MICROBIOLOGICAL AND MOLECULAR METHODS FOR THE STUDY OF OCHRATOXIN A IN FOODS AND ITS CONTROL

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…In memory of my beloved father

Late T. Sreenivasan

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May 10, 2006

CERTIFICATE

I hereby certify that the thesis entitled "**Microbiological and molecular methods for the study of ochratoxin A in foods and its control**" submitted by **Mr. S. Anand** for the Degree of Doctor of Philosophy in Micrbiology to the University of Mysore is the result of research work carried out by him in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, during the period December 2000 to June 2004.

E. RATI RAO`

DECLARATION

I hereby declare that the thesis entitled **"MICROBIOLOGICAL AND MOLECULAR METHODS FOR THE STUDY OF OCHRATOXIN A IN FOODS AND ITS CONTROL"** submitted to the UNIVERSITY OF MYSORE, for the award of the degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY**, is the result of research work carried out by me under the guidance of Dr. E, Rati Rao**,** Scientist, Human Resource Development, Central Food Technological Research Institute, Mysore, during the period December 2000 to June 2004. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Date: May 10, 2006

Place: Mysore (**ANAND. S**)

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CONTENTS

LIST OF ABBREVIATIONS

AFB_1	- Aflatoxin B_1
μg	- Microgram
μl	- Microlitre
ALP	- Alkaline phosphatase
BCIP	- 5-Bromo-3-cresol-3-indolyl phosphate
BF_3	- Boron trifluoride
BSA	- Bovine Serum albumin
CFU	- Colony forming unit
DNA	- Deoxyribonucleic acid
EDPC	- 1-ethyl-3,3- dimethylaminpropyl carbodiimide
ELISA	- Enzyme Linked Immunosorbent Assay
FCA	- Freund's complete adjuvant
FICA.	- Freund's incomplete adjuvant
h	- Hour
HPLC	- High performance liquid chromatography
HPTLC	- High performance thin layer chromatography
HRP	- Horse radish peroxidase
IC_{50}	- Competitor concentration, which inhibits 50% of maximum absorbance in competitive ELISA
IgG	- Immunoglobulin G
IgY	- Immunoglobulin Y
LD_{50}	- Lethal dose which kills 50% of test animals.
mg	- Milligram
min	- Minute
ml	- Milli litre
NBT	- Nitroblue tetrazolium.
ng	- Nanogram
nm	- Nanometer
^oC	- Degree celiseus
OTA	- Ochratoxin A
OTA-BSA	- Ochratoxin A –bovine serum albumin conjugate
OTB	- Ochratoxin B
OTC	- Ochratoxin C
p.s.i	- Pounds per square inch
ppb	- Parts per billion
ppm	- Parts per million
R_f	- Retention factor.
RNA	- Ribonucleic acid
rpm	- Rotation per minute
RT	- Room temperature
Sec	- Seconds
TFA	- Tri fluoro acetic acid
TLC	- Thin layer chromatography
TMB	- 3',3',5',5' -Tetramethylbenzidine.
UV	- Ultraviolet

SYNOPSIS

INTRODUCTION

Processing of food commodities to produce food products, which meet the safety and quality requirements of consumer, has been a challenge. Microbial hazards pose a significant threat to food safety, of which mycotoxin contamination is one of the main concerns of fungal infestation. Aflatoxin B_1, one of the most potent mycotoxin was discovered in England in 1960 as a causative agent of aflatoxicosis in poultry. The discovery of aflatoxin led to screening and identification of more than 100 fungal metabolites, which are toxic to animals (Scudamore, 1994). Ochratoxins are such a group of toxic fungal metabolites, which has drawn much attention in the last few decades due to its implications in the nephropathy conditions in animals and humans in certain regions of the world. It is also studied for other toxic properties such as immunosuppression, teratogenecity and carcinogenicity. Ochratoxin A is classified by IARC as possible human carcinogen. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended monitoring of this toxin and ochratoxigenic fungi in food commodities (WHO, 2002).

The unprecedented growth of human population has necessitated the need for increasing the farm output and minimizing of the preharvest and post harvest losses of food grains. It is estimated that 5 - 10% of the food commodities grown in the world is rendered unfit for human consumption by fungal infestation (Pitt and Hocking, 1985). It is imperative to develop controlling strategies to reduce the pre and post harvest losses and microbial hazard in agricultural produce. Food industries are adopting the HACCP principles for producing quality food products free from hazards associated with food. Monitoring and control of the hazardous microorganism associated

with the food commodities are key factors for ensuring food safety. In this regard the present work is focused on developing methods for the identification of ochratoxigenic fungi and ochratoxins, prediction of the ochratoxigenic fungal behavior in foods, and control of ochratoxin A contamination.

Objectives:

- Evaluation of food commodities for ochratoxigenic fungal contamination and ochratoxin A contamination.
- Identification and evaluation of ochratoxigenic potential of food isolates.
- Immunological and DNA based methods for detection of ochratoxigenic fungi.
- Immunological method for the determination of ochratoxin.
- Microbiological studies on factors affecting ochratoxin A production.
- Control of ochratoxin A contamination.

The review of literature and research findings of the study undertaken to fulfill the objectives summarized here are presented in 8 chapters in the final thesis.

Chapter 1.0
Introduction

Fungal spoilage of food commodities can occur both at the field and during storage. Fungal infestation is one of the important groups of spoilage organism responsible for pre and post harvest losses of agricultural produce. In this chapter consequences of fungal spoilage on loss of agricultural produce and their importance in food safety are emphasized. A list of food borne illness suspected to occur / caused by fungal spoilage is presented. The methods available for analysis of spoilage fungi and toxic metabolites are briefly explained. The chapter gives brief background information on threats and challenges posed by fungal spoilage.

CHAPTER 2.0
Review of literature

The literature survey covering the current research status on ochratoxins and ochratoxigenic fungi is presented under the following major headings.

2.1 History and importance of ochratoxins

2.2 Ochratoxicosis

2.3 Ochratoxigenic fungi and their taxonomy

2.4 Chemistry of ochratoxins

2.5 Natural occurrence of ochratoxigenic fungi

2.6 Natural occurrence of ochratoxins

2.7 Biosynthesis of ochratoxins

2.8 Mycotoxigenic fungal study

2.9 Methods for determination of ochratoxins.

2.10 Factors affecting fungal growth and ochratoxin A production.

2.11 Biological data on ochratoxin A

2.12 Fate of ochratoxin A during food processing

2.13 Degradation of Ochratoxin A

2.14 Prevention of Ochratoxin A Contamination

2.15 Risk assessment, Regulation and Legislation

2.16 Microbiological Safety and Quality

The literature review revealed scanty reports from Indian subcontinent on ochratoxin contamination in food commodities of which only few reports have detailed on ochratoxigenic fungi. No report is available on screening of food mycoflora other than *A.ochraceus* group for evaluation of ochratoxigenic potential from Indian subcontinent. The reports of identification of new species of ochratoxigenic fungi other than *A.ochraceus* group emphasize the need for evaluating food commodities for fungal isolates other than *A.ochraceus* group for ochratoxin elaboration.

The literature review revealed lack of specific microbiological differentiation medium for ochratoxigenic fungi belonging to *A.ochraceus* group. The comparatively slow rate of growth of species belonging to *A.ochraceus* group of fungi also makes it difficult to identify these fungi in conventional general-purpose mycological medium. This indicated ample scope for development of rapid and specific detection method for these fungi for regular monitoring and to study the influence of biotic and abiotic factors on these fungi. The review also revealed ochratoxin A contamination in spices apart from the common major commodities such as maize, coffee and feed commodities. This emphasizes the need for simple, rapid screening and

quantification methods for constant monitoring of various food commodities to ensure food safety. The biocontrol of the *A.ochraceus* group of fungi is limited to use of preservatives, plant extracts and irradiation. The data on degradation/ biotransformation of this toxin is limited to fate of this toxin during food processing environment and degradation by few microbial cultures.

Chapter 3.0

Prevalence of ochratoxigenic fungi and natural occurrence of ochratoxins in foods

Ochratoxins are not only potent nephrotoxin but in recent years are also considered to be a carcinogen, which prompts the food analysts to monitor this toxin in food commodities. The investigation revealed that the distribution of ochratoxin producers is wide spread among the food commodities like cereals, spice, coffee beans and feed materials. This study show that *A.ochraceus* species is the dominant fungus producing ochratoxins along with other members of the *A.ochraceus* group that included *A.sulphureus, A.auricomus, A.melleus and A.ostianus.* The natural occurrence of ochratoxin A was limited to a sample each of poultry feed, groundnut cake and coffee bean among the 73 different food commoties tested representing cereals, oil seed, feed, beverage and spice (4.1%). This is well in agreement with other similar work reported in Indian sub continent, which was in the range of 1 - 6%. However the incidence of *A.ochraceus* fungi was found in 20.5% of food commodities, in which 59.5% of the isolates belonging to *A.ochraceus* group had the potential to produce this toxin ranging from 0.4 ppm to 200 ppm. Distribution of ochratoxigenic fungi with high toxigenic potential in variety of

foods reaffirms the need for constant monitoring of this toxin in food commodities.

Chapter 4.0
Immunological methods for detection of ochratoxigenic fungi

The main hurdle in fungal detection and identification is the need of mycological expertise and need of specific medium that can be used effectively to enumerate and identify the specific toxigenic fungus. The toxigenic fungus varies in their growth requirements such as water activity, temperature, pH etc and hence no single medium can be used for identification of all fungus. Moreover the fungus also differs in growth rate on the common enumeration media such as PDA, Czapak Dox agar, and Malt extract agar and hence the fast growing organisms such as *A.niger* and other fungus dominate and suppress the slow growing fungi. Two immunoassay protocols were optimized for detection of ochratoxigenic fungi, which is presented in chapter 4A and chapter 4B.

Chapter 4A
Microplate immunoassay for monitoring of *A.ochraceus* in foods

Indirect non-competitive microplate immunoassay developed specifically to detect the *A.ochraceus* group of fungi in this study has direct application. Immunological direct detection of fungal biomass is possible and is advantageous over other chemical methods of indirect fungal biomass estimation such as ergosterol, glucose amine etc.

The sensitivity of the method was ≥ 0.2 µg fungal biomass per mg substrate and could be used to detect fungal spore at ≥ 4 $\log_{10}$ cfu/g. The spore enrichment technique was adopted to increase the detection limit for spores in the immunoassay, which enabled detection of spore at ≥ 3 $\log_{10}$ spores/g.

The method was used for monitoring and comparison of *A.ochraceus* growth in different food commodities representing beverage, spice and feed commodities. poultry feed, which is a mixture of various feed ingredient supported highest fungal biomass and toxin production followed by coffee beans and chili in the substrates studied. The method was also applied to estimate fungal biomass in maize under the influence of temperature, moisture and inoculum in the predictive microbiology study of *A.ochraceus* behavior in maize (chapter 7).

Chapter 4B
Dot-binding immunoassay for detection of *A.ochraceus* in foods

The non-competitive dot-binding ELISA developed for the detection of ochratoxigenic fungi has potential to be used as an alternative to microplate immunoassay in field condition. The immunoassay was sensitive to *A.ochraceus* biomass at ≥1 µg/ml in pure culture studies. The immunoassay can be used for detection of *A.ochraceus* infestation at ≥5 µg/mg substrate in chili and poultry feed whereas in coffee beans the detection is possible at ≥10 µg/mg substrate. An indirect competitive dot-binding immunoassay was also optimized, which has a limit of detection at 0.2 µg *A.ochraceus* biomass per ml in pure culture studies.

Chapter 5.0

PCR based detection method for detection of ochratoxigenic fungi

Molecular detection of ochratoxigenic fungi by PCR technique has been optimized. The sequence on the small subunit ribosomal RNA gene of common fungal genera were compared with that of *A.ochraceus* and two primer pairs were designed for specific amplification of 18S rRNA gene in *A.ochraceus*. The designed primer sets OT1 and OT2 targeted 906 bp and 353 bp fragments respectively in the 18S rRNA gene. The PCR was optimized for specific amplification of *A.ochraceus* and related species belonging to *A.ochraceus* group. The method is specific to *A.ochraceus* group of fungi as substantiated in the study by experiments carried out with DNA extracted from common foodborne fungal cultures in pure and mixed culture, as well as directly in food commodities. The minimum quantity of biomass and spore concentration required for successful detection by the PCR was determined. The method was sensitive to *A.ochraceus* spore at ≥ $4\log_{10}$ spores/g and 10 mg biomass. Studies were conducted to increase the sensitivity of the assay for detection of *A.ochraceus* spore by enrichment technique. The *A.ochraceus* spores at concentration of 100 spores/g could be detected by the PCR following the spore enrichment prior to DNA isolation.

Chapter 6.0

Immunological method for determination of ochratoxin A

Immunoassays for detection of ochratoxin A in food have been optimized. Ochratoxin A-BSA conjugate was used as an immunogen to raise antibodies in rabbit and as well as in hens. The sensitivity of antibody (IC_{50}) for OTA was 15ng/ml and 40ng/ml for antibodies elicited in rabbit and egg yolk respectively in the indirect competitive immunoassay. Protocols for ochratoxin estimations by ELISA were optimized using poultry feed as food substrate. Recovery studies on ochratoxin A (OTA) spiked poultry feed had a recovery at 66 - 125% of added OTA with a detection limit of 5ppb in the ELISA. Market samples of poultry feed samples were analyzed by ELISA and HPLC. ELISA estimations compared well with the HPLC method in all the samples analyzed without any false positive or negative results. The results with hen egg yolk antibody indicate the potential of using hen egg as a source for large-scale production of antibodies against mycotoxins.

Chapter 7.0

A predictive microbiological study – Interrelationships of temperature, moisture, and inoculum level on behavior of toxigenic *A. ochraceus*

Influence of moisture, temperature, and inoculum on *A.ochraceus* growth and ochratoxin A elaboration in maize was studied using three factorial Central Rotatory Composite Design. Multiple linear regression analysis of fungal responses under the influence of different combination of statistically designed variables was carried out and polynomial regression equation was

derived to predict the fungal responses at different variables. The study revealed that the fungal biomass correlates more positively with ochratoxin elaboration than with viable count of fungi. The coefficient of correlation between biomass-viable count, biomass-ochratoxin A elaboration and viable count-ochratoxin A elaboration were 0.79, 0.79 and 0.57 respectively. The bias factor of 1.0 and 0.97 for predictions for fungal growth and ochratoxin A elaboration study respectively indicate that the predictions obtained within the experimental domain are dependable. The predictive model when used with the immunological method for determination of fungal biomass has potential for application in food processing industries to predict the behavior of ochratoxigenic fungi in specific food environment.

Chapter 8.0
Control of ochratoxigenic fungi and degradation of ochratoxins by microbiological method

The work relating to screening of organism, which are antagonistic towards ochratoxigenic fungi and organism that can degrade ochratoxin has been presented in chapter 8A and chapter 8B respectively.

Chapter 8A
Identification, Isolation and application of antifungal metabolite

Bacillus species, identified as *Bacillus badius,* which was antagonistic against fungi belonging to *A.ochraceus* group and few other common fungi, was isolated. The organism produced an extracellular metabolite, which had a

molecular weight between 2000–10000 Daltons, was found to be heat stable to temperature up to 80^{o}C and was also found to be active in a wide pH range of 2.5 - 10. The metabolite was resistant to the action of enzymes viz., trypsin, and lysozyme. The metabolite exhibited inhibitory activity against *A.ochraceus* growth in broth culture as well in solid food substrate. The metabolite inhibited 85% of *A.ochraceus* biomass and 100% inhibition of ochratoxin A elaboration in maize compared to a control experiment without the metabolite.

Chapter 8B

Degradation of ochratoxin A by *Pseudomonas* species

About 10 *Pseudomonas* species were screened to evaluate their potential for degradation of ochratoxin A. Cell free extracts from two of the *Pseudomonas fluroscens* strains screened exhibited potential to degrade ochratoxin A. The temperature and time for degradation of ochratoxin A was optimized and degradation of ochratoxin A at different concentration under optimum conditions was evaluated. The cell free extract exhibited potential to degrade 80 -100% of added ochratoxin A at tested concentrations ranging from 0.2 to 500 µg. A bioassay with *Bacillus subtilis,* which was sensitive to ochratoxin A was used as an indicator organism to study the toxicity of the degraded product of ochratoxin A. The microbial assay revealed that the OTA degraded byproducts is not toxic.

