrate for β-galactosidase was assessed using whole cells and permeabilised cells. Media supplemented with 50mM lactose was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2; 150µl of this subculture was added to the wells of a microtitre plate. Xgal was used to determine β-galactosidase activity in permeabilised cells and non-permeabilised cells as detailed below.

Non-permeabilised cells:- 1µl of Xgal in DMF (100mg/ml) was added to each well at the beginning of the experiment. Control wells contained 150µl of subculture and 1µl of DMF and blank wells contained 150µl of media and 1µl of Xgal. The plate was sealed with a Mylar plate sealer and incubated at 35°C in the plate shaker. Cell density and β-galactosidase activity were determined at regular intervals throughout the growth cycle. Cell density was determined as detailed in section 2.2.4.1.2 and β-galactosidase activity was determined by a single measurement of A_{666}-A_{560}.

Permeabilised cells:- the plate was sealed with a Mylar plate sealer and incubated at 35°C in the shaker-incubator. At regular intervals throughout the growth cycle the contents of triplicate wells were transferred to a second microtitre plate and cell density and β-galactosidase activity determined as detailed in section 2.2.4.1.2 & 2.2.4.1.3 respectively.

2.2.4.4 Effect of carbon source on β-galactosidase activity:- Xgal as the chromogenic substrate

Media supplemented with 50mM lactose, galactose or glucose, was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2 and 150µl of the subculture was added to the wells of a microtitre plate. The plate was sealed with a Mylar plate sealer and incubated in the plate shaker at 35°C. At regular intervals throughout the growth cycle, the contents of triplicate wells was transferred to a second microtitre plate and cell density and β-galactosidase activity were determined as detailed in sections 2.2.4.1.2 & 2.2.4.1.3 respectively.

2.2.4.5 Optimisation of the Xgal bioassay

2.2.4.5.1 Effect of concentration of Xgal on the rate of cleavage

Medium supplemented with 50mM glucose was inoculated as detailed in section 2.2.1.1.2. 150µl of this subculture was added to the wells of a microtitre plate and 5µl of 0.1% SDS and 3µl of chloroform was added to each well. Various concentrations of Xgal were added to duplicate wells, in 5µl of DMF, so as to give final concentrations of 0.5, 0.4, 0.3, 0.2, 0.15, 0.1, 0.075, 0.05 & 0.025µg/150µl (see Table 23). The plate was sealed with a Mylar plate sealer and placed in the shaker incubator at 35°C and the cleavage of Xgal was monitored over 60 minutes by measuring

A_{666}-A_{560}.

Assay conc. Xgal (µg/150µl)	0.5	0.4	0.3	0.2	0.15	0.1	0.075	0.05	0.025
Stock conc. Xgal (mg/ml)	100	80	60	40	30	20	15	10	5

Table 23 Stock and final concentrations of Xgal added to assay samples.

The experiment was repeated using solutions of Xgal in various ratios of aqueous DMF as follows:- water:DMF - 1:4, 2:3, 3:2, 4:1.

2.2.4.5.2 Effect of glucose concentration on β-galactosidase activity in *K. marxianus*

Media supplemented with either 25, 50, 100 or 200mM glucose was inoculated with *K. marxianus* as detailed in section 2.2.1.1.2 and 150µl of the subculture was added to the wells of a microtitre plate. The plate was sealed and incubated in the plate shaker at 35°C and cell density and β-galactosidase activity were determined at regular intervals throughout the growth cycle. Cell density was determined as detailed in section 2.2.4.1.2 and β-galactosidase activity as detailed in section 2.2.4.1.3 with the exception of the solution of Xgal used, which was modified as follows:- 5µl of 20mg/ml Xgal in aqueous DMF (3 part water: 2 parts DMF) was added to each well.

2.2.4.5.3 Effect of cell density on β-galactosidase activity in *K. marxianus*

An inoculation culture supplemented with 50mM glucose was prepared as detailed in section 2.2.1.1.2. This was used to inoculate fresh medium with *K. marxianus,* at various cell densities, by dilutions of 1:50, 1:20, 1:10 or 1:5 into the fresh media. 150µl of the subcultures was added to the wells of a microtitre plate. The plate was sealed and incubated in the plate shaker at 35°C and cell density and β-galactosidase activity were determined at regular intervals throughout the growth cycle. Cell density was determined as detailed in section 2.2.4.1.2 and β-galactosidase activity as detailed in section 2.2.4.1.3 with the exception of the solution of Xgal used, which was modified as follows:- 5µl of 20mg/ml Xgal in aqueous DMF (3 part water: 2 parts DMF) was added to each well.

2.2.5. TOXICITY OF METHANOL AND ETHANOL TO *K. MARXIANUS*

2.2.5.1 Media and reagents

Medium (50mM YEPG) contained 1% (w/v) yeast extract, 1% (w/v) bacteriological peptone and 50mM glucose. An inoculation culture was prepared by adding a scc of *K. marxianus* from an agar plate to 50ml of medium, and incubating it for 16 hours at 35°C and 200rpm.

2.2.5.2 Experimental procedures

124µl of 50mM YEPG media was added to the wells of a microtitre plate, and methanol or ethanol was added to give a final concentration of 0, 2.5, 5, 7.5, 10, 12.5% (v/v) (Table 24). 16µl of yeast inoculum was added to each well. Blank wells for each solvent concentration were identical to the sample wells with the exception of 16µl of medium was added in place of 16µl of inoculum. Plates were sealed with a Mylar plate sealer and incubated in the plate shaker at 35°C. Cell density was determined at regular intervals throughout the growth cycle as detailed in section 2.2.5.2.1 below; β-galactosidase activity determined following 10 hours of growth as detailed in section 2.2.5.2.2 below.

% Solvent (v/v)	0	2.5	5	7.5	10	12.5
Volume of media (µl)	124					
Volume of solvent (µl)	0	4	8	12	16	20
Volume of water (µl)	20	16	12	8	4	0
Volume of inocula (µl)	16					

Table 24 Volume added to each well of the microtitre plate in the solvent experiment.

2.2.5.2.1 Determination of cell density

Cell density was determined by the measurement of absorbance at 560nm, using a Titertek Multiscan Plus Mk II microtitre plate reader (Labsystems), interfaced with an Amstrad microcomputer and Titresoft 1.01 software (Labsystems).

2.2.5.2.2 Determination of β-galactosidase activity

A working solution of 20mg/ml Xgal in aqueous DMF (2 parts water:3 parts DMF) was prepared immediately before each assay from a stock of 100mg/ml in DMF. 8µl of the working solution was added to each well followed by 5µl of 0.1% SDS and 3µl of chloroform. The plates were mixed and incubated at 35°C in the plate shaker for 30 minutes. The plates were then read on the microtitre plate reader using a test filter at 666nm and a reference filter at 560nm. β-galactosidase activity is expressed as the cleavage of Xgal (A_{666}-A_{560}) as a function of cell density (A_{560}).

2.2.6 ENHANCEMENT OF THE SENSITIVITY OF THE BIOASSAY BY THE USE OF MEMBRANE MODULATING AGENTS (MMAs)

2.2.6.1 Effect of MMAs on growth and β-galactosidase activity of *K. marxianus*

2.2.6.1.1 Media

50mM YEPG (section 2.2.5.1) was supplemented with a selected MMA at a concentration 1.176 times greater than that required in the final assay (the addition of the yeast inocula and toxin in methanol reduced the MMA concentration by a factor of 0.85) Three MMAs were assessed:- cetyltrimethyl ammonium bromide (CTAB), polymyxin B sulphate (PMBS) and polymyxin B nonapeptide (PBN); the concentrations used are shown in Table 25.

Final assay concentration of MMA (μg/ml)	Concentrations at which each MMA was assessed		
	CTAB	PMBS	PBN
250			✓
200			✓
175			✓
150			✓
100		✓	✓
50		✓	✓
35		✓	
25		✓	
20		✓	
15		✓	
10	✓	✓	✓
7.5	✓		
5	✓	✓	
2.5	✓		
1	✓	✓	✓
0.5	✓		
0.1	✓		
0	✓	✓	✓

Table 25 Concentrations of MMAs assessed (✓) in the bioassay system.

2.2.6.1.2 Assay procedure

136µl of 50mM YEPG medium supplemented with the various concentrations of the MMAs was added in triplicate to the wells of a microtitre plate. 8µl of methanol and 16µl of inoculum was added to each well. Blank wells contained 152µl of media and 8µl of methanol. Plates were sealed with a Mylar plate sealer and incubated in the shaker-incubator at 35°C throughout the experiment. Cell density was determined at regular intervals throughout the growth cycle as detailed in section 2.2.5.2.1 and β-galactosidase activity determined at the end of the growth cycle (8 hours) as detailed in section 2.2.5.2.2.

2.2.6.2 Effect of MMAs on verrucarin A toxicity to *K. marxianus*

2.2.6.2.1 Media

50mM YEPG (section 2.2.5.1) was supplemented with either CTAB, PMBS or PBN at 1.176, 17.64 and 176.4µg/ml respectively to give a final assay concentration of 1µg/ml CTAB, 15µg/ml PMBS and 150µg/ml PBS (see section 2.2.6.1.1).

2.2.6.2.2 Verrucarin A

A stock solution of 100mg/ml verrucarin A was prepared in methanol and was diluted further in methanol to give a series of standards to be used in the assay (section 2.3.1.3, below). As the toxin is diluted by a factor of 20 upon addition to the assay mixture each standard was prepared at a concentration 20-times greater than the final concentration required in the assay (Table 26).

Final assay conc. (ng/ml)	**0.1**	**0.25**	**0.5**	**0.6**	**0.8**	**1**	**2.5**	**5**	**7.5**	**10**
Conc. of standard (ng/ml)	2	5	10	12	16	20	50	100	150	200
Final assay conc. (ng/ml)	**15**	**20**	**30**	**40**	**50**	**75**	**100**	**150**	**250**	
Conc. of standard (ng/ml)	300	400	600	800	1000	1500	2000	3000	5000	

Table 26 Concentration of verrucarin A standards.

2.2.6.2.3 Assay procedure

136µl of media supplemented with one of each of MMAs (section 2.2.6.2.1) was added to the wells of a microtitre plate. 8µl of each verrucarin A standard was added to the wells in triplicate, followed by 16µl of the inoculum. Control wells contained methanol in place of verrucarin A and blank wells contained 152µl of medium and 8µl of methanol. The plate was sealed with a Mylar plate sealer and placed in the shaker incubator at 35°C for the duration of the experiment. Cell density was measured at regular intervals throughout the growth cycle as detailed in section 2.2.5.2.1 and β-galactosidase activity determined at the end of the growth cycle (10 hours) as detailed in section 2.2.5.2.2.

2.3 DETECTION OF MYCOTOXINS USING THE YEAST BIOASSAY

2.3.1 BIOASSAY METHODOLOGY

2.3.1.1 Media

Medium (50mM YEPG) consisted of 1% (w/v) yeast extract, 1% (w/v) bacteriological peptone and 50mM glucose. This was supplemented with 17.65µg/ml polymyxin B sulphate (PMBS) prior to each assay; this gave a final assay concentration of 15µg/ml PMBS after the addition of the mycotoxin standard and yeast inoculum (see section 2.2.6.1.1).

2.3.1.2 Preparation of yeast inocula

50ml of 50mM YEPG was inoculated with a single cell colony of *K. marxianus* from an agar plate and was incubated for 16 hours at 35°C, 200 rpm.

2.3.1.3 Preparation of mycotoxin stock solutions

Stock solutions of each mycotoxin were prepared by the addition of 10ml of spectroscopic grade methanol to 1mg of crystalline material. Absolute concentrations were determined by UV absorbance (Lambda 3, Perkin-Elmer) using equation 1.

$$\text{Conc.} = \frac{A \times CF}{MA \times pl} \qquad \text{Equation 1}$$

Where:-

Conc. = Concentration in moles/l. Multiply by M.wt x 10^3 to convert into µg/ml.

M.wt = Molecular weight of mycotoxin (Table 27).

A = Absorbance at λ_{max} (see Table 27 for λ_{max} for individual mycotoxins).

pl =Path length of cuvette (1cm).

MA = Molar absorbtivity of the mycotoxin (Table 27) or potassium dichromate.

CF = Correction factor for the instrument and cuvettes used.