CHAPTER 1.0
INTRODUCTION

INTRODUCTION

Agriculture is the major source of livelihood in most of the countries of the world. In India agriculture provides employment to 57% of population and contributes to about 25% of national economy. The exports from agricultural commodities contribute to about 12% of total national exports (Directorate of economic statistics, 2004). Although the country is self sufficient in its food grain requirement, there is a need for increasing food and fodder production at least by 5 - 10 million tons per year to overcome the demands of burgeoning population, which is increasing at a rate of 2.1% per annum. The productivity of crop tremendously increased during green revolution in the late sixties, which involved four major inputs viz., the use of chemical fertilizers and pesticides, irrigation of the agriculture land, the introduction of high-yielding hybrid seed strains, and the mechanization of agriculture, wherein at least four folds increase in crop yield was achieved. The increase in food production was not sustainable by conventional approaches due to inherent limitation such as small land holdings, pest resistance to synthetic pesticides and natural calamities. Emphasis has been laid on improving agricultural practices, application of biopesticides and biofertilizers in order to increase the crop productivity. It is estimated that 10 - 15% of food grains produced is lost during post harvest process, which may increase upto 50% in certain developing countries (http://www.fao.org/docrep/t0522e/t0522e00.html). It is apparent that with the stagnant crop productivity, prevention of preharvest and post harvest losses of agricultural produce is very important for overcoming the growing food demand.

Fungi contribute significantly to the storage losses either alone or together with insects. It is estimated that 5 - 10% of food grains produced is lost due to fungal spoilage (Pitt and Hocking, 1985). Food spoilage by fungi is cause of concern as they may bring about changes in food such as unpleasant appearance, odour, discolouration, caking, reduction or loss of germination, dry matter loss, chemical and nutritional changes, reduction in processing quality and they may cause allergy and infection. Physical attributes of mold spoilage such as visible sign of mold infection and discoloration are important grading factors of food commodity and hence determine the price of commodity (Sauer, 1988).

Mycotoxin elaboration is an important concern of mold infestation due to its implications in a range of human and animal diseases. The disease caused by consumption of moldy food dates back to the ergotism reported way back in 18^{th} century. Worldwide attention to the mold infestation and mycotoxins was drawn after the outbreak of Turkey-X disease in England in 1960, which led to the death of one lakh poults due to the consumption of moldy Brazilian peanuts (Bullerman, 1979). The causative agent was identified as *A.flavus* and the metabolite produced by the fungus was identified as aflatoxin. Aflatoxin is considered as one of the most potent natural carcinogen discovered till now. The discovery of aflatoxins led to intensive work on other toxic metabolites produced by molds. Henceforth various new human and animal toxicoses have been established that are caused by consumption of mycotoxin contaminated foods. It is estimated that at least 300 – 400 fungal metabolites are potentially toxic to humans and animals (Cole and Cox, 1981). Mycotoxins are diverse in their structure and

biological activity. Beardall and Miller (1994) reviewed various diseases and toxicoses caused or considered probably due to the mold activity in foods. A relevant reference on major outbreak of mycotoxicoses at the world level is presented in Table.1.

Fungi in food commodities have been ecologically classified into two main categories based on their moisture requirements for growth as field fungi and storage fungi (Christensen, 1991). The field fungi require moisture in equilibrium with relative humidity of 95 - 100%, equivalent to 20 - 25% moisture for their growth. They characteristically invade seeds before harvest and colonize the ripening grain. They cease to grow once the seeds are harvested and seldom develop in stored food. Major field fungi in foods include *Alternaria*, *Cladosporium*, *Helminthosporium* and *Fusarium* species (Bullerman, 1979). The storage fungi on the other hand require moisture in equilibrium with relative humidity of 65 - 90%, equivalent to moisture levels of 13 - 18% for their growth. The storage fungi, with few exceptions like *A.flavus* do not invade seeds before harvest and some of them will not grow in grains whose moisture contents are in equilibrium with relative humidity of 85 - 90%. Major storage fungi in food grains include *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor* and *Wallemia*.

The mycoflora in foods varies depending on the type of food, environmental factors and climatic conditions. In temperate climate *Penicillium* species dominate and constitute important mycoflora responsible for mycotoxin contamination (Pitt, 1987). *Aspergillus* species are known to dominate the mycoflora in tropical climate (Krogh, 1987). More than 150 species of *Aspergillus* are known to be associated with food grains of which

45 species are mycotoxin producers and many *Aspergilli* are capable of growing in foods with low water activity (Li, *et al*., 2000).

The foodborne illness and toxic outbreaks among human and animal population has increased concern regarding health hazards caused by microbial contaminants in foods. With increased safety concerns and quality consciousness among consumers various regulations have been enforced to prevent human exposure to these contaminants. According to available report the regulation for mycotoxins exists in about 98 countries with varying levels of maximum permissible limits depending upon the commodity, consumption pattern of the commodity and safety concern of the countries (Van Egmond, 2003). The number of countries having regulation on mycotoxin has increased by about 30% than that existed in 1995. Different level of stringent regulation in the developed countries is detrimental to international trade affecting economy especially of developing and less developed countries. The need for coordinated efforts and assistance in developing countries for effective control of microbial hazards is on the rise.

In this regard there is need for rapid, accurate and effective methods for monitoring of pathogens and their toxic metabolites. Early detection of the pathogens and effective monitoring of its incidence is the key factor for preventing the microbial hazard. Conventional methods such as plating gives little or insufficient information regarding the microbial activity and quality of the food commodity. Rapid microbiological methods that can give accurate result in shortest period of time are required for effective control of microbial hazard. Modern methods of mycological examination give food analyst and

food technologist tools for accurate determination of general mold flora and selective detection of specific fungi.

Mycological methods that can be used for monitoring fungi include the use of selective and differential media, biochemical methods, electrical impedance, immunological method and molecular biological methods (Gourama and Bullerman, 1995a). Immunological methods are currently employed by which selective identification of microbes of interest can be performed in short duration of time. The method is also advantageous as it can be automated for large-scale routine screening of samples. The immunological methods have already revolutionized the detection of clinically important microorganisms. Kits based on various immunoassay formats such as latex agglutination test, microplate ELISA, dipstick immunoassay, immunocapture etc are available in the market for detection of foodborne pathogens and their metabolites. In other words, it can be applied for identification and quantification of both the fungi and their toxic metabolites.

Recently molecular biological approaches are gaining prominence for monitoring of mycotoxigenic fungi in foods. The rapid improvement made in sophistication and automation of instruments used in molecular biology has increased the rate of sequencing of genes and genomes of microorganism. The free availability of sequence data of various microorganisms and availability of various bioinformatics tools for analyzing sequence data has given impetus to the development of new methods for selective detection of microorganism. The molecular methods have enabled to develop sensitive methods that can distinguish between microorganisms differing even in single nucleotide base. The phylogenetic studies of various fungi by these

techniques have made taxonomist to reexamine the taxonomical placement of various fungal genera and species. Advancements made in molecular approaches and techniques have also given new tools for improving the quality traits of crop plants such as disease resistance, drought resistance etc

Importance to safety concerns regarding the use of pesticides, herbicides and chemical agents in agriculture has led to the impetus for other methods of pest control such as bio control of weeds, pests, and pathogens. In this regard microbial antagonism has gained prominence as hazard posed by one microorganism can be controlled by another organism or by its metabolites without the use of harmful chemicals. Microorganism can play an important role yet in another area, the biotransformation of toxic metabolites into byproducts that are safe for human or animal consumption, thereby ensuring effective use and disposal of contaminated food commodities.

In the present scenario advances in the field of microbiological sciences has gained prominence in developing identification, monitoring and controlling strategies for microbial hazards to ensure food safety. Mold and mycotoxin contamination is one such important concern wherein development of rapid, and sensitive methods for detection of toxigenic fungi and their metabolites is needed for monitoring and controlling mycotoxin contamination. Development of monitoring and preventive strategies for mold infestation in foods is needed to check the mycotoxin hazard. The food technologists need to adopt such systems like HACCP (Hazard analysis and critical control point) to ensure safety and quality of the food products.

PREAMBLE

Mycotoxins are important health hazard associated with the food commodities. Understanding the behavior of the fungus in field and storage environments is very important for monitoring and controlling the mold infestation. The modern methods of analysis of microbes are becoming more and more specific, rapid and may be automated for routine and large-scale sample analysis. Some of the methods also find application in detection of unculturable microorganism. Of late, ochratoxin A has emerged as a toxin of concern amongst the lesser-known mycotoxin. The Joint FAO/WHO expert committee on food additives made the following recommendations regarding ochratoxin A (WHO, 2002):

- Studies should be conducted to clarify the mechanisms by which ochratoxin A induces nephrotoxicity and carcinogenicity.
- Appropriate sampling procedures should be developed for food commodities likely to be contaminated with ochratoxin A.
- Better surveys are needed, particularly in regions of the world other than Europe, in order to assess intake of ochratoxin A in these regions.
- Epidemiological investigations should be encouraged to explore the role of ochratoxin A in chronic renal disease.
- Studies should be conducted to improve understanding of the occurrence and ecology of the fungi that produce ochratoxin A, especially in fresh produce.

OBJECTIVES OF THE STUDY

In India reports to assess the status of ochratoxin A contamination in food commodities is limited and inadequate. However, there are few reports of ochratoxigenic *Aspergilli* that have been isolated from general airspora, food processing environments and food commodities. In some cases toxigenic potential of these isolates have been evaluated. At international level many countries have opted for regulation of ochratoxin A in food commodities. However, in India with least support from literature, regulation of this mycotoxin is not feasible in near future. However, in today's competitive global and domestic scenario the food industries are compelled to adopt HACCP systems to provide quality products, free from microbiological hazard. Monitoring of food commodities for presence of microbial hazards is primary step in ensuring food safety. In this context molecular methods like Immunological and PCR have gained precedence over the conventional methods, which are time consuming, labour intensive and costly. In this background the present investigation was carried out with the following objectives.

- Evaluation of food commodities for ochratoxigenic fungal contamination and ochratoxin A contamination.
- Identification and evaluation of ochratoxigenic potential of food isolates.
- Immunological and DNA based method for detection of ochratoxigenic fungi.
- Immunological method for determination of ochratoxin A
- Microbiological studies on factors affecting ochratoxin A production.
- Control of ochratoxin A contamination.

Table. 1 Fungal infestation leading to toxicity outbreaks amongst human/animal populations.

Disease/ Illness/ toxic effects	Fungi involved/ suspected	Toxin/s responsible	Regions	Symptoms and organs affected	Food/s involved
1. Fusarium toxicosis[1]	*Fusarium species*	Deoxynivelenol	China	Nausea, abdominal pain, diarrhea, dizziness	Wheat
2. DON toxicosis in India[3]	*Fusarium* species	DON	Kashmir, India 1987	Abdominal pain, diarrhea, vomiting blood in stool	Wheat
3. Red mold disease in Japan[1]	*Fusarium species*	Trichothecene Deoxynivelenol	Japan and Korea	Nausea, vomiting, diarrhea	Moldy wheat and maize.
4. Esophageal cancer[1]	*Fusarium species*	Fumonisins	Southern Africa, north eastern Iran and china	Esophageal cancer	Moldy cereals, and millets
5. Kashmir Beck Disease[1]	*Fusarium species*	T-2 toxin	Russia, china, Korea Sweden Holland	Symmetric stiffness and swelling in finger and wrist joints	Moldy wheat and maize

Disease/ Illness/ toxic effects	Fungi involved/ suspected	Toxin/s responsible	Regions	Symptoms and organs affected	Food/s involved
6. Onyalai[1]	*Alternaria* and *Phoma species*	Unknown	Southern Sahara, European countries	Hemorrhagic bullae in oral cavity and skin, haematuria, bleeding from mouth	Mold millet and sorghum
7. Eendemic Familiar Arthritis of malnad[1]	Not identified	Unknown	Karnataka, India	Osteoarthritis Bilateral symmetrical lesion on hip	
8. Sago Hemolysis[1]	Not Identified	Unknown	Papua New Guinea`	Severe anemia, sudden onset of jaundice	Stale and moldy sago
9. Frontoethmoidal Encephalomeningocele[1]	Not identified	Unknown	Myanmar, England, United states, German,	Teratogen, Tumor protrusion at the base of nose and or eyes	Moldy grain
10. Arthrinium Sugarcane Poisoning[1]	Not identified	Unknown	South Africa, Morocco, India	Torsion, spasms and may lead to permanent disability	Sugarcane contaminated with Arthrinium
11. Ergotism[1]	*Claviceps Purpurea*	Ergotamine Ergot alkaloids	Russia, India, Central Europe	Gangrene and convulsions	Millets, Maize

Disease/ illness/ toxic effects	Fungi involved/ suspected	Toxin/s responsible	Regions	Symptoms and organs affected	Food/s involved
12. Mseleni joint Disease[1]	*Fusarium species*	Unknown	Southern Africa	Osteoarthritis, Disorder in hip, knee and ankle joints	Maize and groundnut
13. Alimentary Toxic Aleukia[1]	*Alternaria Fusarium species*	T-2 toxin	U.S.S.R	Leukopenia, agranulcytosis Hemorrhage	
14. Aflatoxicosis[1]	*Aspergillus flavus*	Aflatoxin	Gujarat India, 1974	Swollen abdomen, Liver cancer, cirrhosis	Moldy maize
15. Balkan Endemic Nephropathy[2]	*Penicillium verrucossum Aspergillus ochraceus*	Ochratoxin A	Balkan states (Romania, Bulgaria, Yugoslavia), Egypt, Tunisia	Renal impairment, protenuria, creatininuria, anemia,	Cereals
16. Fusarium Toxicosis [1]	*Fusarium species*	Deoxynivelenol	China	Nausea, abdominal pain, diarrhea, dizziness	Wheat
17. Red mold disease in Japan [3]	*Fusarium species*	Trichothecene Deoxynivelenol	Japan and Korea	Nausea, vomiting, diarrhea	Moldy wheat and maize.

1 Beardall & Miller, 1994
2 Krogh, 1972
3 Bhat, *et al.*, 1989.

Chapter 2.0

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

2.0 Ochratoxins

2.1 History and importance

The outbreak of aflatoxicosis and subsequent isolation of aflatoxins in the year 1960 led to screening of other fungi for their toxic metabolites. Ochratoxin was isolated and identified as toxic metabolite in one such screening study conducted by Van der Merwe and co-workers in the year 1965 (Van der Merwe, *et al*., 1965). Since its discovery as nephrotoxic agent ochratoxin A has been shown to have various other toxic effects such as immunosuppression, teratogenic effects and carcinogenicity (Mantle, 2002) and has been categorized into a possible human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Ochratoxin contamination has been considered as serious health hazard owing mainly to its nephrotoxic effect and its implication as an etiological agent, particularly affecting renal function, which is endemic in certain, Balkan states (Krogh, 1992).

Ochratoxin contamination has also received attention due to the fact that more than one group of fungi has the ability to produce this toxin in agricultural commodities in both temperate and tropical climates. *Penicillium* species is considered the principal organism responsible for ochratoxin contamination in temperate environment whereas the *Aspergillus* species is considered as the important organism responsible for ochratoxin contamination in tropical countries. Moreover, in the recent years new species belonging to *Aspergillus* group, especially *A.niger* has been reported as ochratoxin A producers. It is of great concern that *A.niger,* which find various industrial applications and the products of which are labeled as generally

recognized as safe (GRAS) by the Food and Drug administration may produce ochratoxin A.

Ochratoxin A (OTA) is the principal toxin of *A.ochraceus* group and is one of the actively studied mycotoxin. OTA has been evaluated by various regional and international agencies for its natural occurrence, distribution, toxicity, regulation and for control measures. Many countries have adapted legislation to limit this toxin in foods in the last decade. Study on the various aspects of this toxin contamination has been emphasized by various international agencies.