The correction factor was calculated using a known concentration of potassium dichromate ($K_2Cr_2O_7$) solution as shown in equation 2.

$$CF = \frac{MA(K_2Cr_2O_7) \times Conc.\ (K_2Cr_2O_7)}{A} \qquad \text{Equation 2}$$

where:- MA ($K_2Cr_2O_7$) = 3160

Stock solutions were stored in the dark at -20°C. Prior to each assay an appropriate series of dilutions of each stock was made in methanol. The concentration series of mycotoxins was twenty times the final concentration required in the bioassay to account for dilutions that occurred on adding the toxin to the media and cells. The concentration series of each toxin used in the bioassay is detailed in the relevant sections.

Mycotoxin	Molecular weight	Molar absorbtivity (mol/cm/l)	λ_{max} (nm)
Aflatoxin B_1	312	25600 (ethanol)	223
Aflatoxin M_1	328	23100 (ethanol)	226
Citrinin	250	23000 (ethanol)	222
Cyclopiazonic acid	336	20417	284
Deoxynivalenol	296	6395	219
Diacetoxyscirpenol	366	2487	203
Fusarenon-X	354	6500	220
HT-2 toxin	424	End absorbtion	
Neosolaniol	382	2644	203
Nivalenol	312	End absorbtion	
Ochratoxin A	403	36800	215
Patulin	154	16600	276
Roridin A	532	18600 (ethanol)	263
Scirpentriol	282	End absorbtion	
Sterigmatocystin	324	13100 (ethanol)	329
Tenuazonic acid	197	5248	217
T-2 tetraol	298	End absorbtion	
T-2 toxin	466	3681	202
T-2 triol	381	End absorbtion	
Verrucarin A	502	17700 (ethanol)	260
Zearalenone	318	29700	236

Table 27 Spectrophotometric parameters of some mycotoxins in methanol, unless otherwise stated.

2.3.1.4 Assay Procedure

136µl of PMBS supplemented media was added to the wells of a microtitre plate. 8µl of mycotoxin stock solution or control (methanol) was added, followed by 16µl of yeast inoculum. Blank wells contained 152µl of medium and 8µl of methanol. The plate was mixed and the absorbance at 560nm (A_{560}) measured. The plate was sealed with a Mylar plate sealer and incubated in a plate shaker at 35°C for the duration of the assay. Cell density was determined at regular intervals throughout the growth cycle as detailed in section 2.3.1.4.1. When the control cultures reached stationary phase (this corresponded to an A_{560} of ca. 1.2 and usually took 10 hours), the cultures were assayed for β-galactosidase activity as detailed in section 2.3.1.4.2.

2.3.1.4.1 Determination of cell density

Cell density was determined by the measurement of absorbance at 560nm (A_{560}), using a Titertek Multiscan Plus Mk II microtitre plate reader (Labsystems), interfaced with an Amstrad microcomputer and Titresoft 1.01 software (Labsystems).

2.3.1.4.2 Determination of β-galactosidase activity (Xgal assay)

A working solution of 20mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) in aqueous DMF (2 parts water:3 parts DMF) was prepared immediately before each assay from a stock of 100mg/ml in DMF. 8µl of the working solution was added to each well followed by 5µl of 0.1% SDS and 3µl of chloroform. The plates were mixed and incubated at 35°C in the plate shaker for 20 minutes. The plates were then read on the microtitre plate reader using a test filter at 666nm and a reference filter at 560nm. β-galactosidase activity is expressed as the cleavage of Xgal (A_{666}-A_{560}) as a function of cell density (A_{560}).

2.3.1.4.3 Determination of cell viability (MTT assay)

Cell viability was determined using an adaptation of the method of Mossman, 1983. The assay is based on the capacity of mitochondrial enzymes of viable cells to transform the MTT tetrazolium salt into MTT formazan.

3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline (PBS) (Dulbecco's formula) at a concentration of 5mg/ml and was filter sterilised prior to use to remove any insoluble residue present. 16µl of MTT in PBS was added to the each well of the microtitre plate and the plate incubated statically at 35°C for 4 hours, after which the media was removed from each well and 200µl of DMSO added to all wells. The samples were thoroughly mixed by repeated pipetting to dissolve the blue product formed and the absorbance determined using the microtitre plate reader using a test wavelength of 560nm and a reference wavelength of 666nm.

2.3.1.5 Data Processing

2.3.1.5.1 Construction of dose response curves

Dose-response curves were constructed for the inhibition of growth, β-galactosidase activity and cell viability after 10 hours of growth. Percentage inhibition of a given endpoint was determined by comparison to methanol controls using equations 3, 4 or 5, as appropriate. For each toxin concentration at least two replicate wells were used and for the methanol controls at least 12 replicates were used.

Percentage inhibition of growth

$$100 - \left(\frac{\Delta A_{(toxin)}}{\Delta A_{(control)}} \times 100\right) \qquad \text{Equation 3}$$

where

$\Delta A_{(toxin)}$ = average change in absorbance of wells containing a given toxin concentration (final A_{560} - start A_{560}).

$\Delta A_{(control)}$ = average change in absorbance of control wells (final A_{560} - start A_{560}).

Percentage inhibition of β-galactosidase activity

$$100 - \left(\frac{A_{666} - A_{560\,(toxin)}}{A_{666} - A_{560\,(control)}} \times 100\right) \qquad \text{Equation 4}$$

where

A_{666}-A_{560}(toxin)= average absorbance of the indigo product of cleaved Xgal at a given toxin concentration

A_{666}-A_{560}(control)= average absorbance of the indigo product of cleaved Xgal for the control wells

Percentage inhibition of cell viability

$$100 - \left(\frac{A_{560} - A_{666\,(toxin)}}{A_{560} - A_{666\,(control)}} \times 100\right) \qquad \text{Equation 5}$$

where

A_{560}-A_{666} (toxin)= average absorbance of the formazan product of cleaved MTT at a given toxin concentration

A_{560}-A_{666} (control)= average absorbance of the formazan product of cleaved MTT for the control wells

2.3.1.5.2 Use of dose-response curves

Dose-response curves can be used to obtain a number of parameters that can be used to determine toxicity. In this study three parameters were calculated from the dose-response curves. These were:-

1. **NEL** - nil-effect level; the concentration of toxin at which no inhibition is detected.
2. **EC_{50}** - the concentration of toxin at which 50% inhibition is detected.
3. **MIC** - minimum inhibitory concentration; the concentration of toxin at which 100% inhibition is detected.

These parameters are shown on a representative dose-response curve in Figure 12.

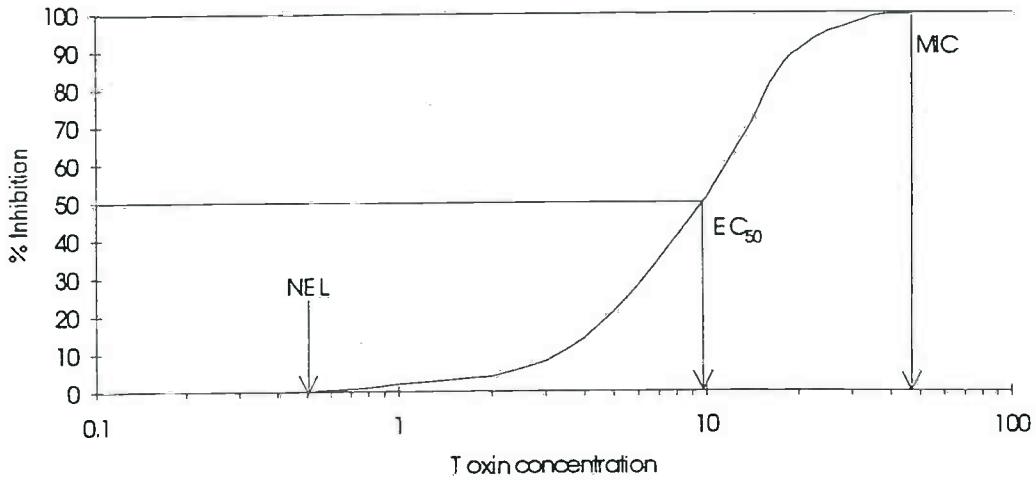


Figure 12 Estimation of NEL, EC_{50} and MIC concentrations of mycotoxins using dose-response curves.

2.3.2 REPRODUCIBILITY OF THE BIOASSAY

The reproducibility of the bioassay was assessed using T-2 toxin. The assay procedure is detailed in section 2.3.1.4, and concentrations of T-2 toxin used are shown in Table 28. Each toxin concentration was added to triplicate wells; six triplicate control wells (18 wells in total) were also added to each plate. Cell density was determined throughout the growth cycle as detailed in section 2.3.1.4.1 and β-galactosidase activity and cell viability were determined after 10 hours growth as detailed in sections 2.3.1.4.2 & 2.3.1.4.3 respectively. Dose-response curves for the inhibition of growth, β-galactosidase activity and cell viability were constructed as detailed in section 2.3.1.5.1. The assay was repeated on three different occasions so as both within-assay and between-assay variation could be determined.

Concentration of stock solution (ng/ml)	Final assay concentration (ng/ml)
2	0.1
20	1
50	2.5
100	5
150	7.5
200	10
500	25
1000	50
1500	75
2000	100
5000	250

Table 28 Concentrations of T-2 toxin used to assess the reproducibility of the bioassay.

2.3.3 DETECTION OF MYCOTOXINS THAT ARE KNOWN NATURAL CONTAMINANTS OF FOODS AND FEEDS

The toxicity of various mycotoxin standards to the bioassay system was examined using the assay methodology detailed in section 2.3.1.4. The mycotoxins tested are listed in Table 29, and a range of concentrations of each standard was prepared by dilution in methanol (as detailed in section 2.3.1.3). The concentrations used in this experiment are shown in Table 30. Cell density was determined throughout the growth cycle as detailed in section 2.3.1.4.1 and β-galactosidase activity was determined at the end of the growth cycle as detailed in section 2.3.1.4.2. Dose-response curves for the inhibition of growth and β-galactosidase activity were constructed as detailed in section 2.3.1.5.1, and were used to determine NEL, EC_{50} and MIC concentrations for each toxin as detailed in section 2.3.1.5.2.

Aflatoxin B_1	Aflatoxin M_1	Citrinin
Cyclopiazonic acid	Deoxynivalenol	Diacetoxyscirpenol
Fumonisin B_1	Ochratoxin A	Patulin
Roridin A	Sterigmatocystin	T-2 toxin
Tenuazonic acid	Verrucarin A	Zearalenone

Table 29 Toxins assessed using the yeast bioassay system.

Final assay conc.	25µg/ml *	10µg/ml	1µg/ml	100ng/ml	10ng/ml	1ng/ml	0.1ng/ml
Conc. of standard.	500µg/ml *	200µg/ml	20µg/ml	2µg/ml	200ng/ml	20ng/ml	2ng/ml

* Concentration tested using aflatoxin B_1 and M_1 only.

Table 30 Concentration of mycotoxins tested using the yeast bioassay system.

2.3.4 STRUCTURE-ACTIVITY RELATIONSHIPS AMONGST THE TRICHOTHECENE MYCOTOXINS

A study was made to determine the structure-activity relationships amongst a number of trichothecene mycotoxins. The trichothecenes assessed are shown in Table 31; the concentrations of each toxin used in the bioassay varied, and are shown in Table 32. The assay procedure is as detailed in section 2.3.1.4, and cell density and β-galactosidase activity were determined as detailed in section 2.3.1.4.1 & 2.3.1.4.2 respectively. Dose-response curves for the inhibition growth and β-galactosidase activity by each trichothecene were constructed as detailed in section 2.3.1.5.1 and were used to determine NEL, EC_{50} and MIC concentrations for each toxin as detailed in section 2.3.1.5.2.

Acetyl T-2 toxin	(AcT-2)	Deoxynivalenol	(DON)	Diacetoxyscirpenol	(DAS)
Fusarenon-X	(FUS)	HT-2 toxin	(HT-2)	Neosolaniol	(NEO)
Nivalenol	(NIV)	Roridin A	(ROR)	Scirpentriol	(SCR)
T-2 tetraol	(TET)	T-2 triol	(TRI)	T-2 toxin	(T-2)
Verrucarin A	(VER)				

Table 31 Trichothecene mycotoxins studied using the bioassay system.