2.2 Ochratoxicosis

The diseases or ill effects caused by consumption of toxin-contaminated foods are referred as mycotoxicoses. The ill effect caused by consumption of ochratoxin A is referred as ochratoxicosis. Most of the data available regarding ochratoxin toxicity is through laboratory experimentations involving various animals. However, two naturally occurring diseases observed in humans and pigs, endemic to certain regions of Europe, have been associated with this toxin (Krogh, 1992).

2.2.1 Balkan Endemic Nephropathy (BEN)

Balkan Endemic Nephropathy is a fatal renal disorder in humans, discovered in 1950's (Austwick, 1975). This was considered to be endemic to certain regions of Balkan such as Romania, Croatia, Bosnia and Herzegovina, Yugoslavia and Bulgaria. Later similar disorders have been reported in Tunisia, Algeria and Egypt (Maaroufi, *et al*., 1996; Waffa, *et al*., 1998). The

disease progress slowly with atypical symptoms and manifests by renal impairment. The disease manifestations are preceded by repeated findings of anemia, protenuria and creatinanaemia. The prevalence of the disease was more in females than in males and onset corresponds with age 30 – 50 years but patients of 10 – 19 years were also reported (Stoyanov, *et al*., 1978).

Ochratoxin has been implicated as one of the possible determinants of the BEN based on the incidence of ochratoxin A in food stuffs and serum OTA level which is relatively high in endemic areas than that of non endemic regions of BEN (Krogh, 1987). The symptoms of BEN and mycotoxic porcine nephropathy (MPN), a disease observed in pigs, endemic in regions endemic to BEN, was similar to ochratoxin A induced nephropathy observed in experimental animals such as impairment of proximal tubular function, atrophy of tubules, hyalinization of glomeruli, interstitial fibrosis of adrenal cortex and sex specific distribution of disease. The epidemiological outbreaks of the BEN and MPN are also correlated with unusual climatic changes favouring mold infestation during 1961-1971 in Denmark and Croatia (Krogh, 1976).

2.2.2 Mycotoxic Porcine Nephropathy (MPN)

Ochratoxin A consumption at a level of 200 ppb has been demonstrated to induce nephrotoxic symptoms in all animal models tested (Krogh, 1974). Porcine nephropathy has been reported in Denmark since 1928. The disease was found to be endemic in Denmark but was unevenly distributed with prevalence rates varying from 0.6 to 65.9 cases per 10,000 pigs in 1971. Epidemic outbreak of porcine nephropathy has been reported in 1963 and 1971. The disease is more frequent in females than in male pigs. A high

incidence of ochratoxin contamination (58%) has been reported in cereals grown in area, affected by this disease (Krogh, 1978).

2.3 Ochratoxigenic fungi and their taxonomy

Ochratoxin A was originally discovered as a metabolite of *A. ochraceus* var Wilhelm in the laboratory experiments conducted by Van Der Merwe and co-workers (Van der Merwe, *et al*., 1965). The discovery was followed by report of potential of ochratoxin A elaboration by few more isolates belonging to *A.ochraceus* group. These species are *A.sulphureus*, *A.sclerotiorum*, *A.melleus*, *A.alliaceus*, *A.alliaceus, A.ostianus, A.auricomus and A.albertensis* (Hesselentine *et al.*, 1972; Ciegler, 1972; Varga *et al.,* 1996). Subsequently, ochratoxin A was shown to be produced by species identified as *P.viridicatum* Westling (Van Walbeek *et al*, 1969). The ochratoxin A producing *Penicillium* species which was identified as *P.viridicatum* was questioned and later it has been shown that only *P verrucossum* could produce ochratoxin A (Pitt, 1987). *P.verrucossum* has been now classified as *P.verrucossum* Diertz and is considered the principal ochratoxin A producer in temperate climates. It has been divided into two chemotypes based on the mycotoxins they produce (Frisvad and Filtenborg, 1989). Species that produces ochratoxin A, verrucolon and citrinin are grouped under Chemotype I and species that produces ochratoxin A along with verrucolon only are grouped as chemotype II.

A.ochraceus was referred by some workers earlier as *A.alutaceus* var *alutaceus* (Kozakiewicz, 1989). However, later on the original name has been

restored for the species and it has been placed under genus *Aspergillus* sub genus *Circumdati* section *Circumdati* (Gams, *et al.,* 1985).

Table.2 Fungus reported as ochratoxin A producers

Group	Section	Species	Reference
Aspergillus	*Circumdati*	*A.ochraceus* *A.melleus* *A.sclerotiorum* *A.auricomus* *A.ostianus* *A.petrakii* *A.sulphureus* *A albertensis* *A.alliaceus*	Hesselentine *et al.* 1972. Ciegler, 1972. Varga *et al.* 1996.
	Aspergillus	*Eurotium herbariorum*	Chelkowski *et al*. 1987.
	Fumigati	*A.fumigatus*	Abarca *et al*. 1997. Szebiotko *et al*. 1981.
	Nigri	*A.niger* *A.carbonarius*	Abarca *et al.* 1994. Heenan *et al* 1998. Ueno *et al*. 1991.
	Terrei	*A.terreus*	Ueno *et al*. 1991.
	Wentii	*A.wentii*	Varga *et al*. 1996.
	Versicolores	*A.versicolor* *A.sydowii*	Abarca *et al.* 1997. Ueno *et al.* 1991.
	Usti	*A.usti*	Ueno *et al.* 1991.
Penicillium	*Penicillium*	*P. verrucossum*	Pitt 1987.

The sexual stage of *A.ochraceus* is known as *Petromyces alliaceus*. *A.ochraceus* is considered the most frequent fungus that is responsible for ochratoxin A contamination in tropical countries especially in cereals and coffee beans. Abarca *et al.* (2001) reviewed the reports on ochratoxin producing *Aspergilli* and had summarized that OTA is produced by other

species of *Aspergillus* belonging to different infrageneric group such as those belonging to section *Aspergillus, Nigri, Versicolores, Usti, Wentii, Terreus, and Fumigati.* The various ochratoxin A producing species reported are summarized in Table. 2.

However in the genus *Aspergillus* apart from species belonging to section Circumdati, only species belonging to section Nigri are considered to produce ochratoxin A in foods under natural conditions (WHO, 2002). However, only small percentage of *A.niger* isolates is shown to produce ochratoxin A. In recent years *A.carbonarius* has been reported to produce ochratoxin A. *A.carbonarius* is considered the major source of ochratoxin A in grapes and grape products. *A.ochraceus* is an important ochratoxin A producing fungi occurring in wide variety of foods, among the genus *Aspergillus*.

2.4 Chemistry of ochratoxins

2.4.1 Ochratoxic metabolites

Ochratoxins are classified as pentaketide group of mycotoxins and have isocoumarin moiety linked to phenylalanine through amide linkage (Fig.1). The ochratoxins exists in β-conformation, where the amide (NH) is hydrogen bonded to the phonetic oxygen. The other toxic metabolites identified include the ochratoxin acids and ochratoxin esters. The ochratoxin acids include ochratoxin A (OTA) and ochratoxin B (OTB), which is a dechloro analogue of ochratoxin A. The ochratoxin esters include the ethyl ester of ochratoxin A which is known as ochratoxin C and methyl and ethyl esters of ochratoxin A &

B. The functional groups and molecular weights of different ochratoxins are shown in Table 3.

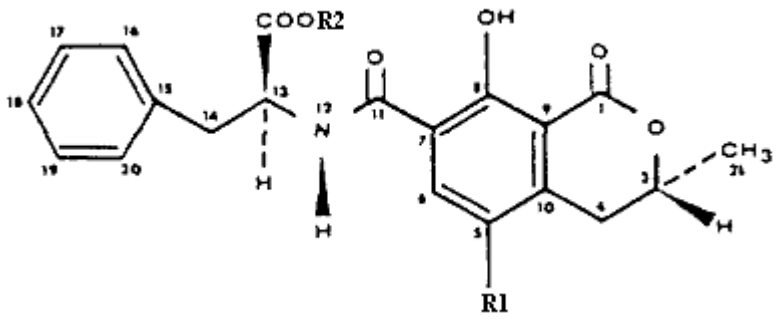

Fig.1 General structure of ochratoxins

Table. 3 Types of ochratoxins and their functional groups

Ochratoxin	R1	R2	MW
Ochratoxin A	Cl	H	403
Ochratoxin B	H	H	369
Ochratoxin C	Cl	C_2H_5	431
Methyl ester of OTA	Cl	CH_3	417
Methyl ester of OTB	H	CH_3	383
Ethyl ester of OTB	H	C_2H_5	397

2.4.2 Ochratoxin A

Ochratoxin A (OTA) is the important toxic metabolite elaborated by ochratoxin producing species of fungi and other toxic metabolites are produced in minor quantities. The chemical nomenclature of ochratoxin A is 3-methyl-5-chloro-8-hydroxyl-3, 4-dihydro-3-methyl isocoumarin-7-L-β-Phenylalanine. Ochratoxin A is a colorless crystalline compound with a molecular weight of 403 Daltons having a melting point of 90^{o}C and 169^{o}C when crystallized in benzene and in xylene respectively. OTA is slightly soluble in water and dissolves well in polar organic solvents and dilute sodium bicarbonate solution. The UV absorption spectrum in methanol has absorption maxima at 213 nm (ε 36800) and 332 nm (ε 6600) and has fluorescence emission maxima at 467nm in 96% ethanol and 428nm in absolute ethanol. The infrared spectrum of ochratoxin A in chloroform has been reported to have peaks at 3380, 2988, 1723, 1674, 1612, 1528, 1425, 1381, 1304, 1260, 1170, 1140, 1107, and 827 cm^{-1}. Ochratoxin A is stable in organic solvent and can be stored for over one year under refrigerated condition.

2.5 Natural occurrence of ochratoxigenic fungi

A.ochraceus and other ochratoxin A producers have been reported from various food commodities. *A.ochraceus* is widely distributed in dried foods like various kinds of beans, dried fruits, nuts including peanuts, pecans, betel nuts, wide range of cereals including barley, wheat flour and rice (Pitt and Hocking, 1985). *A.niger* and *A.carbonarius* are considered important ochratoxin A producers in coffee and grapes (WHO, 2002). Species belonging to *A.ochraceus* group is the important OTA producers in cereals and other

food grains in tropical environment. *A.ochraceus* has been isolated from various stored grains, air and working environments (Rao, 1975; Jayaprakash, 1983). Occurrence of ochratoxin A in wide variety of foods such as cereals, wine, fruit juices, vine fruits, spices such as coriander, ginger, turmeric, chili and coffee beans indicates occurrence of ochratoxigenic fungal infection in wide variety of foods all over the world.

2.6 Natural occurrence of ochratoxins

Occurrence of ochratoxin A has been reported all over the world, mainly in stored cereals, coffee beans, vine fruits, pork and meat products, etc. Numerous reports on ochratoxin A contamination in coffee have been reported in many countries. Natural occurrence of ochratoxin A available from the published literature has been summarized in the report of Joint FAO/WHO Expert Committee on Food Additives (WHO, 2002). In the report data obtained from 23,167 samples has been summarized, comprising 85% samples from Europe (Croatia, Denmark, Finland, France, Germany, Italy, Netherlands, Norway, Spain, Sweden, Switzerland, and the United Kingdom), 7% from South America (Brazil and Uruguay), 6% from North America (Canada and the USA), 1% from Africa (Sierra Leone and Tunisia), and 1% from Asia (Dubai and Japan). According to which the ochratoxin A contamination is known to occur in wide variety of foods which include cereal and cereal products, green and roasted coffee, dried fruits, wine, grape juice, cocoa and chocolate, herbs and spices, canned foods, oils, olive, pulses, chickpeas, lentils, soya products, sweets, milk and milk products, meat, kidney, liver, beer, tea, vinegar, mustard, baby foods and house dust.

According to the data Ochratoxin A concentration in different commodities were highly variable and 1.4% of the total samples had ochratoxin A contamination in excess of 5 ppb, 0.3% of cereals, 0.05% of cereal products had toxin in excess of 20 ppb and in 1.2% of cereals and 0.3% cereal products had contamination exceeding 5 ppb level.

Table. 4 Natural occurrence of ochratoxin A (in India)

Sample	No. of samples contaminated	OTA (ppb)	% Contaminated	Reference
Maize	3	10-20	1.5	1
Corn Wheat Sorghum Groundnut Ragi Pulses	3 2 3 2 1 0	30 - 50 30 - 50 50 - 70 50 -2000 70 0	6	2
Chilies	26	10 -120	26	3
Black pepper Coriander Ginger Turmeric	14 20 2 9	15 - 69 10 - 51 23 - 80 11 - 102	36	4

[1] Janardhan *et al.* 1999.

[2] Rao *et al.* 1979.

[3] Thirumala-Devi *et al.* 2000.

[4] Thirumala-Devi *et al.* 2001.

Patel *et al.* (1996) reported occurrence of ochratoxin A in wide variety of ethnic foods, wheat noodles, bread, chapatti, sesame oil, chili sauce, pickles and herbs and spices ranging from 0.2 to 50 ppb. An unexpectedly

high mean concentration of ochratoxin A at ≤ 33 000 µg/kg has been reported in foods (wheat, barley, mixed cereals, dried vegetables, and olives) collected in Tunisia (Maaroufi, *et al.*, 1995).

In India limited number of reports is available on natural occurrence of ochratoxin A in food commodities. In recent years, the occurrence of ochratoxin A in different spice commodities such as chilies, ginger, black pepper, ginger and turmeric has been reported. The reports on the natural incidence of ochratoxin A in variety of food commodities from Indian sub-continent are summarized in Table.4. The data on natural occurrence of ochratoxin A is limited to few studies. However the available literature shows that the ochratoxin A contamination level in foods from Indian subcontinent is well above the regulatory limit of 5 ppb set by European Union. There is need for coordinated efforts from various agencies to assess the risk associated with high level of ochratoxin A contamination in foods from India.

2.7. Biosynthesis of ochratoxins

2.7.1 Biosynthesis of ochratoxin A

One of the earliest reports on biosynthetic pathway for ochratoxin was the work of Steyn *et al.* (1970). They proposed that the carbon skeleton of isocoumarin moiety of ochratoxin A comes from the condensation of five or one acetate unit with four malonate units. They proposed that the phenylalanine moiety is synthesized via shikimic acid pathway and it is linked to isocoumarin moiety. Similar hypothesis was also proposed by Yamazaki *et al.* (1971). The proposed biosynthetic pathway of ochratoxins is shown in

Fig.2. They also proposed that the carboxyl group of the isocoumarin moiety at C-4 is from C-1 carbon molecules such as methionine.

The chlorination of the isocoumarin moiety has been shown by Ferreira and Pitout (1969) and Wei *et al.* (1971) to occur as a penultimate step in ochratoxin A biosynthesis by an enzyme requiring ATP and Mg^{+2}. The proposed pathway of condensation of acetate units and malonate units is now also supported by the work of O'Callaghan *et al.* (2003). They reported cloning and sequencing of polyketide synthase gene (PKS) involved in ochratoxin A biosynthesis, which is expressed only in ochratoxin A permissive

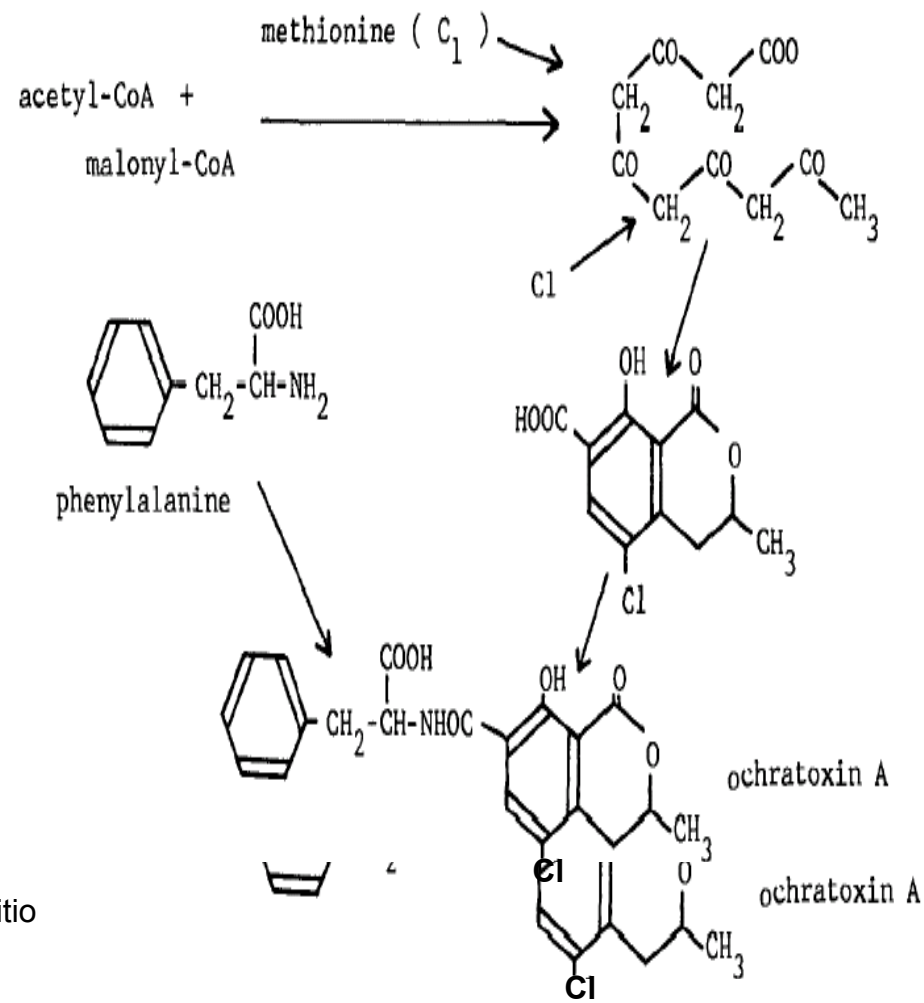

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Fig.2. Schematic diagram of biosynthetic pathway of ochratoxins

2.7.2 Biosynthesis of ochratoxin B

Ochratoxin A is considered as the principal metabolite of ochratoxigenic fungi but other analogues such as ochratoxin B (OTB) is produced in smaller quantities. Harris and Mantle (2001) proposed that the biosynthesis of ochratoxin B essentially occurs via OTβ $\rightarrow$ OTα $\rightarrow$ OTA with a branch for ochratoxins β $\rightarrow$ OTB. They also proposed that the ochratoxin A is converted to ochratoxin B by dechlorination.

2.8 Mycotoxigenic fungal study

Molds are common microbial contaminants in food and agricultural commodities. Occurrence of toxin producing molds in food and feed commodities is cause for concern and hence suitable methods for monitoring is necessary to control and implement regulating strategies. Various methods are in place for detecting the mold infestation in food. Conventional methods involve the plating of sample on culture medium, enumeration and identification based on morphology and microscopy. The plating methods give an overall mold count, which only represents the degree of sporulation rather than actual fungal biomass and are subject to high degree of variations (Jarvis, *et al.,* 1983). Despite these drawbacks, most of the laboratories still use the cultural methods for assessing the fungal growth and their quantification in foods. Common microbiological methods followed routinely

includes direct examination of foods, general plating methods and use of specific diagnostic medium.

2.8.1 General-purpose enumeration media

Food samples vary in their type, composition, and mycoflora hence, no single medium is suitable for all types of foods. The foods is classified into foods with high water activities, foods with low soluble solids, high sugar foods such as confectionary, dried foods and salted foods (Pitt and Hocking, 1994). Hocking and Pitt (1980) developed DG18 (Dichloran 18% glycerol agar) media for enumeration of xerophilic fungi from intermediate and low moisture foods such as stored grains, nuts, flour and spices. DG18 is the recommended media for general examination of mycoflora of dried foods, which is most susceptible for spoilage of storage fungi, which are known to elaborate mycotoxins. DG18 has been recommended for satisfactory enumeration of *A.ochraceus* in food grains (ICMSF, 1996).

2.8.2 Selective/differential medium

The level and type of mold contamination determines the quality attributes of food commodities. The type of mold contaminant is more important since food contaminated with mycotoxigenic fungus even at low level may be more hazardous than food with higher infestation with other common mycoflora. Hence various specific morphological and biochemical characteristics of fungus are used for formulating media for specific fungus.

2.8.3 Selective medium for ochratoxigenic fungi

Frisvad (1983) developed Pentachloronitrobenzene rose bengal yeast extract sucrose (PRYES) agar medium for differentiation of ochratoxin A and citrinin producing *P.viridicatum* (now known as *P.verrucossum)* from *P.aurantogriseum* which produces closely related toxins viomellein and xanthomegnin. *P.verrucossum* produces a violet brown reverse colony whereas the closely related species *P.aurantogriseum* produces a yellow reverse coloration.

2.8.4 Media for Ochratoxin production

Davis *et al.* (1969) developed medium with 2% yeast extract and 4% sucrose for ochratoxin A production. Lai *et al.* (1970) studied effect of different nitrogen sources on ochratoxin A production and found glutamic acid and sucrose as the best nitrogen and carbon source respectively for ochratoxin A production. The same workers also reported that some trace elements like zinc, copper, boron, manganese and molybdenum stimulate OTA production in some members of *A.ochraceus.* Later Ciegler (1972) suggested that 2% yeast extract and 15% sucrose medium for OTA production and screening media for ochratoxin A producers. Nefisa *et al.* (1990) reported carbon sources other than sucrose such as galactose, mannitol and lactose are poor carbon source for ochratoxin A production. They also reported that 15% sucrose and 2% yeast extract as the optimal concentration for ochratoxin A production.

2.8.5 Biochemical / chemical methods

Various biochemical methods are available for identification and estimation of filamentous fungi. These methods refer to the techniques used to detect specific physiological activity/metabolites or components of the fungus, thereby estimating the fungal biomass.

2.8.5.1 Chitin/glucoseamine estimation

Chitin is a polymer of N-acetyl-D-glucoseamine occurring in fungal spores and mycelia. Chitin is also present in insects (exoskeleton) and it is absent in other microorganism and food. Ride and Drysdale (1972) described a method of estimation of chitin, in which the chitin is hydrolyzed into its monomer by alkaline hydrolysis at 130^{o}C and glucoseamine released is deaminated to produce 2,5-anhydromannose, which is reacted with 3-methyl-2-benzothiazolone hydrozone hydrochloride for estimating colorimetrically. Lin and Cousin (1985) described an improved method for detection of glucosamine by derivatizing glucoseamine with O-pthaladehyde and separation of derivatized products by HPLC followed by spectrofluorometric detection of fluorescent compounds. Patel and Haynes (1993) demonstrated the potential of glucoseamine specific lectins in detection of food spoilage molds. Fungal biomass can be measured within 4 - 6 h by chitin assay.

Although glucoseamine measurement gives an indication of presence / past contamination of mold in the food material, it may give false results in food grains with insect particles or insect infested food. Glucoseamine assay is said to be of low sensitivity and reproducibility. Hagglbom (1982) and

Madhyastha *et al.* (1990) used glucoseamine estimation for determination of *A.ochraceus* and *P. verrucossum* biomass in cereals and oilseeds.

2.8.5.2 Ergosterol measurement

Ergosterol is component of fungal cell membrane and is the major steroid in fungi, except in certain phycomycetes and rust fungi and is minor component of plant sterol and certain insects (Weete, 1980). Major plant sterols are β-sitosterol and stigmasterol, which can be distinguished from ergostrerol by differences in UV absorption. Ergosterol has double bonds between carbon atoms 5-6 and 7-8, hence absorbs strongly at 240 – 300 nm and weakly below 240nm. Plant sterols are known to have weak absorbtion above 240nm and hence ergosterol is measured by UV absorbtion at 282 nm where plant sterols have little or no absorbance.

Seitz and coworkers (1977, 1979) proposed ergosterol measurement as a method of estimation of fungal biomass in grains. They developed HPLC method for determination of ergosterol with a detection limit of 0.05 µg/g, which corresponds, to 0.25 µg fungal dry weight/g. The ergosterol can also be measured by UV spectrophotometry with a detection limit of 0.1 µg/g. Shasidhar *et al.* (1989) developed a rapid method, which involve iodination of ergosterol to make it fluorescent under long wavelength UV light. Ergosterol is considered one of the rapid and sensitive methods of detection of mold spoilage in food grains and can be performed within 1h compared to 4 – 6 h required by chitin assay. Gouramma and Bullerman (1995b) reported correlation of ergosterol biosynthesis with mycotoxin biosynthesis and suggested that ergosterol measurement may be used for predicting mycotoxin

production in food grains. Saxena *et al.* (2001) studied on correlation of ergosterol content with mold plate count and ochratoxin A production by *A.ochraceus* and *P.verrucossum* in rice. They suggested that ergosterol measurement as a useful method to detect *A.ochraceus* activity.

2.8.5.3 Secondary metabolite

Secondary metabolites are compounds that are not required for growth. The secondary metabolite profile has been used as an important aid in identification and classification of fungal species. Some of the important secondary metabolites used are steroids, terpenes, alkaloids, cyclopeptides, coumarins etc. Mycotoxins are important secondary metabolites, which have been used for identification of toxigenic fungi. Many simple TLC techniques have been developed for direct extraction and analysis from the petri plates or culture tubes. Filtenborg *et al.* (1983) developed simple screening method for molds producing mycotoxins. Secondary metabolite profile has been used as an important taxonomic marker to differentiate *Pencillium viridicatum* from *Pencillium verrucossum* (Pitt, 1987).

2.8.5.4 Volatile compounds

Deterioration of store grain by fungal infestation brings about various physical and biochemical changes due to their growth and metabolic activity. Fungi produce different volatile compounds in food commodity and volatile metabolites are used as markers for detection of fungi. Olsson *et al.* (2002) reported that barley samples with OTA levels below 5 µg/kg had higher concentration of aldehydes (nonanal, 2-hexenal) and alcohols (1-penten-3-ol,

1-octanol) and samples with OTA levels above 5 µg/kg had higher concentrations of ketones (2-hexanone, 3-octanone).

2.8.6 Impedimetry and conductimetry

Impedimetry and conductimetry detection of microorganisms is based on measurement of alteration in impedance and capacitance of media, due to the growth and metabolic activity of the organism. This method was used in studying bacterial growth (Hadley and Senky, 1975) and later on adapted to molds (Jarvis, *et al.,* 1983). They reported that electrical impedance correlated well with level of conidia and impedance changes for about 30 storage fungi. Watson-Craik *et al.* (1990) reported that conductance and capacitance were both medium and species specific. Williams and Woods (1986) reported that Blakeslee's malt extract agar gives the best signal to detect *A.ochraceus* using a bactometer and showed that an inverse linear relationship between colony count and detection time.

2.8.7 Immunological methods

Li *et al.* **(**2000) has reviewed immunological methods used for detection of molds. The common antigen source used for raising antibodies includes extracellular water-soluble compounds, which are generally called as exoantigens, extracellular polysaccharides (EPS) and mycelium-soluble antigens. Notermans and Heuvelman (1985) developed ELISA method for identification of *P.verrucossum*, *Mucor* and *Fusarium species* in foods, using polyclonal antibodies raised against antigens isolated by simple water extraction. They reported that the antigens so extracted were genus specific

and heat stable. Notermans *et al.* (1987) evaluated the antigenicity of EPS extracted from *P.verrucossum* and described an ELISA method to detect *Penicillium* species in foods. Lu *et al.* (1995) reported an ELISA method for detection of *A.ochraceus* in wheat, using antibodies raised against exoantigens of *A.ochraceus*.

2.8.8 Nucleic acid based methods

In the last decade there is increase in publication on DNA based detection method for toxigenic and pathogenic fungi. The most of species/genus specific detection methods are based on the primers for specific amplification of unique gene such as toxin biosynthetic pathway gene, ribosomal RNA gene. The chromosomal and mitochondrial ribosomal DNA has been used frequently in fungal diagnostics.

Varga *et al.* (2000a) studied genetic variability among *A.ochraceus* strains and had shown that by restriction fragment length polymorphism (RFLP) of mitochondrial DNA, random amplified polymorphic DNA (RAPD) and telomeric PCR amplification, the *A.ochraceus* can be differentiated into two distinct groups. The strains showing type I mitochondrial DNA restriction profiles were ochratoxin producing strains and the type II comprised strains which does not produce ochratoxin. Similarly Castella *et al.* (2002) differentiated the strains identified as *P.verrucossum* into two chemotaxonomic group by applying molecular methods like RFLP, AFLP of internal transcribed spacer (ITS) regions of rRNA gene. They distinguished two chemotaxonomic groups, the group one consisting of high ochratoxin producing strains isolated from cheese and meat products and the group two

consisting of moderate to non-producing strains isolated from plant source. Schmidt and co-workers (2004) reported a real time PCR method for detection of *A.ochraceus* in coffee based on species-specific SCAR (Sequence Characterized Amplified Region) primers. They reported that the method could detect coffee beans infected as low as 0.1% (w/w).

2.9 Methods for determination of ochratoxins

2.9.1 Analytical methods

The thin layer chromatographic (TLC) method developed by Neishem *et al.* (1973) has been adopted by the Association of Analytical chemist (AOAC) for analysis of ochratoxin A in coffee beans. Trenk and Chu (1971) described an improved TLC method of quantification based on increase in fluorescence after exposure of toxin to ammonia. The liquid chromatographic method (AOAC, 1997) is the validated standard method of analysis in cereals and coffee beans. Various methods like HPLC (Zimmereli and Deck, 1995), HPTLC, reverse phase HPTLC (Frohlich *et al.*, 1988), and GC-MS (Jiao *et al.*, 1992) method have been developed for the estimation of OTA in cereals, wine, blood and other food commodities with sensitivity up to 0.01 ng/ml depending upon the various cleanup methods involved such as Sep-Pak silica gel cartridge clean up or immuno affinity column clean up.

Hult and Gatenbeck (1976) developed a spectrophotometric method for determination of ochratoxin A in barley with a sensitivity of 4 ppb, based on difference in fluorescence excitation spectra of ochratoxin A and ochratoxin α. In the method the conversion of ochratoxin A to ochratoxin α is achieved by treatment of ochratoxin with carboxypeptidase enzyme and shift in

fluorescence spectra is recorded. Scott *et al.* (1979) developed a convenient TLC method for screening 18 mycotoxins including ochratoxin A from fungal extracts based on two acidic developing solvent system, toluene: ethyl acetate: formic acid (6:3:1) and benzene: methanol: acetic acid (24:2:1), for effective separation of mycotoxins and use of P-anisaldehyde spray reagent for visualization of non fluorescent mycotoxins.

2.9.2 Immunoassays for Ochratoxin A

Immunoassays are increasingly used for screening of food commodities for mycotoxin contamination. Different types of immunoassay methods developed for mycotoxins have been reviewed by various authors (Chu, 1984; Pestka, 1988). Few commercial immunoassay kits and immunoaffinity columns are available for determination of ochratoxin A in foods. Abouzied and Pestka (1994) reported a line blot immunoassay for simultaneous detection of multiple mycotoxins with a detection limit of 0.5 – 500 ng/ml for fumonisin, aflatoxin and zearalenone. Number of enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibody are reported for estimation of ochratoxin A in food commodities, serum, and meat products. Chu (1976) described a method for conjugation of ochratoxin A to bovine serum albumin (BSA) and production of polyclonal antibody in rabbits. Clarke *et al.* (1993) described an ELISA for ochratoxin A using antibodies raised in hens and reported a sensitivity of the assay as 50 ppb in feed commodity. The same authors (Clarke, *et al*., 1995) compared the egg yolk antibodies with antibodies raised in rabbit and reported that rabbit antiserum is superior to hen antiserum in terms of sensitivity, which was 3 ppb in antibodies raised in

rabbits compared to 50 ppb in egg yolk antibodies. Saegar and Peteghem (1999) reported a flow through membrane based ELISA for rapid detection of ochratoxin A in wheat. Gaag *et al.* (2003) developed an immunochemical biosensor assay for the rapid detection of multiple mycotoxins including ochratoxin A in food commodities with a sensitivity of 0.1 ppb. The information on the commercial availability of immunoassay kits for the detection of ochratoxin A is presented in Table.5.