Final conc. in assay.	VER	ROR	T-2	DAS	HT-2	ALL OTHERS
0.1ng/ml	✓	✓				
0.5ng/ml	✓	✓	✓			
1ng/ml	✓	✓	✓		✓	
5ng/ml	✓	✓	✓	✓		
10ng/ml	✓	✓	✓	✓	✓	
25ng/ml	✓	✓	✓	✓		
50ng/ml	✓	✓	✓	✓	✓	
100ng/ml	✓	✓	✓	✓	✓	✓
250ng/ml			✓	✓		
500ng/ml				✓	✓	✓
1µg/ml				✓	✓	✓
2.5µg/ml						✓
5µg/ml					✓	✓
10µg/ml					✓	✓
20µg/ml						✓
25µg/ml						✓

Table 32 Concentrations of trichothecene mycotoxins tested (✓) using the yeast bioassay.

2.4 UPTAKE, BINDING AND METABOLISM OF AFLATOXIN B_1 AND T-2 TOXIN BY *K. MARXIANUS* AND *B. MEGATERIUM*

2.4.1 GENERAL METHODOLOGIES

2.4.1.1 Media

K. marxianus was grown on medium containing 1% (w/v) yeast extract, 1% (w/v) bacteriological peptone and 1% (w/v) glucose (1% YEPG) supplemented with 15µg/ml PMBS. *B. megaterium* was grown on 3% (w/v) Trypticase Soy Broth (3% TSB).

2.4.1.2 Preparation of inoculation cultures

An inoculation culture was prepared by adding a single cell colony of *K. marxianus* or *B. megaterium* from an agar plate to 50ml of medium. The culture was incubated for 16 hours at 35°C and 200 rpm.

2.4.1.3 Mycotoxin solutions

Stock solution of AFB_1 at 200µg/ml in benzene:acetonitrile (98:2) and T-2 toxin at 250µg/ml in methanol were prepared as detailed in section 2.3.1.3. All solutions were stored in the dark at -20°C until required.

2.4.1.3.1 Standard solutions

A single concentration standard at 1µg/ml in benzene acetonitrile (B:A) (98:2) was prepared for the estimation of aflatoxin B_1 as detailed in section 2.3.1.3.

Four standard concentrations at 1, 2.5, 5 & 10µg/ml in B:A (9:1) were prepared for the estimation of T-2 toxin as detailed in section 2.3.1.3.

All solutions were stored in the dark at -20°C until required.

2.4.1.3.2 Spiking solutions

25µl of aflatoxin B_1 (200µg/ml in B:A (98:2)) was used to spike 5ml cultures of *K. marxianus* and *B. megaterium*. A control spiking solution was prepared by adding 25µl of AFB_1 to 5ml of B:A (98:2).

250µl of T-2 toxin (200µg/ml in methanol) was used to spike 4.75ml cultures of *K. marxianus* and *B. megaterium*. A control spiking solution was prepared by drying down 250µl of T-2 toxin (200µg/ml in methanol) under nitrogen and resuspending in 5ml of B:A (9:1).

2.4.1.4 Uptake and binding procedure

Cells were harvested from 5ml of inoculation culture by centrifugation at 4°C for 20 minutes, and resuspended in 5ml of fresh medium. Spiking solutions of AFB_1 or T-2 toxin were added to the samples as detailed in section 2.4.1.3.2. A control sample was prepared in a similar manner, but was spiked with appropriate solvent and not toxin. All samples were incubated for 16 hours at 35°C and 200rpm. At the end of the incubation period, cells were harvested by centrifugation, and toxin extracted from the supernatant as detailed in section 2.4.1.5. Cells were washed by resuspending in 5ml of sterile water, followed by centrifugation, and toxin was extracted from the wash solution. The wash procedure was repeated five times, after which cells were disrupted by ultrasonic treatment (30 minutes) and toxin was extracted from the ruptured cells.

2.4.1.5 Extraction of mycotoxins

Mycotoxins were extracted from the supernatant, wash solutions and ruptured cells using liquid:liquid partitioning. Chloroform (2 x 5ml) was used as the organic phase. Chloroform extracts were evaporated to dryness at 45°C under a stream of nitrogen using a sample concentrator.

2.4.1.6 Estimation of aflatoxin B_1

2.4.1.6.1 Sample preparation and application

Aliquots (0.1-5.0ml - see Table 34) of benzene:acetonitrile (B:A) (98:2 v/v) were used for the dilution of sample extracts, which were then mixed in a vortex mixer for 15 seconds. Standards and samples were applied to Kieselgel 60 silica gel HPTLC plates (Merck 5547), 30mm from the bottom of the plate, using a Nanomat III semi-automated TLC plate spotter (Camag). 5 standards and up to 12 duplicated samples were applied to each plate and a typical spotting pattern is shown in Figure 13. The volume of B:A used to resuspend each sample and the volume of each sample spotted varied according to the concentration of toxin in that sample and is summarised in Table 33.

a b I c d e f g I h i j k l I d a c g e I i b h l j I f k

Figure 13 Typical spotting pattern for aflatoxin B_1.
a-l = duplicated samples, I = replicated standard (1µg/ml AFB_1 in B:A (98:2)).

2.4.1.6.2 HPTLC development

Aflatoxin was chromatographed using two 20 minute developments with 20ml of chloroform:xylene:acetone (6:3:1 v/v) and a conventional vee-bottomed TLC tank. The solvent was evaporated from the plate between developments under a stream of air for two minutes. After the second development, the plate was dried in a fan-assisted oven for one minute at 100°C.

2.4.1.6.3 Densitometric determination of aflatoxin B_1

AFB_1 was detected by UV fluorescence in the reflectance mode as detailed in section 2.4.1.8.

2.4.1.6.4 Detection of metabolites of AFB_1

HPTLC plates were examined under long-wave UV light for the presence of metabolites of AFB_1. Metabolites being defined as any spots in the tracks containing sample extracts that did not correspond with spots in either the standard or control tracks (extracts from cells dosed with solvent instead of mycotoxin).