Table. 5 Commercial Immunoassay kits for the detection of ochratoxin A

Company	Name of the kit	Commodities
Diffchamb AB	Transia Plate Ochratoxin A	Wheat, barley, corn, oats, dried fruits
Editek / Diagnostix	EZ Screen, EZ-QUANT	Cereals, grains
Euro-Diagnostica B. V.	Ochratoxin ELISA	Cereals
Reidel-de Haen AG	ELIZA System for Ochratoxin A	Cereal, feed
r-Biopharm GmbH	RIDASCREEN Ochratoxin A	Cereals, feed, coffee beans
TECNA S.r.l.	Immunoscreen OCHRA	Cereals, feeds, wine, green coffee and cocoa
Tepnel BioSytems Ltd.	BioKits Ochratoxin A Assay	Cereal, dried fruits, green coffee, and white wine
VICAM	OchraTest	Coffee beans
Romer Labs	AgraQuant Ochratoxin Assay	Barley, corn, green coffee milo, soybeans and wheat

2.10 Factors affecting fungal growth and ochratoxin A production

Factors that affect mycotoxin production may be broadly divided into abiotic and biotic factors. Important abiotic factors likely to affect mycotoxin formation include water activity, temperature, time, damage to the seed, oxygen and carbon dioxide level, and composition of substrate. Biotic factors include the fungal abundance, prevalence of toxigenic strains, spore load, microbial interactions, and invertebrate vectors (Hesseltine, 1976). The fungal growth, spoilage and mycotoxin contamination result from the complex interaction of the biotic and abiotic factors. Most of the available reports relates to the effect of individual factors on growth and toxin production. Only in few reports different ecological parameters have been studied simultaneously. However an understanding of individual factor involved is essential for comprehension of the overall process for prediction and prevention of mycotoxin formation. The fungi can grow in a broader range of temperature and water activity than that required for toxin production. Similar findings have been reported for ochratoxin A production by *A.ochraceus* and *P.verrucossum* (Northolt, *et al,* 1979).

2.10.1 Water activity and water content

Water activity (a_w) is defined as the ratio of the vapor pressure of the product to that of pure water. The lower the water activity the less the water available to the fungi. Adequate water for fungal growth in grain may result from inadequate drying before storage, penetration of rain into storage structures or moisture migration because of temperature gradient within a bin (Lacey, 1989). All the fungi require a minimum, optimum and maximum water activity

for growth. Storage fungi require a moisture content of 13 - 18% for growth (Lillehoj and Elling, 1983). *A.ochraceus* has the ability to grow at aw as low as 0.79 and reported optimum a_w for growth is 0.95. The minimum a_w for toxin production by *A.ochraceus* varies from 0.83 to 0.87 with an optimum of 0.99. *P.verrucossum* requires a minimum aw of 0.80 for growth and minimum water activtiy for toxin production varies ranging from 0.83 to 0.86 with optimum ranging from 0.95 to 0.99 (Northolt, *et al*., 1979).

2.10.2 Temperature

Similar to water requirement each fungal species has characteristic minimum, maximum and optimum temperature requirement for growth and toxin production. Temperature has considerable influence on water requirements. *A.ochraceus* can grow within the range 8 - 37^{o}C with an optimum varyingly reported as ranging from 25 to 31^{o}C (Sansing, *et al.,* 1973; Northolt, *et al*., 1979). *A.ochraceus* can produce ochratoxin A within temperature range of 15 - 37^{o}C with an optimum ranging from 28 to 31^{o}C. *P.verrucossum* has the ability to grow within temperature range of 0 - 31^{o}C. *P.verrucossum* can produce ochratoxin in temperature ranging from 4 to 31^{o}C, with an optimum temperature of 24^{o}C.

2.10.3 Substrate

Substrate with other ecological factors like temperature and moisture determines the type and amount of mycotoxin produced. Substrates differ in their ability to support toxin production. Madhyastha *et al.* (1990) studied effects of different cereal and oilseed substrates on the growth and ochratoxin production by *A.ochraceus* and *P.verrucossum*. Peanuts and soyabean

supported maximum OTA by *A.ochraceus*, whereas wheat supported maximum OTA by *Pencillium verrucossum*.

The growth and toxin production occurs as a result of complex interaction among all the biotic and abiotic factors prevailing over the substrate. The minimum water activity for growth can be lowest at the optimum temperature and highest near minimum and maximum growth temperature (Bullerman, 1986). Bacon *et al.* (1973) reported that low temperature (15/22^{o}C) and low moisture favoured production of penicillic acid and high temperature (30^{o}C) and high moisture favoured ochratoxin A production by *A.ochraceus* in poultry feed. They also reported that at 15^{o}C *A.ochraceus* developed conidial heads only at high water activity (0.99) whereas at 30^{o}C the fungus produced conidial heads at all levels of water activities ranging from 0.75 to 0.99. Ramakrishna *et al.* (1996) studied the effect of different competing fungi on growth and toxin production by *P.verrucossum*. They reported that significant decrease in seed infection and ochratoxin A production by *P.verrucossum* in presence of certain competing fungi such as *A.flavus*.

2.11 Biological data on ochratoxin A

The main toxic metabolites produced by the *Aspergillus* and *Penicillium* species include acids and esters (ethyl and methyl) of ochratoxin A & B and isocoumarin moiety of ochratoxin - OTα. Although all these metabolites are isolated from fungal cultures, only ochratoxin A and rarely ochratoxin B has been reported as natural food contaminant. Moreover substitution of a chlorine atom converts ochratoxin B to ochratoxin A and thereby protects the

nephrotoxin from carboxypeptidases in digestive system of monogastric animal (Mantle, 2002). Hence among the ochratoxins, ochratoxin A is the most important toxin of the group. Most of the data available is from the laboratory studies conducted using ochratoxin A.

2.11.1 Toxicological studies

The primary target of ochratoxin A is the kidney in all the mammalian species tested. Laboratory data on the LD_{50} values when the toxin was administered through the oral route is presented in Table.6. The LD_{50} value varies from 0.2 – 58 mg/kg bw with sensitivity in decreasing order in animals dog>pig>chicken>rat>mouse (Harwig, *et al*., 1983).

Table 6. LD_{50} values for ochratoxin A in animals

Species	LD_{50} (mg/kg bw)
Mouse	46–58
Rat	20–30
Dog	0.2
Pig	1
Chicken	3.3

2.11.2 Nephrotoxicity

Ochratoxin A is a nephrotoxin to all animal species studied to date and is most likely toxic to humans, who have the longest half-life for its elimination than any of the species examined (Creepy, 1999). The main renal effect of

ochratoxin A in rats was found in the 'postproximal' nephron, where it affects by a reduced glomerular filtration rate, increased fractional water, Na^+, K^+, and Cl^- excretion, and increased dependence of osmol clearance on urine flow. In addition, ochratoxin A blocked membrane anion conductance in canine kidney cells in vitro (Gekle, *et al*., 1993).

2.11.3 Renal carcinogenicity

Ochratoxin A is considered as renal carcinogen in rats but female pigs exposed to OTA for 2 years did not exhibited any sign of renal carcinogenicity. The IARC has concluded that data on carcinogenic risk of ochratoxin A is inadequate and has classified ochratoxin A as group 2B carcinogen indicating it to be a possible human carcinogen. In pigs and dogs when fed with >5 mg/kg feed other effects including renal lesions and also lesions in intestine, spleen, lymphoid tissue and leucocytes have been reported (Szuzech *et al*., 1973a, b).

2.11.4 Immunotoxicity

Dwivedi and Burns (1984) reported a decrease up to 66% serum immunoglobulins in chickens fed with 2 – 4 ppm OTA for 20 days. Chang *et al.* (1979) reported development of leucocytopaenia in chickens fed with ochratoxin A up to 8 mg/kg feed. Huff and coworkers (1980) reported decrease in bone strength in chicks fed with OTA at 2 – 4 ppm. In mice at higher dose of OTA (20 – 40 mg/kg) over a period of 8 days exhibited decreased thymic mass, myelotoxicity of bone marrow and decrease in granulocyte-macrophage progenitors (Boorman, *et al*., 1984). Lea *et al.*

(1989) reported in vitro inhibition of human peripheral B & T cell lymphocyte proliferation and abolition of IL-2 and IL-2 receptor production.

Immunosuppressive effects have also been linked to renal carcinogenicity by some researchers. The possibility of carcinogenicity by immunosuppressive effects of ochratoxin A is hypothesized because:

1. The dose for tumor induction is similar to that required for immunosuppression.
2. Immunosuppression precedes development of detectable tumors.
3. Immune alterations are consistent with those involved in tumor immunity and correlate with changes in resistance to transplantable syngeneic tumor cells.

Manolova *et al.* (1990) reported OTA induced in vitro induction of chromosomal aberration in human peripheral lymphocytes in the presence or absence of a kidney microsomal metabolic activation system, similar to that observed in patients suffering from endemic nephropathy. Lusker *et al.* (1987) suggested that the ochratoxin A induced renal carcinogenicity might be due to suppression of endogenous interferon levels, which lead to suppression of natural killer cell activity.

2.11.5 Teratogenecity

Ochratoxin A induced malformation in fetus characterized by hydrocephaly, ompholocete and anopthalmia and shift in position of oesophagus has been reported in mice administered with OTA at 1.75 mg/kg bw on days 5, 6 and 7 of gestation (Mayura, *et al*., 1989). Cerebral necrosis has been reported in fetuses from dams treated on 15 – 17 days of gestations. In hamsters

administered with OTA intraperitonially at 5 – 20 mg/kg bw on day one of gestation, increased prenatal mortality and malformation such as hydrocephaly, micrognathic and heart defects has been reported (Hood, et *al*., 1976).

2.11.6 Synergistic toxic effects of ochratoxin A

Ochratoxin A in combination with other toxins is known to have a synergistic action on toxic effects in animals. OTA fed along with either citrinin or penicillic acid exhibited a decrease in LD_{50} values in mice (Sansing, *et al*., 1976). In chicken embryos an additive effect on embryotoxicity has been reported when fed with OTA and citrinin (Vesela, *et al*., 1983). Similarly synergism has been reported in terms of mortality and disease severity in dogs fed with OTA along with citrinin at 0.1 - 0.2 mg/kg and 5 – 10 mg/kg bw respectively (Kitchen, *et al*., 1977). When OTA was fed along with aflatoxin B_1 in low dose over long-term, liver and kidney tumors were reported in rats (Rao, et *al*., 1991).

2.11.7 Adsorption

Ochratoxin has been reported to adsorb mainly from the stomach and small intestine. Ochratoxin A adsorbed differs with the species and ranges from 40 to 66% in chickens, rats, rabbits and pigs (Roth, *et al*., 1988).

2.11.8 Distribution

The concentration of ochratoxins in different tissues and plasma depends upon the length of feeding, the route, the degree of serum binding and the

half-life of ochratoxin A (Kuiper-Goodman & Scott, 1989). The serum protein is considered as the important reservoir of the toxin, which is responsible for retention, and supply of mycotoxins. The ochratoxin A in the blood is known to bind to serum albumin and an affinity constant ranging from 4×10^4 - 7×10^4 in chickens, rats and pigs has been reported. The work of Stojkovic *et al.* (1984) and Hult & Fuchs (1986) has revealed that ochratoxin bind strongly with an unknown serum macromolecule with a molecular mass of 20 KDa, with association constants of 2.3×10^{10} per mol in human serum and constants of 0.59×10^{10} in porcine serum and has reported to be saturated with 10 – 20 ng/ml serum. The work of Hagelberg *et al.* (1989) revealed two plasma proteins which can bind ochratoxin A ranging from 78 to 99.9% in work involving humans, rats, monkeys and fish The half-life of ochratoxin A in serum depends on species, degree of binding to serum macromolecules. Various workers (Hagelberg, *et al*., 1989, Sreemannarayana, *et al*., 1988; Galtier, *et al*., 1979; Ballinger, *et al*., 1986) have calculated the serum half-life of ochratoxin A fed by oral route varying from 4.1 to 510 h in chickens, quail, rats, calves and monkeys. The data on the metabolic disposition of ochratoxin A in humans is scanty.

The distribution of ochratoxin A in tissue of pigs, rats, chickens, and goats generally follow the order kidney > liver > muscle > fat (Harwig, *et al*., 1983). The ochratoxin A fed at 10 mg/kg bw to chickens was found to be transferred to egg yolk in 6h and was present even 4 days after administration (Juszkiewicz, *et al*., 1982). However no detectable ochratoxin A was found in egg yolk when fed <1 mg/kg bw (Piskorska-Pliszczynska & Juszkiewicz, 1990).

Ochratoxin A fed to lactating rats up to 25 µg/kg bw was found to be transferred to milk. Ochratoxin A is also reported to be transfered to placenta within 20 min in mice when administered intravenously before 10 days of gestation period (Appelgren & Arora, 1983a, b).

2.11.9 Excretion

Glomerular filtration and biliary excretion is considered important routes of plasma clearance of ochratoxin A in rats. Up to 33% of ochratoxin A fed to rats orally was found to be excreted into bile of rats up to 6h after dosing and only trace amounts is excreted as ochratoxin α (Suzuki, *et al*., 1977). Storen, *et al.* (1982) reported that the major excretory product apart from ochratoxin α (25 - 27%) was ochratoxin A and 4R-OH-ochratoxin A epimer at 6% and 1 - 1.5% of administered OTA respectively. In pre-ruminant calves, 85 - 90% of orally administered ochratoxin A was excreted as ochratoxin α (Sreemannarayana, *et al*., 1988).

2.11.10 Biotransformation

Ochratoxin A is hydrolyzed to the non-toxic ochratoxin α at various sites. In rats the biotransformation of ochratoxin A is carried out by bacterial microflora of the caecum (Galtier, 1978). The enzymes carboxypeptidase and chymotrypsin is considered the important enzymes, which transforms ochratoxin A into ochratoxin α (Pittout, 1969a, b; Pitout & Nel, 1969). The biotransformation into ochratoxin α was low in the liver and kidney tissue (Suzuki, *et al*., 1977).

The effective hydrolysis of ochratoxin A into ochratoxin α was observed in ruminant stomach by the ruminant protozoa and it is estimated that up to 12 mg/kg of feed could be degraded in the stomachs of cow (Hult, *et al.,* 1976; Peterson, *et al*., 1982). The studies in mice suggest that ochratoxin A circulates from the liver into the bile and into the intestine, where it is hydrolyzed to ochratoxin α (Moroi, *et al*., 1985).

The 4R-OH epimer, which is considered less toxic than ochratoxin A, is the main metabolite formed in human and rat liver microsomal systems (Stormer, *et al*., 1981), whereas the 4S-OH epimer is more prevalent in pig liver microsomes. No data were available on its toxicity (Moroi, *et al*., 1985).

Ochratoxin B, a dechloro derivative of ochratoxin A may occur along with it in cereal products. In rats, it is less toxic than ochratoxin A and is metabolized to 4-OH-ochratoxin B and ochratoxin β (Stormer, *et al*., 1985).

2.12 Fate of ochratoxin A during food processing

The effect of food processing on fate of OTA in coffee beans and wheat has been studied by various workers. Ochratoxin A is relatively less stable to heat than aflatoxin B_1. Van der Stegen *et al.* (2001) carried out experiments on effect of ochratoxin A during roasting in coffee beans, wherein they have reported 69 - 96% reduction. Boudra *et al.* (1995) conducted experiment to determine half-life of ochratoxin A in wheat under dry and wet conditions. They reported half-life of OTA in wheat, corresponding to 50% reduction, at 200^{o}C as 12 and 19 min under dry and wet conditions respectively. Furthermore they found that destruction of only 2% when treated at 100^{0}C for 20 min. Similar findings has been reported by El-Banna and Scott (1984) in

studies involving polished wheat, wherein they reported a reduction of about 6% of OTA when polished wheat was treated at 100^0C for 30 min. Osborne *et al.* (1996) studied the effect of milling and processing of wheat contaminated with ochratoxin A and they reported that scouring of wheat to reduce 70 - 80% ochratoxin A in wholemeal flour for both the soft and hard wheat.