2.4.1.7 Estimation of T-2 toxin

2.4.1.7.1 Sample preparation and application

Aliquots (0.1-5.0ml - see Table 34) of benzene:acetonitrile (B:A) (9:1 v/v) were used for the dilution of sample extracts, which were then mixed in a vortex mixer for 15 seconds. Standards and samples were applied to Kieselgel 60 silica gel HPTLC plates (Merck 5547), 30 mm from the bottom of the plate, using a Nanomat III semi-automated TLC plate spotter (Camag). To each plate 4 duplicated standards and up to 12 duplicated samples were applied. A typical spotting pattern is shown in Figure 14. The volume of B:A (9:1) used to resuspend each sample and the volume of each sample spotted varied according to the concentration of toxin in that sample and is summarised in Table 33.

a b c 1 d e 2 f g 3 h i 4 j b 1 a c 2 i h 3 e d 4 g f j

Figure 14 Typical spotting pattern for T-2 toxin.
a-j = duplicated samples, 1, 2, 3, 4 = duplicated standards (1, 2.5, 5 & 10µg/ml T-2, respectively, in B:A (9:1))

2.4.1.7.2 HPTLC development

Trichothecenes were chromatographed using a single eleven minute development with 20ml of toluene:ethyl acetate:formic acid:water (1500:700:198:7 v/v). The solvent was evaporated from the plate under a stream of air for two minutes, and the plate dipped in 2.5% sulphuric acid in methanol for 3 seconds. The plate was then placed in a fan-assisted oven for 10 minutes at 100°C.

Sample	Volume of B:A used for sample extract dilution (ml)		Volume of sample spotted on the HPTLC plate (µl)	
	Aflatoxin B_1	T-2 toxin	Aflatoxin B_1	T-2 toxin
Supernatant	5.0	5.0	2	2
Wash 1	1.5	1.0	2	2
Wash 2	0.2	0.2	2	5
Wash 3	0.2	0.2	2	5
Wash 4	0.2	0.2	2	5
Wash 5	0.2	0.2	2	5
Ruptured Cells	0.1	0.1	5	10
Spike	n/a	n/a	2	2
Standards	n/a	n/a	2	2

n/a = not applicable

Table 33 Volume of benzene:acetonitrile (B:A) used for sample extract dilution and the volume of each sample spotted on the HPTLC plate.

2.4.1.7.3 Densitometric detection of T-2 toxin

T-2 was detected by UV fluorescence in the reflectance mode as detailed in section 2.4.1.8.

2.4.1.7.4 Detection of metabolites of T-2 toxin

HPTLC plates were examined under long-wave UV light for the presence of metabolites of T-2 toxin. Metabolites being defined as any spots in the tracks containing sample extracts that did not correspond with spots in either the standard or control tracks (extracts from cells dosed with solvent instead of mycotoxin).

2.4.1.8 Densitometry

AFB_1 and T-2 toxin were detected by UV fluorescence in the reflectance mode using a Camag monochromatic densitometer (TLC scanner II, No. 76610), a Camag electronic integrator (SP 4270 TLC integrator, No. 76650) and an Elonex PC425X computer with CATS3 software (Camag). Parameters used varied according to the toxin being evaluated and are summarised in Table 34. Equation 6 was used to calculate the toxin mass in each sample.

Parameter	AFB_1	T-2 toxin
Light source:	high pressure mercury lamp	high pressure mercury lamp
Wavelength:	360nm filter	360nm filter
Half band width:	30nm	30nm
Optical slit dimensions:	3 x 0.5mm	4 x 0.4mm
Scan speed:	0.5mm/s	0.5mm/s
Peak evaluation:	peak area.	peak area.
Calibration:	external standard; single level-2ng	external standards; four levels - 2, 5, 10, & 20ng

Table 34 Scanner parameters used for the densitometric estimation of AFB_1 and T-2 toxin.

Calculation of toxin mass in the samples:

$$Mass = \frac{P_1 \times C \times V_1 \times D}{P_1 \times V_2}$$ Equation 6

Where:-

Mass = Toxin mass in each sample (ng)

P_1 = Sample peak area (mm^2)

C = Concentration of standard (ng/ml)

V_1 = Volume of standard applied (µl)

D = Volume of benzene:acetonitrile used for sample extract dilution (µl)

P_2 = Average peak area of replicate standards (mm^2)

V_2 = Volume of sample extract applied (µl)

2.4.1.8.1 Data Processing.

For all experiments the amount of AFB_1 or T-2 toxin in a given extract is expressed as mass (ng for AFB_1 and µg for T-2 toxin) (section 2.4.1.8) and as a percentage of the total amount of mycotoxin to which the microorganisms in the 5ml cultures were exposed; this was calculated using equation 7:

$$\% \text{ Toxin} = \frac{E}{S} \times 100 \qquad \text{Equation 7}$$

Where:-

% Toxin = Percentage of AFB_1 or T-2 toxin in a given extract.

E = amount of AFB_1 or T-2 toxin in a given extract (ng or µg respectively).

S = amount of AFB_1 or T-2 toxin in the control spiking solution (ng or µg respectively) (see section 2.4.1.3.2).

2.4.2 METHODOLOGIES FOR INDIVIDUAL EXPERIMENTS

2.4.2.1 Sensitivity of *B. megaterium* to AFB_1 and T-2 toxin

The effect of T-2 toxin and AFB_1 on the growth of *B. megaterium* was determined, using a similar protocol to that of the bioassay using *K. marxianus* (section 2.3.1). An inoculation culture of *B. megaterium* was prepared as detailed in section 2.4.1.2, and was diluted 10-fold into fresh TSB medium. Standards of AFB_1 (20µg/ml) and T-2 toxin (200µg/ml) in methanol were prepared as detailed in section 2.3.1.3. 136µl of 3% TSB medium was added to the wells of a microtitre plate, 8µl of the mycotoxin standard or methanol control was added to six of the wells and 16µl of the 10-fold diluted inoculum was added to all the wells. Blank wells contained 152µl of medium and 8µl of methanol. The plate was sealed with a Mylar plate sealer and incubated in the shaker-incubator at 35°C; cell density was determined at regular intervals throughout the growth cycle by the measurement of absorbance at 560nm (A_{560}) using a Titertek Multiscan Plus Mk II microtitre plate reader (section 2.3.1.4.1).