2.13. Degradation of Ochratoxin A

Ochratoxin A is reported to be degraded by chemicals. McKenzie *et al.* (1997) reported degradation of ochratoxin A by ozone treatment (for 15 sec) and found significant reduction in toxicity in bioassay. Nefisa and El-Shayab (1994) studied effect of food additives on *A.ochraceus* growth and ochratoxin A production. They reported that complete inhibition of the fungal growth and ochratoxin A production in broth with the treatment of benzoic acid, sodium benzoate, para amino benzoic acid, propionic acid, crystal violet, boric acid and potassium fluoride at 1, 1, 2, 0.2, 0.0008, 2, 4% level respectively. Baxter *et al.* (2001) studied the behavior of ochratoxin A during brewing. They reported loss of up to 40% ochratoxin A in grist during mashing and little degradation during fermentation. Turbie *et al.* (2002) reported in vitro binding of ochratoxin A and other mycotoxins, ranging from 33 to 76% by *Lactobacillus rhamnosus* strains.

2.14 Prevention of Ochratoxin A Contamination

Several general methods for prevention of mycotoxin contamination have been known. These are reduction of moisture content after harvest, drying, fumigation, proper ventilation in storage structures, physical segregation of

infected lot etc. Majumder (1974) suggested fumigation with methyl bromide for effective control of fungi and insect infestation. Most of the reports available are on prevention of ochratoxin production in liquid media, on food substrates and control of *A.ochraceus* growth. Paster *et al.* (1999) reported complete inhibition of *A. ochraceus* growth in media containing propionic acid (0.05%) and nisin (1000 ppm). However the physical methods like exposure to sunlight and biological methods have not been worked out for decontamination of OTA contaminated foods. Refai *et al.* (1996) studied the use of gamma radiation as a means of inhibition of *A.ochraceus* growth and ochratoxin A destruction. They reported at a radiation level of 4 kGy complete inhibition of *A.ochraceus* growth and radiation level of 15 and 20 KGy was sufficient for complete destruction of ochratoxin A in yellow corn and soyabean respectively, whereas up to 47% destruction was achieved in poultry feed concentrates, broiler concentrate and cotton seed.

2.15 Risk assessment, Regulation and Legislation

The WHO and Joint Expert Committee on Food Additives (JECFA) assessed the toxicological implication of dietary ochratoxin A intake based on data obtained from reported natural occurrence of ochratoxin A in food commodities at the international level. The intake of OTA was estimated based on weighted mean concentrations of ochratoxin A in foods, which were estimated as 0.94 µg/kg for cereals, 0.19 µg/kg for cereal products, 0.32 µg/kg for wine, 0.86 µg/kg for coffee, 2.3 µg/kg for dried vine fruit, and 0.44 µg/kg for grape juice. The committee considering the concentration of OTA in foods and food consumption in Europe proposed a PTWI (Permissible

Tolerable Weekly intake) of 100 ng/kg bw based on the No Adverse Effect Level (NO(A)EL) for nephrotoxic effects, which corresponds to 0.008 mg/kg bw and a safety factor of 500, which corresponds to a safety factor of 1500 applied to the NOEL for carcinogenicity in male rats, the most susceptible species for ochratoxin A carcinogenicity (WHO, 2002). The European Union based on PTWI recommended by JECFA implemented a regulatory limit of 5 µg/kg in cereals, 3 µg/kg in cereal products and 10 µg/kg in dried vine fruits (currants, raisins and sultanas) with effect from April 2002 (European Commission, 2002). Recently it has been agreed to extend the regulatory limits to roasted coffee beans and ground roasted coffee at 5.0 µg/kg, soluble coffee at 10 µg/kg, wine, grape based drinks, grape juice and grape juice ingredients in other drinks at 2.0 µg/kg and baby foods at 0.5 µg/kg (European commission, 2005).

2.16 Microbiological Safety and Quality

Microbial contamination is considered the major risk associated with food safety. Regular monitoring and surveillance of food commodities for microbial hazards is necessary. Many of the countries realizing the risk of foodborne illnesses have set stringent regulatory limits for microorganisms and their metabolites. The food commodities free from all contaminants is highly desirable, but it is understood that complete prevention of mold contamination is very difficult to achieve. Although legislation barring trading of toxin contaminated commodities is one of the methods to control human exposure to toxins, but it has its own repercussions on international trade. Strict legislation may cause more damage to economy of poor countries, which are

dependent on agriculture as the main source of income. Hence efforts are needed to ensure reduction of these contaminants as low as reasonably achievable (ALARA).

The growing concern of consumer towards toxic metabolites in food products has emphasized the need for application of efficient product quality management practices for ensuring safety of food products. The stringent legislations for limiting toxic metabolites in food grains have compelled industries and various trading agencies to monitor microbial hazards and their toxic metabolites. The hazard analysis critical control point (HACCP) system is becoming increasingly adapted for controlling microbial hazards in foods. HACCP has been applied to limited number of agricultural commodities for preventing preharvest and post harvest mold infestation and mycotoxin contamination. Rapid microbiological methods can be used nearly at all steps of HACCP system including verifying critical control limits (Vanne, *et al*., 1996). In this regard immunological techniques and nucleic acid based detection methods are advantageous over conventional methods for routine and rapid analysis of microorganisms and their metabolites in foods.

CHAPTER 3.0

PREVALENCE OF OCHRATOXIGENIC FUNGI AND NATURAL OCCURRENCE OF OCRHATOXINS IN FOODS

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RESULTS AND DISCUSSION

3.1 INTRODUCTION

Historically, Ochratoxin A was isolated from a strain of *A. ochraceus* way back in 1965 (Van Der Merwe, *et al.,* 1965). Later six other species of *Aspergillus* section *Circumdati* (*A.ochraceus* group) have been identified as ochratoxin A producers. These species are *A.alliaceus*, *A.melleus*, *A.sulphureus*, *A. ostianus*, *A.petrakii* and *A.sclerotiorum*. It is also established that this toxin is also produced by *Penicillium verrucossum*. The fungus *A.ochraceus* is widely distributed in foods like various kinds of beans, dried fruits, nuts including peanuts, pecans, betel nuts, wide range of cereals including barley, wheat flour and rice (Pitt and Hocking, 1985). The production of ochratoxin A by fungal strains other than *A.ochraceus* group has caused alarm in the international community (Abarca, *et al.,* 2001). Testing of raw food commodities provide assurance for further processing of raw materials to finished product with safety aspects built into the system.

The present chapter deals with determination of prevalence of ochratoxigenic fungi and ochratoxin A contamination in food commodities. Market samples of various food commodities were screened for ochratoxin A contamination. Common fungal isolates belonging to *A.ochraceus* group, *Penicillium* species and other common food borne fungal isolates were also screened to evaluate the ochratoxigenic potential.

3.2 MATERIALS AND METHODS

3.2.1 Microbiological analysis of foods and isolation of ochratoxigenic fungi

3.2.1.1 Food samples

Market samples of maize, sorghum, groundnut, rice, chilies, coffee beans, groundnut cake, poultry feed, cotton seed and dried coconut were purchased from various grocery shops and feed manufacturers in and around the city of Mysore, Karnataka, India. The samples were collected in sterile polythene covers and microbiological analysis was performed within 24 h of sample collection.

3.2.1.2 Diluent

The diluent used was normal saline (0.85%), which was dispensed in requisite quantities in suitable glass containers and sterilized at 121^{o}C, 15 p.s.i. for 15 min.

3.2.1.3 Media

The following media were used for enumeration and isolation of molds from food commodities.

3.2.1.3.1 Potato Dextrose agar (PDA)

Composition	g/l
Potato	200
Dextrose	40
Agar	15
Chloramphenicol	100mg
or	
10% Tartaric acid	10ml
pH	5 ± 0.2

Two hundred gram peeled potato was boiled in distilled water and filtered. The filtrate was made up to 1 liter and other ingredients were added. The medium was sterilized at 121^{0}C, 15 p.s.i for 15 min. The medium was cooled to 50^{0}C and was supplemented with chloramphenicol or 10 ml tartaric acid (10%) per litre. The PDA was used for the enumeration of total viable yeast and mold counts present in the food commodities and for the routine maintenance of cultures.

3.2.1.3.2 Czapek Dox agar

Composition	g/l
Sucrose	30.0
Sodium nitrate	3.0
Casein hydrolyasate	3.5
Di potassium hydrogen phosphate	1.0
Potassium chloride	0.5
Magnesium sulphate	0.5
Ferrous sulphate $7H_2O$	0.01
Agar	15.0
pH	6.0 ± 0.2

The requisite quantity of media was dissolved in distilled water and sterilized at 121^{o}C, 15 p.s.i for 15 min. The medium was used for identification of *Aspergillus* isolates.

3.2.1.3.3 Yeast Extract Sucrose agar (YES)

Composition	g/l
Yeast extract	20.0
Sucrose	40.0
Agar	15.0
pH	6.0 ± 0.2

The requisite quantity of media was dissolved in distilled water and sterilized at 121 ^{o}C, 15 p.s.i for 15 min. The medium was used for screening of fungal isolates for ochratoxin A production.

YES broth medium was used for confirmation of ochratoxin A production and evaluation of toxigenic potential of isolates which was giving positive result in the above assay.

3.2.1.4. Food sampling and isolation of fungi

Sample homogenate of individual food samples (3.2.1.1), were prepared by mixing 11 g of ground sample in 99 ml of sterile saline (0.85%). Appropriate serial dilutions in aliquots of 0.1 ml were surface plated in triplicate on prepoured plates of PDA (HiMedia, Mumbai, India) for enumeration and isolation of mycoflora. The plates were incubated at 28 ± 2°C for 3 - 5 days. The characteristic colonies of *Aspergillus*, *Penicillium*, and other fungal genera were counted and expressed as mean colony forming units per gram (cfu/g).The representative isolates belonging to different genera were isolated, purified and maintained on PDA at 4°C.

3.2.1.5 Identification of Fungi

All *Aspergillus* isolates were grown on Czapek Dox agar plates and identified at the genera level and the isolates belonging to *A.ochraceus* group were identified at the species level by using the manual for the identification of the genus *Aspergillus* (Raper and Fennel, 1965).

3.2.2. Screening of isolates for ochratoxin A potential

3.2.2.1 Culturing of isolates

The isolates belonging to *A.ochraceus* group, *Penicillium* group and other fungal genera were screened for the production of ochratoxins by agar plug method (Filtenborgh, *et al*., 1983). All isolates of *A.ochraceus* group and other representative genera from different food commodities were sub cultured on PDA. The 7 day old cultures of all the isolates were point inoculated at three spots per plate on prepoured yeast extract sucrose agar plates and incubated at 28 ± 2 ^{o}C for 5 - 7 days.

3.2.2.2 Extraction of ochratoxins from fungal isolates

Three agar plugs (0.5 cm) were removed from central colony area and taken in sterile glass vials. Chloroform (1ml) was added into each vial and shaken for 30 min at 150 rpm. The extracts were decanted and 10 µl of extract were spotted on TLC plate. The TLC plates were run along with ochratoxin (OTA) standards in benzene: methanol: acetic acid (18:1:1) for 30 min. The developed plates were air dried and observed under longwave UV light (366nm) for presence of characteristic fluorescence with similar R_f value matching with that of OTA standard.

3.2.2.3 Evaluation of ochratoxigenic potential

3.2.2.3.1 Media

The isolates that were positive for ochratoxin A production as analyzed by the agar plug method (Section 3.2.2.2) were further analyzed for ochratoxin A production and confirmation in yeast extract sucrose broth (Davis, *et al.,* 1972). YES broth were prepared and pH was adjusted to 6.0 with 1N HCl.

YES broth in 50 ml quantities was dispensed into 250 ml conical flasks and sterilized at 121^{o}C, 15 p.s.i for 15 min.

3.2.2.3.2 Fungal inoculum

All the isolates were sub cultured on PDA and 7 day old cultures were used for preparation of inoculum. Spore suspension was made in 0.5% peptone broth containing 0.85% sodium chloride. 1ml spore suspension (10^{6} spores/ml) was inoculated into YES broth and incubated at 30^{o}C for 7days.

3.2.2.3.3 Extraction of ochratoxins

The mycelial mat was separated on Whatman No.1 filter paper and culture filtrate was collected. The pH of the culture filtrate was adjusted to 4.0 using HCl. The culture filtrate was measured and taken in a separating funnel and extracted thrice with chloroform (25 ml x 3). The chloroform extracts were combined and passed through a bed of anhydrous sodium sulphate. The extract was evaporated to dryness under reduced pressure in a rotary evaporator (Buchi, Switzerland). The extract was dissolved in known quantity of chloroform. Ochratoxin A was quantified by TLC and densitometry. The ochratoxin content was expressed as concentration of ochratoxin A per ml of medium.

3.2.2.3.4 Thin Layer Chromatography and Densitometry

3.2.2.3.4.1 TLC plates

Precoated silica gel G-60 TLC plates with fluorescence indicator was used.

3.2.2.3.4.2 TLC developing solvent systems

The following developing solvents were used for separation of toxin on TLC. The developing solvents were prepared fresh and TLC plates were developed in unlined equilibrated tank.

1. Benzene: methanol: acetic acid (18:1:1)
2. Toluene: ethyl acetate: formic acid (6:3:1).

3.2.2.3.4.3 Calibration of Scanner

Camag HPTLC scanner S/N 0302A005 and associated software CATS4 (version V4.06) was used for densitometry measurement of mycotoxins. Mycotoxin standard solutions with concentrations ranging from 0.2 to 2 µg/ml were made in chloroform. Toxin standard solution (10 µl) was spotted on TLC plate with concentration ranging from 2 to 20 ng/spot. The TLC plate was developed in benzene: methanol: acetic acid solvent system (18:1:1). The plates were air dried and scanned in Camag Scanner in fluorescence/reflectance mode set at 360 nm at a scan speed of 5 mm/sec and slit width 4 mm x 0.1 mm. The area for different concentration of mycotoxin was analyzed for linearity with respect to concentration of toxin. The area of absorbance was linear for 2 - 10 ng per spot for aflatoxin B_1 and ochratoxin A.

3.2.2.3.4.4 Quantification of mycotoxins

All positive samples were appropriately diluted to give a concentration of about 0.2 - 2 µg/ml and spotted on TLC plate (5 -10 µl) along with mycotoxin standard solution. The TLC plate was developed in benzene: methanol: acetic acid (18:1:1) solvent system as described in the method of Scott *et al*. (1979).

The TLC plate was scanned at 360nm as described earlier. The R_f value and fluorescence intensity of mycotoxins standards were compared with sample and quantified using the following formula

$$\text{Toxin Concentration (µg/g)} = \frac{X}{Y} \times \frac{S}{V} \times \frac{D}{G}$$

Where X = Area given by sample

V = volume of sample spotted.

Y = Area given by standard

S = Quantity of standard spotted (ng)

D =Sample dilution (ml)

G = Corresponding weight of the sample equivalent to the volume of extract taken for analysis.

3.2.2.4 Confirmation of ochratoxin A

Ochratoxin A was confirmed by formation of methyl ester (Neishem, *et al*., 1973). Ochratoxin A standard (250 ng) was taken in a glass vial and evaporated to dryness. Boron tri fluoride (BF_3, 10 ml) was added into the vial containing standard and heated to boiling for 5 min on a water bath. The treated sample was taken in a separating funnel containing 30 ml water and extracted thrice with 10ml chloroform. The chloroform extracts were combined and evaporated to dryness under reduced pressure. The derivatized sample was dissolved in 250 µl of chloroform. Aliquots of sample extract was taken in a vial and derivatized in similar manner as that of standard. Derivatized sample (10µl), underivatized sample, derivatized standard (10µl), and underivatized standard (10µl) were spotted on TLC plate and the plate was

developed in benzene: methanol: acetic acid (18:1:1) solvent system. The plate was air dried and observed under longwave UV light (366 nm) for characteristic fluorescence at derivatized and underivatized ochratoxin A standard R_f.

3.2.3. Mycotoxin analysis

3.2.3.1 Food samples

The samples were aseptically ground to pass through 20 BSM (British standard Mesh) sieve and stored under refrigerated condition at –20^{o}C until analyzed. Samples in 50 g quantity were processed for mycotoxin analysis.

3.2.3.2 Chemicals

Standard Aflatoxin B_1, Ochratoxin A, Citrinin, Trifluoroacetic acid (TFA), Boron trifluoride (BF_3) 14% (w/w) were procured from Sigma Aldrich, USA. Precoated silica Gel G-60 20 x 20 cm TLC F_{254} plates were purchased from E. Merck chemicals, Mumbai, India. All other reagents were of analytical grade.

3.2.3.3.Mycotoxin standards

Mycotoxin standard were weighed accurately and taken in standard volumetric flasks. The mycotoxin standards were dissolved in appropriate solvents, checked for purity and quantified by spectrophotometry. The mycotoxin solutions were stored under refrigerated condition in dark. The following parameters and constants mentioned in Table 3.1 was used for determination of purity and concentration. The absorbtion of toxins at maximum absorbtion wavelength (λ_{max}) were recorded in a spectrophotometer

(Schimadzu UV-160, Japan). Toxin concentration was determined using the formula

Toxin concentration (ug/ml) = (A x MW x 1000) / ε

Where A - Absorbance at wavelength (λ_{max}) given for each toxin at Table 3.1

MW - Molecular weight of the toxin

ε - Molar absorptivity

3.2.3.4 Ochratoxin A

Ochratoxin A was analyzed by following AOAC procedure (AOAC, 1997). Sample (50 g) was extracted with 25 ml 0.1 M phosphoric acid and 250 ml of chloroform for 30 min in a wrist-action shaker. The extract was filtered and 50ml filtrate was mixed with 40 ml hexane and passed through 2 g diatomaceous earth with 1 ml 1.25% sodium bicarbonate solution in 700 x 17 mm chromatographic tube. Esters were removed by eluting with 75ml chloroform. Ochratoxin A was eluted with 75 ml freshly prepared formic acid: chloroform (1:99). The eluate was evaporated to dryness under reduced pressure and dissolved in known quantity of chloroform and analyzed by thin layer chromatography (TLC). Quantification of ochratoxin A was by TLC and densitometry.

3.2.3.5 Citrinin

Citrinin was extracted and analyzed by the method of Jackson and Ciegler (1978). Food sample (50 g) was mixed with 200 ml chloroform, 10 ml hydrochloric acid and 25 ml water for 30 min in a wrist-action shaker and filtered through Whatman No.1 filter paper.

Table 3.1 Molecular weights and molar absorbtivities of mycotoxins

Mycotoxin (MW)	Solvent	Molar absorption (ε)	λ Maximum (nm)
Ochratoxin A (403)	Benzene–acetic acid (99+1)	5550	333
Ochratoxin B (369)	Benzene–acetic acid (99+1)	6000	320
Aflatoxin B_1 (312)	Benzene-acetonitrile (98+2)	19800	348
Citrinin (250)	Ethanol	22280	222

Fifty ml filtrate was taken and citrinin was partitioned into 0.1 M sodium bicarbonate solution. The aqueous extract was acidified to pH 2.5 with hydrochloric acid and extracted with chloroform (25 ml x 3). The citrinin estimation was by TLC and densitometry.

3.2.3.6 Aflatoxin

3.2.3.6.1 Aflatoxin B_1 Extraction and quantification

Aflatoxin B_1 in the sample was analyzed by CB method (AOAC, 1996). Food sample (50 g) was extracted with mixture of 25 ml water, 25 g diatomaceous earth and 250 ml chloroform for 30 min in a wrist-action shaker. The extract was filtered through Whatman No. 1 filter paper and 50 ml filtrate was purified on silica gel (10 g) placed in glass column (300 mm x 20 mm). The extract was loaded on the column and it was washed with hexane (150 ml) followed by diethyl ether (150 ml). Aflatoxin was eluted with methanol: chloroform (3:97). The eluate was evaporated to dryness. The residue was dissolved in known quantity of chloroform, and analyzed by TLC and densitometry.

3.2.3.6.2 Confirmation of Aflatoxin B_1

Confirmation of identity of aflatoxin was by the method of Przybylski (1975). Silica gel plate was divided into 2 equal vertical sections. On each section two 10 µl aliquot of sample extract and 10 µl of aflatoxin B_1 standard solution was spotted. On one of the sample extract spot AFB_1 (10 µl) was superimposed. One section was covered with glass plate. TFA (1 µl) was spotted on all the three spots in the uncovered section and allowed to react for 5 min. The plate was heated (from lower side) with hot air, maintaining the temperature of the plate at 35 - 40^{o}C for 10 min. The covered section was now uncovered. The

plate was developed in chloroform: acetone (85:15) and air-dried. Aflatoxin B_1 reacts with strong acid to give a highly fluorescent heavier compound that has a lower R_f value. The plate was observed under longwave UV (366 nm) for characteristic aflatoxin B_{2a} derivative, which has R_f of approximately ¼ of AFB_1 where the samples are reacted with TFA. Co-migration of aflatoxin B_1 in the sample along with superimposed standard aflatoxin B_1 and characteristic blue fluorescence with similar R_f confirms the presence of aflatoxin B_1 in the sample.

RESULTS AND DISCUSSION

3.3 RESULTS

3.3.1 Mycological quality of food commodities

The quality and safety of food depends on the presence of fungal flora, nature of the flora harboring the commodities and the distribution of these fungi on various food commodities. In the present study a total of 73 food commodities were screened for the presence of *A.ochraceus* group and *Penicillium* species. The total fungal flora for unprocessed food commodities were in the range of $2 \times 10^2 - 26 \times 10^4$ cfu/g. The load of *A.ochraceus* group of fungi was in the range of $1 \times 10^2 - 2 \times 10^4$ cfu/g (Table 3.2). The incidence of species belonging to *A. ochraceus* group in food commodities is presented in Table 3.3. The various species of *A.ochraceus* group of fungi isolated from food commodities are shown in Fig. 3.1. The incidence of *A.ochraceus* group of fungi was 20.5%, Of the 73 food samples screened 15 samples harbored *A. ochraceus* group of fungi. Highest incidence being in maize (50%) followed by coffee beans (33.3%) and poultry feed (33.3%). A total of 22 number of *A. ochraceus* group of fungi was isolated, purified and identified. *A.ochraceus group* of fungi were also identified to their species level. Distribution of molds belonging to *A.ochraceus* group in foods tested is shown in Table 3.4. It is evident that *A.ochraceus* species was the most dominant. *A.melleus* and *A.sulphureus* were the next common species present on food commodities. In the present investigation the incidence of *A.flavus* in the food commodities were also recorded (Table 3.5). The results indicate that incidence of ochratoxigenic fungi is less common than aflatoxigenic fungi, the most studied mycotoxigenic fungi.

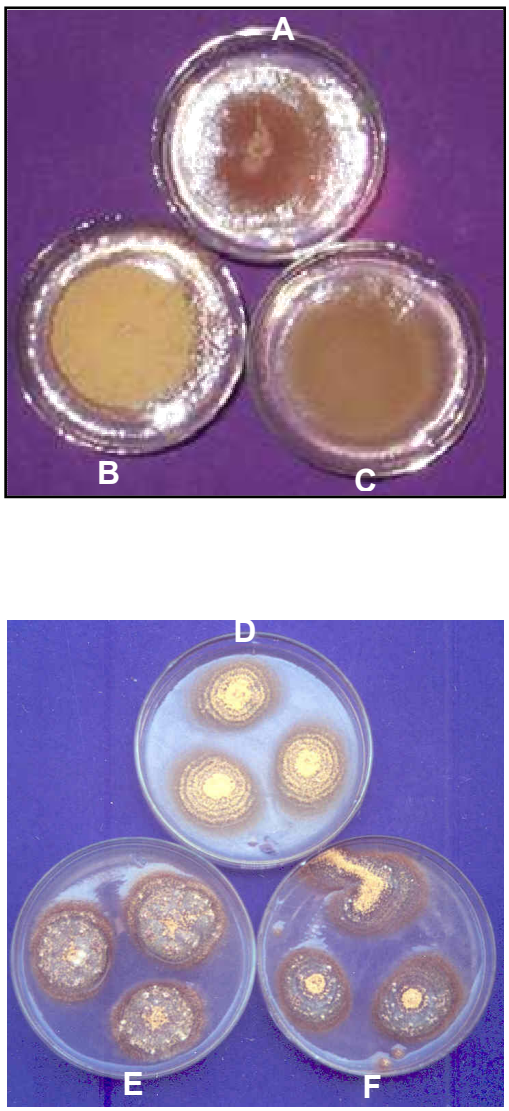

Fig. 3.1 *Aspergillus ochraceus* group

A. *A. melleus*.
B. *A. ostianus*
C. *A. ochraceus*
D. *A. auricomus*
E. *A. sulphureus*
F. *A. sclerotiorum*

Table 3.2 Mycoflora and their range in different food commodities.

Commodity	Viable counts	
	Yeast and mold count (cfu / g)	*A.ochraceus* group (cfu / g)
Maize	4×10^3 - 26×10^4	2×10^2-3×10^3
Sorghum	8×10^3 - 14×10^4	1×10^2-1×10^3
Rice	15×10^2 - 19×10^4	1×10^2
Groundnut	8×10^3 - 15×10^4	Nil
Chili	9×10^2 - 9×10^4	2×10^2-1×10^4
Coffee bean	14×10^2 - 8×10^3	2×10^2-1×10^3
Poultry feed	1×10^3 - 14×10^4	1×10^2-2×10^4
Groundnut cake	3×10^2 - 15×10^3	2×10^2
Cotton seed	6×10^3 - 14×10^4	Nil
Copra	2×10^2 - 12×10^3	1×10^2

Table 3.3 Incidence of species belonging to *A.ochraceus* group in food commodities

Food commodities	Incidence %	No of isolates of *A.ochraceus* group
Maize	50.0 (3/6)*	7
Coffee beans	33.3 (3/9)	6
Poultry feed	33.3 (2/6)	2
Sorghum	25.0 (2/8)	2
Chili	22.2 (2/9)	2
Rice	16.6 (1/6)	1
Copra	16.6 (1/6)	1
Groundnut cake	11.0 (1/9)	1
Cotton seed	0	-
Groundnut	0	-
Total	20.5 (15/73)	22

- Figures in parenthesis indicate number of samples harboring *A.ochraceus* group of fungi / total number of commodity screened

Table 3.4 Distribution of species belonging to *A.ochraceus* group in food commodities.

Food	Species identified
Maize	*A.ochraceus* (3) *A.melleus* (3) *A.sulphureus* (1)
Coffee beans	*A.ochraceus* (1) *A.sulphureus* (3) *A.melleus* (1) *A.sclerotiorum* (1)
Poultry feed	*A.ochraceus* (1) *A.auricomus* (1)
Chili	*A.sclerotiorum* (1) *A.ostianus* (1)
Sorghum	*A.ochraceus* (1) *A.ostianus* (1)
Rice	*A.ochraceus* (1)
Copra	*A.auricomus* (1)
Groundnut cake	*A.ochraceus* (1)
Cotton seed	-
Groundnut	-

* Number in the parenthesis indicates total number of isolates.

Table 3.5 Incidence of *A.flavus* group isolates in food commodities

Food commodities	Incidence %	
Groundnut cake	100.0	(6/6) *
Chilli	62.5	(5/8)
Poultry feed	55.5	(5/9)
Cotton seed	50.0	(4/8)
Maize	50.0	(3/6)
Groundnut	50.0	(3/6)
Sorghum	44.4	(4/9)
Coffee beans	16.6	(1/6)
Rice	11.1	(1/9)
Copra	0	(0/6)
Total	43.83	(32/73)

- * Figures in parenthesis indicate number of samples harboring *A.flavus* group of fungi / total number of commodity screened

3.3.2 Natural contamination of mycotoxins

All the food samples were analyzed for the presence of ochratoxin A by HPTLC. Only three samples, one each of groundnut cake, poultry feed and coffee bean was contaminated with ochratoxin A at 0.4, 0.2 and 1.25 ppm respectively (Table 3.6). The densitometric chromatographs of ochratoxin A standard and feed sample are presented in Fig. 3.2 and Fig. 3.3 respectively. The sample of coffee bean showed highest amount of ochratoxin A contamination (1.25 ppm). In these samples other mycotoxins were also present. Aflatoxin was present in two samples, one each of groundnut cake and poultry feed at 3.4 and 10 ppm respectively. In the coffee bean sample citrinin mycotoxin at 0.5 ppm was present.

3.3.3 Characterization of isolates

The fungal isolates belonging to *A.ochraceus* group were screened for their potential to elaborate ochratoxins. The chromatogram of culture extracts belonging to *A.ochraceus* group of fungi is shown in Fig. 3.4. A total of 22 isolates belonging to *A.ochraceus* group were screened to evaluate ochratoxigenic potential. The toxigenic potential of isolates is presented in the Table 3.7. The isolates having toxigenic potential belong to the species of *A.ochraceus* (7), *A.sulphureus* (2), *A.auricomus* (2), *A.ostianus* (1), and *A.melleus* (1) (Table 3.8). Of these 13 isolates (59.2%) produced ochratoxin A. *A.ochraceus* was the dominant toxigenic species present on food

commodities, out of the eight isolates screened for production of ochratoxin A, seven of them elaborated OTA (Table 3.8).