2.4.2.2 Uptake and binding of AFB_1 by *K. marxianus*

2.4.2.2.1 Precision of the methodology

Three separate inoculation cultures were prepared as detailed in section 2.4.1.2; cells were harvested from 5ml of each inoculation culture, and the pellet resuspended in 5ml of 1% YEPG medium. Each culture was spiked with 5µg of AFB_1 (section 2.4.1.3.2) and the uptake and binding of AFB_1 by *K. marxianus* determined as detailed in section 2.4.1.4. AFB_1 was extracted from the supernatant, wash solutions

and ruptured cells as detailed in section 2.4.1.5 and the amount of AFB_1 in each extract determined as detailed in section 2.4.1.6.

2.4.2.2.2 Effect of methanol and PMBS on the uptake and binding of AFB_1 by *K. marxianus*.

Cells were harvested from 5ml of inoculation culture (section 2.4.1.2) and resuspended in 5ml of one of the following:-

1. 1% YEPG
2. 1% YEPG supplemented with 15ug/ml PMBS
3. 1% YEPG supplemented with 15ug/ml PMBS and 5% (v/v) methanol
4. 1% YEPG supplemented with 15ug/ml PMBSand 8.5% (v/v) methanol
5. 1% YEPG supplemented with 15ug/ml PMBS and 15% (v/v) methanol.

Each culture was spiked with AFB_1 (5µg in 25µl of B:A 98:2) or solvent (25µl of B:A 98:2) in the case of controls (section 2.4.1.3.2) and the uptake and binding of AFB_1 by *K. marxianus* determined as detailed in section 2.4.1.4. AFB_1 was extracted from the supernatant, wash solutions and ruptured cells as detailed in section 2.4.1.5, and the amount of AFB_1 in each extract was determined as detailed in section 2.4.1.6. HPTLC plates were examined for metabolites of AFB_1 as detailed in section 2.4.1.6.4.

2.4.2.3 Uptake and binding of AFB_1 by *B. megaterium*

2.4.2.3.1 Precision of the methodology

Three separate inoculation cultures were prepared as detailed in section 2.4.1.2; cells were harvested from 5ml of each inoculation culture and the pellet resuspended in 5ml of 3% TSB medium. Each culture was spiked with 5µg of AFB_1 (section 2.4.1.3.2) and the uptake and binding of AFB_1 by *B. megaterium* determined as detailed in section 2.4.1.4. AFB_1 was extracted from the supernatant, wash solutions

and ruptured cells as detailed in section 2.4.1.5 and the amount of AFB_1 in each extract determined as detailed in section 2.4.1.6.

2.4.2.3.2 Effect of methanol on the uptake and binding of AFB_1 by *B. megaterium*
Cells were harvested from 5ml of inoculation culture and resuspended in 5ml of one of the following:-

1. 1% TSB
3. 1% TSB supplemented with 5% (v/v) methanol
4. 1% TSB supplemented with 8.5% (v/v) methanol
5. 1% TSB supplemented with 15% (v/v) methanol.

Each culture was spiked with AFB_1 (5µg in 25µl of B:A 98:2) or solvent (25µl of B:A 98:2) in the case of the controls (section 2.4.1.3.2) and the uptake and binding of AFB_1 by *B. megaterium* was determined as detailed in section 2.4.1.4. AFB_1 was extracted from the supernatant, wash solutions and ruptured cells as detailed in section 2.4.1.5, and the amount of AFB_1 in each extract was determined as detailed in section 2.4.1.6. HPTLC plates were examined for metabolites of AFB_1 as detailed in 2.4.1.6.4.

2.4.2.4 Linearity of the detection of T-2 toxin by densitometry

2µl of T-2 standard solutions of 1, 2.5, 5, 10, & 15µg/ml were spotted, in quadruplicate, across a HPTLC plate. The plate was developed as detailed in section 2.4.1.7.2 and T-2 toxin quantified as detailed in section 2.4.1.7.3.

2.4.2.5 Uptake and binding of T-2 toxin by *K. marxianus* and *B. megaterium*

Inoculation cultures of *K. marxianus* and *B. megaterium* were prepared as detailed in section 2.4.1.2. Cells were harvested from 5ml of the inoculation cultures and resuspended in 4.75ml of fresh medium (1% YEPG for *K. marxianus* and 3% TSB for *B. megaterium*). Cultures were spiked with T-2 toxin (50µg in 250µl of methanol) or

solvent (250µl of methanol) for the control, as detailed in section 2.4.1.3.2, thereby giving a final methanol concentration of 5% (v/v). The uptake and binding of T-2 toxin by *K. marxianus* and *B. megaterium* was determined as detailed in section 2.4.1.4. T-2 toxin was extracted from the supernatant, wash solutions and ruptured cells as detailed in section 2.4.1.5 and the amount of T-2 toxin in each extract was determined as detailed in section 2.4.1.7. HPTLC plates were examined for metabolites of T-2 toxin as detailed in section 2.4.1.7.4.

2.5 CHARACTERISATION OF CYTOCHROME P450 OF *K. MARXIANUS*.

Microsome samples prepared from *K. marxianus* (strain GK1005) and a control strain of *Saccharomyces cerevisiae* (NCYC 754) were used to investigate the concentration of cyt P450, and its ability to bind to benzo(*a*)pyrene and aflatoxin B_1.

2.5.1 REAGENTS.

Medium consisted of 1% yeast extract, 1% bacteriological peptone and 5% glucose (5% YEPG). Microsome buffer was 100mM phosphate buffer (pH 7.2), containing 20% (v/v) glycerol and 1mM EDTA.

2.5.2. PREPARATION OF YEAST MICROSOMES

2l of yeast culture in 5% YEPG medium was grown at 30°C for *S. cerevisiae,* or 37°C for *K. marxianus,* and 150 rpm on an orbital shaker until the culture reached late logarithmic phase of growth (ca. 1 x 10^8 cells/ml). The cells were harvested by centrifugation, and resuspended in 10ml of microsome buffer. 40g of 0.45-0.55mm diameter glass beads were added, and the cells were broken using a Braun cell homogeniser, cooled with liquid CO_2. The cell lysate was centrifuged at 1,500g for 10 minutes at 4°C, to remove unbroken cells, large fragments of cell wall and glass beads. The resulting supernatant was centrifuged at 12,000g for 10 minutes at 4°C, to remove mitochondria and smaller pieces of cell debris and the supernatant was then centrifuged at 100,000g for 90 minutes at 4°C, so sedimenting the microsomes (the endoplasmic reticulum membrane fraction formed into vesicles by the cell homogenisation process). The pellet was removed from the tube using a spatula, and resuspended using a pre-chilled hand-held Potter homogeniser containing 2ml of microsome buffer. The microsome preparation was frozen at -80°C, until used for analysis.

2.5.3 CYTOCHROME P450 QUANTIFICATION.

The concentration of cytochrome P450 (cyt P450) in the microsome samples was determined as follows, using the method of Omura and Sato (1964). 1ml of the sample was reduced by the addition of a few grains of sodium dithionite. The sample was equally divided between two restricted cuvettes, and, using a scanning spectrophotometer (Lambda 2, Perkin Elmer), the baseline absorption was recorded between 390 and 500nm. Carbon monoxide was then bubbled through the sample cuvette at a rate of 1 bubble per second for 60 seconds and the difference spectrum between 390 and 500nm was recorded. The presence of cyt P450 was revealed by a peak at or around 450nm. The concentration of cyt P450 was calculated from the absorbance change between 450 and 490nm. An extinction coefficient of $91mM^{-1}\ cm^{-1}$ (King *et al.*, 1983a) was used to calculate the enzyme concentration.

2.5.4 TOTAL PROTEIN DETERMINATION.

Total protein content of microsome samples was determined using a Pierce BCA Protein Assay Kit (Sigma), in accordance with manufacturer's protocol.

2.5.5 SUBSTRATE SPECTRAL BINDING ANALYSIS

The binding of two substrates, benzo(*a*)pyrene (B(*a*)P) and aflatoxin B_1 (AFB_1) was investigated using the method of Jefcoate (1979). A pair of divided cuvettes were used, which each have two chambers. To each cuvette, 1ml of microsome buffer was added to one chamber and 1ml of microsome sample to the other. The baseline was recorded between 350 and 500nm. B(*a*)P and AFB_1 were dissolved in DMSO at a concentration of 1mg/ml and 1-10µl of either B(*a*)P or AFB_1 solution was added to the microsome sample in the sample cuvette and to the buffer in the reference cuvette; an equivalent amount of solvent (DMSO) was added to the buffer in the sample cuvette and to the microsome sample in the reference cuvette (see Figure 15).

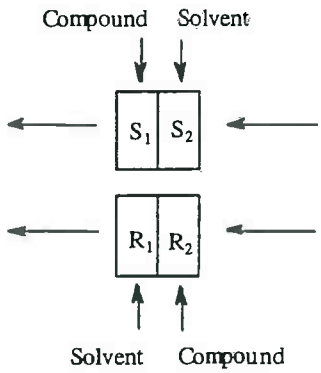


Figure 15 The use of divided cuvettes for the measure of difference spectra. S = sample cuvette R = reference cuvette. 1 = microsome sample 2 = buffer.

After mixing thoroughly, the absorbance spectrum was recorded between 350 and 500nm. Increasing amounts of substrate were added until there was no longer an increase in absorbance. The changes in absorbance with alterations in substrate concentration were analysed using the approach developed by Schenkman *et al.*, (1967), who performed spectral titrations of this sort using hepatic microsomes and a variety of cyt P450 substrates, and found that the resultant titration curves - absorbance change at a fixed wavelength verses substrate concentration - were hyperbolic in shape, suggesting that change in absorbance and substrate concentration were related in the same way as enzyme reaction velocity and substrate concentration, and could be similarly analysed. The Michalis Menten equation is

$$V_o = \frac{V_{max}\ [S]}{K_m + [S]} \qquad \text{Equation 8}$$

Where V_o = velocity at a given substrate concentration

V_{max} = maximal rate of velocity

[S] = substrate concentration

K_m = Michalis constant (substrate concentration where the velocity is half maximal).

Schenkman *et al.*, in effect replaced V_o with A (change in absorbance between λ_{max} and λ_{min}), V_{max} with A_{max} (maximal absorbance (enzyme is saturated)) and K_m with K_s

i.e.

$$A = \frac{A_{max}\ [S]}{K_s + [S]} \qquad \text{Equation 9}$$

and K_S = substrate concentration ([S]) when $A = \frac{A_{max}}{2}$. That is they defined the "spectral dissociation constant", K_s, as the substrate concentration required for half-maximal spectral change. K_s is the ratio of dissociation and association of the enzyme and substrate and can be thought of as a measure of the affinity of the enzyme for a substrate. A large K_s means the enzyme has a weak affinity for the substrate whereas a low K_s means the enzyme has a high affinity for the substrate.

For easier determination of K_s a Lineweaver-Burk type transformation of equation 9 was used:

Lineweaver-Burk equation

$$\frac{1}{A} = \frac{K_s}{[S]} \times \frac{1}{A_{max}} + \frac{1}{A_{max}} \qquad \text{Equation 10}$$

Where A = change in absorbance (absorbance between λ_{max} and λ_{min})

A_{max} = maximal absorbance (enzyme is saturated)

[S] = substrate concentration

K_S = spectral dissociation constant

A plot of $\frac{1}{A}$ against $\frac{1}{[S]}$ gives a straight line of slope $\frac{K_s}{A_{max}}$ with intercepts on the x and y axes of $-\frac{1}{K_s}$ and $\frac{1}{A_{max}}$, respectively.

Because of the non-uniform distribution of error in Lineweaver-Burk type plots, Eadie-Hofstee and Hanes plots were also used to analyse the data:

Eadie-Hofstee equation

Rearrangement of equation 10 gives:-

$$\frac{A}{[S]} = \frac{A_{max}}{K_s} - \frac{A}{K_s} \qquad \text{Equation 11}$$

and a plot of $\frac{A}{[S]}$ against A gives a straight line slope of $-\frac{1}{K_s}$ and an x-axis intercept of A_{max}.

Hanes equation

Rearrangement of equation 10 gives:-

$$\frac{[S]}{A} = \frac{[S]}{A_{max}} + \frac{K_s}{A_{max}} \qquad \text{Equation 12}$$

and a plot of $\frac{[S]}{A}$ against [S] gives a straight line slope of $\frac{1}{A_{max}}$ and an x-axis intercept of $-K_S$.