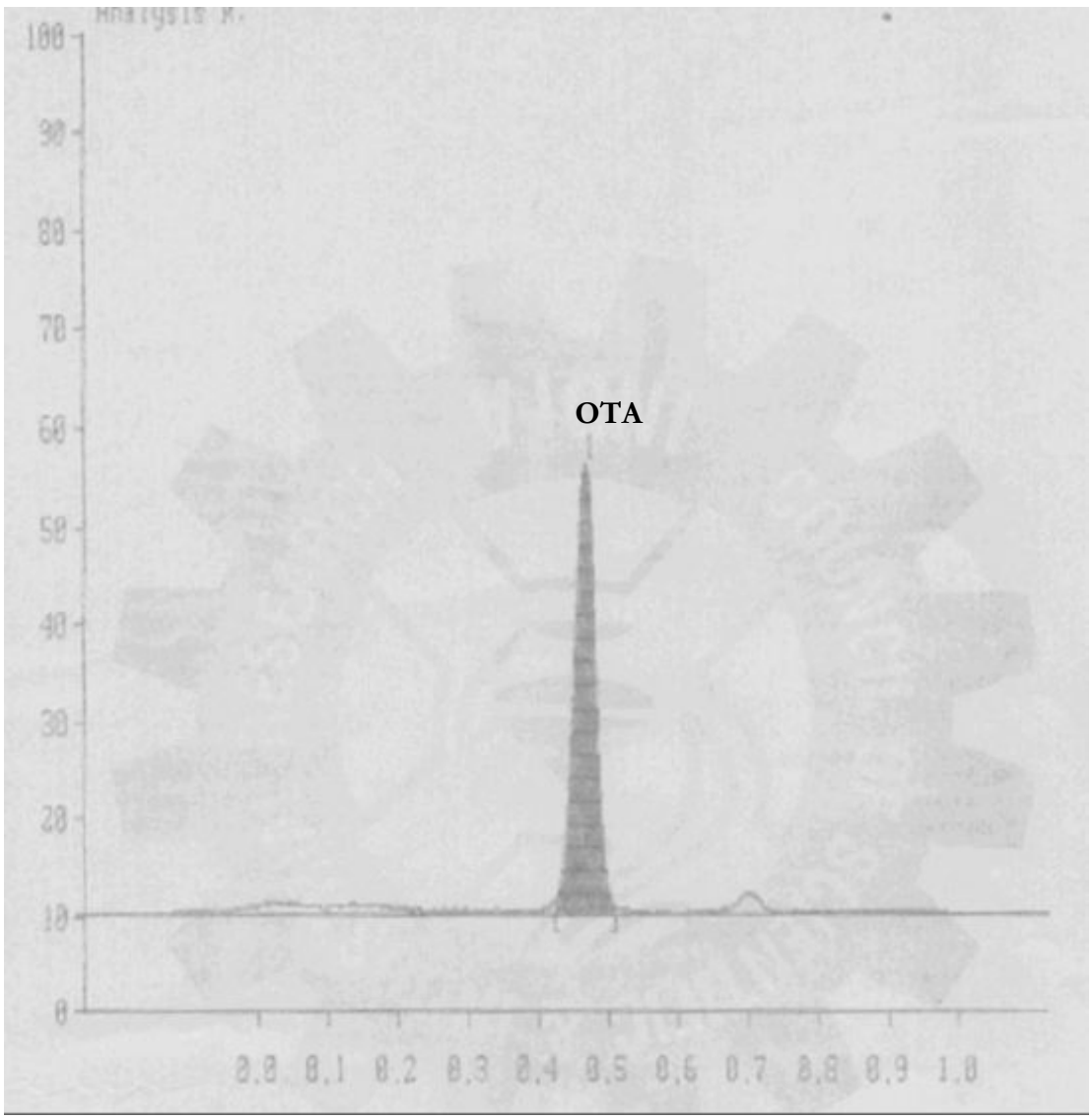

Fig. 3.2 Densitometric pattern of ochratoxin A standard

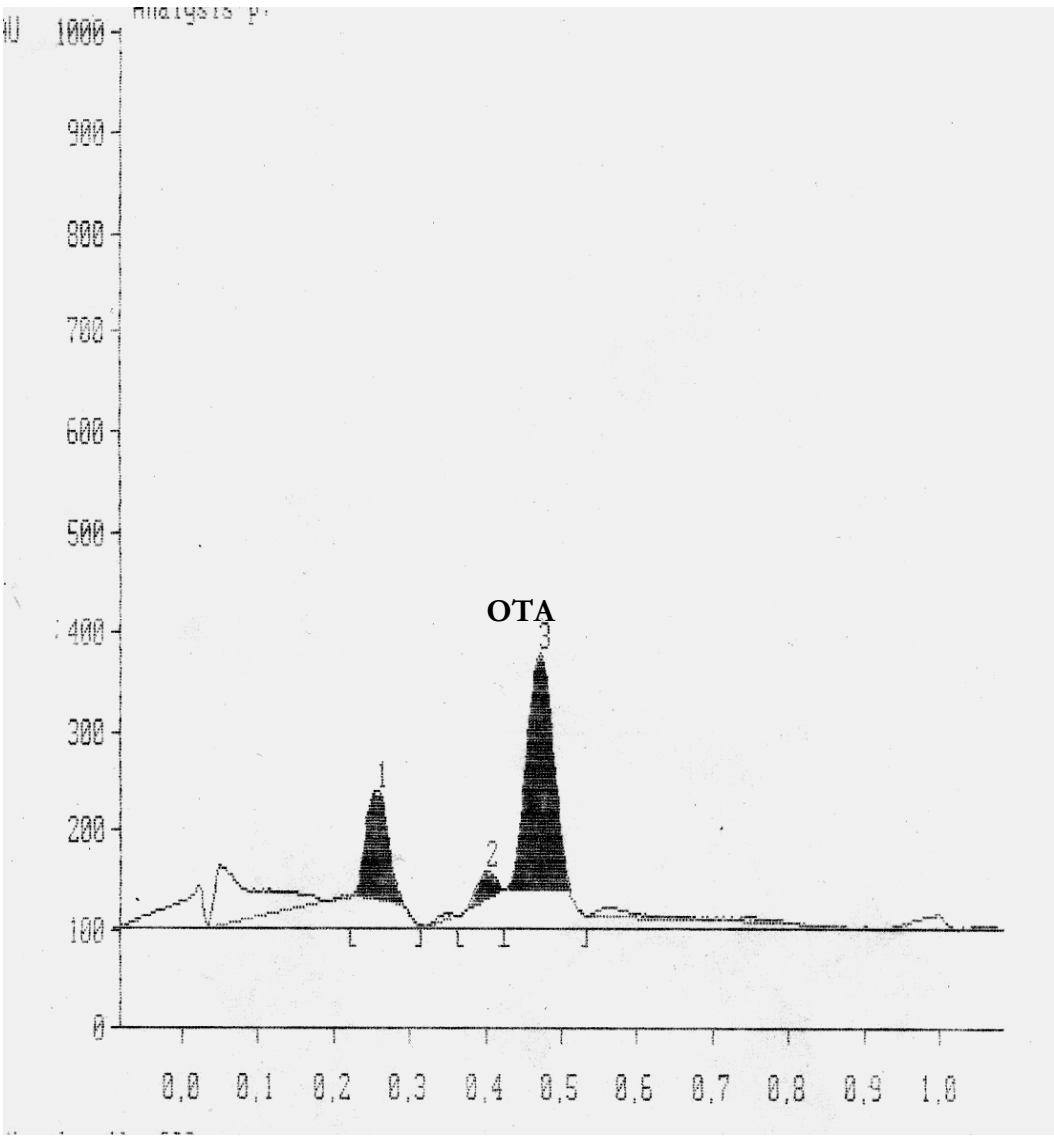

Fig. 3.3 Densitometric pattern of ochratoxin A from feed sample

Table 3.6 Mycotoxin contamination of food samples

Commodity	Ochratoxin A (ppm)	Aflatoxin B_1 (ppm)	Citrinin (ppm)
Groundnut cake	0.4	3.4	-
Poultry feed	0.2	10	-
Coffee beans	1.25	-	0.5

The screening of these isolates for the production of ochratoxin A in liquid medium showed that an isolate from poultry feed elaborated highest amount of ochratoxin A (200 ppm), followed by an isolate from the dry coconut (50 ppm) and sorghum (5 ppm) as presented in Table 3.7.

Ninety-nine fungal species other than *A.ochraceus* group isolated from these commodities, belonging to various common genera were screened for the elaboration of ochratoxin A. The fungi belong to *A.flavus* (35), *A.niger* (14), *A.oryzae* (4), *A.sydowii* (8), *Penicillium* species (16), *Alternaria* species (8), *Rhizopus* species (2) and unidentified white fungus (12) were screened. However none of them produced ochratoxins.

3.4 DISCUSSION

In the recent times reporting of ochratoxigenic fungi amongst common foodborne fungal genera has disturbed the scientific community. Ochratoxin A production by other group of *Aspergillus* belonging to section *Nigri*, *Terrei*, *Usti*, *Versicolores*, *Wentii* and *Fumigati* has been reported (Abarca, *et al.,* 2001). Moreover, the black *Aspergilli* are worldwide in distribution occurring on wide variety of substances and are widely used in food, enzyme and other related Industry and have also been identified as source of protein for human and animal consumption (Beuchat, 1987; Mathot, *et al*., 1992). Therefore, an effort was made to screen all the common fungal isolates to evaluate their potential to produce ochratoxins. In the present investigation none of the fungal species other than *A.ochraceus* group produced ochratoxins.

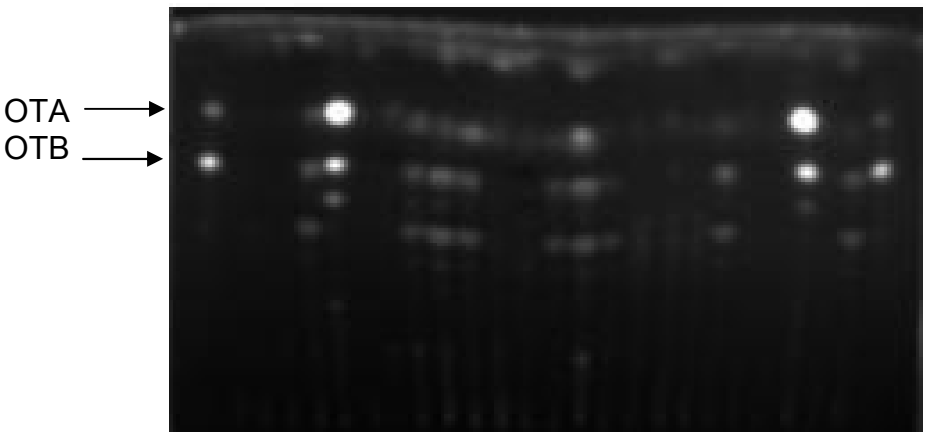

Fig. 3.4 TLC chromatogram of culture extracts from *A.ochraceus* group

Table 3.7 Toxigenic potential of food isolates belonging to *A.ochraceus* group.

Source (No. of isolates)	Toxigenic * Potential %	Ochratoxin A range (in ppm)
Maize	42.8 (3/7)	1.1 - 2.5
Coffee beans	50 (3/6)	1.0 - 2.5
Poultry feed	100 (2/2)	1.0, 200
Sorghum	50 (1/2)	5.0
Rice	100 (1/1)	2.5
Copra	100 (1/1)	50
Chili	50 (1/2)	1.2
Groundnut cake	100 (1/1)	0.4

***** Figures in parenthesis indicate number of isolates producing ochratoxin A / total number of *A.ochraceus* group screened.

Table 3.8 *A.ochraceus* group of fungi and their toxigenic potential

Species identified	Total isolates	No. of toxigenic isolates	Toxigenic potential %
A. ochraceus	8	7	87.5
A. melleus	4	1	25
A. sulphureus	4	2	50
A. auricomus	2	2	100
A. ostianus	2	1	50
A. sclerotiorum	2	0	0
	22	13	59

Among 22 isolates belonging to *A.ochraceus* group screened for ochratoxin A production 59.2% (13/22) produced this mycotoxin. All the food commodities tested, except groundnut and cottonseed were harboring species belonging to *A.ochraceus* group with incidence ranging from 11 - 50%. Moldy coffee bean, poultry feed and groundnut cake was naturally contaminated with ochratoxin A. Incidence of viable population of *A.ochraceus* group of fungi was present in all the ochratoxin A contaminated samples except in coffee sample contaminated with ochratoxin A (1.25 ppm), wherein the incidence of viable population of ochratoxigenic was absent. This indicates the initial abuse of coffee sample by ochratoxigenic strain that did not survive coffee processing conditions.

Surveillance of ochratoxin A in UK revealed wide spread low level OTA contamination of cereal and pork products (MAFF, 1994b; 1995). The survey of moldy green coffee (267 bags) revealed only one sample with high level at 0.36 ppm of ochratoxin A (Levi, *et al.,* 1974). Poultry feed, pork and coffee have been reported to be contaminated with ochratoxin A (Jorgensen, 1998).

Ochratoxin A contamination in different food commodity from India has been reported by Rao, *et al*. (1979). They reported highest contamination in a sample of groundnut seed (2 ppm) followed by 0.07 ppm level in two samples each of finger millet and pearl millet and in others the level was <0.05 ppm. Of the 180 food grains surveyed from India only 6%, comprising groundnut, corn, wheat, finger millet and pearl millet were naturally contaminated with ochratoxin A. In recent years ochratoxin A contamination has been reported in chilli, coriander, black pepper, turmeric and ginger, which were above the regulatory limit of 5 ppb implemented by European Union (Thirumala-Devi, *et*

al., 2000; 2001). The moldy coffee sample in the present survey had highest level (1.25 ppm) of ochratoxin A. These reports are of concern and emphasize the need for monitoring of food commodities and fungal strains of industrial application for ochratoxin A and ochratoxigenic potential respectively. The investigation showed that the natural occurrence of ochratoxin A is not very common. However frequency of the occurrence of the toxigenic strain are rather common in food commodities, which often require constant monitoring by food analyst.

CHAPTER 4.0

IMMUNOLOGICAL METHODS FOR STUDYING OCHRATOXIGENIC FUNGI

CHAPTER 4A

MICROPLATE IMMUNOASSAY FOR MONITORING OF *A.OCHRACEUS* IN FOODS

CHAPTER 4A

MICROPLATE IMMUNOASSAY FOR MONITORING OF *A.OCHRACEUS* IN FOODS

RESULTS AND DISCUSSION

4.1 INTRODUCTION

A.ochraceus has been implicated as the main source of ochratoxin A contamination in tropical countries. The presence of ochratoxigenic fungi in foods indicates possible contamination of ochratoxin A in the commodities. Occurrence of toxin producing molds in food and feed commodities is of concern and hence suitable methods for monitoring the toxigenic fungi is necessary to control and implement regulatory strategies. The plating methods routinely used in most of industries and laboratories indicate an overall mold count, which only represents the degree of sporulation rather than actual fungal biomass and are subject to high degree of variations (Jarvis, *et al*., 1983). Moreover the plate count technique is time consuming and does not detect dead fungi, which is a sign of past fungal contamination (Gourama and Bullerman, 1995a). The various methods based on biochemical analysis such as ergosterol and glucoseamine estimation gives an indirect measure of fungal biomass on solid substrate and cannot be used for comparison in foods differing in composition (Desgranges, *et al*., 1991). In the field of agriculture, immunoassays have been widely used as screening method for molds and their toxins (Chu, 1984; Li, *et al*., 2000). ELISA methods are rapid, sensitive and require very little sample preparations. Although various ELISA methods have been developed for detection of mycotoxigenic fungi, the data on application of these for estimation of fungal biomass on solid substrate is limited and are not adequate (Tsai and Yu, 1999). This chapter describes an immunoassay for estimation of fungal biomass and its application in monitoring of *A.ochraceus* growth in samples of coffee, chili and poultry feed.

4.2 MATERIALS AND METHODS

4.2.1 Cultures

The cultures used in the present study are presented in Table 4.1. *A.ochraceus* CFR 221, an isolate from coffee beans, maintained at the culture collection at C.F.T.R.I. was used for raising antibody. The cultures were grown on PDA slants and maintained at 4 ± 2 ^{o}C.

4.2.2 Chemicals

Anti-rabbit IgG HRP conjugate, Ochratoxin A standard, 3,3,5,5-Tetra methyl Benzidine (TMB), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were purchased from Sigma, USA. Microplates were from NUNC, Denmark. Hydrogen peroxide (H_2O_2), Commassie Brilliant blue G-250 was from SRL chemicals, India. All other chemicals were of analytical grade.

4.2.3 Assessment of viable count:

Conventional dilution plating technique was employed to assess the viable count of *A.ochraceus* in food commodities as described in section 3.2.1.4.

4.2.4 Preparation of antigen

The mycelial antigen of *A. ochraceus* CFR 221 was prepared following the method of Tsai and Yu (1997). Spore suspension from 7day old *A.ochraceus* CFR 221 culture grown on PDA slants was made in 0.5% peptone broth with 0.05% tween-20. Spore suspension (A_{440} 0.6) at 1% level was inoculated into Brain Heart Infusion broth (Himedia, Mumbai, India) in 250ml Erlenmeyer flasks and grown under shake culture condition (150 rpm) for 3 -10 days at 30^{o}C.

Table 4.1 List of cultures.

Culture	Source *
Aspergillus ochraceus CFR 221	CFTRI, India.
Aspergillus ochraceus MTCC 1877	MTCC, India
Aspergillus ochraceus MTCC 1877	MTCC, India
Aspergillus sclerotiorum CFR 227	Groundnut cake
Aspergillus ostianus CFR 228	Chili
Aspergillus melleus CFR 226	Maize
Aspergillus auricomus CFR229	Dried coconut
Aspergillus sulphureus CFR230	Coffee beans
Phoma CFR 231	CFTRI, India.
Aspergillus niger CFR 1398	CFTRI, India.
Pencillium verrucossum MTCC *2007*	MTCC, India.
Penicillium viridicatum MTCC 1758	MTCC, India
Aspergillus oryzae NCIM 665	NCIM, India
Fusarium NCIM 3329	NCIM, India

*MTCC - Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

CFR - Culture collection, CFTRI, Mysore, India.

NCIM – National culture collection center of Industrial Microorganism. National Chemical Laboratory, Pune, India.

The fungal biomass was separated and washed with cold sterile distilled water and freeze-dried. Freeze-dried mycelium was resuspended at 1% level in phosphate-buffered saline (PBS; 0.001 mol/L KH_2PO_4, 0.008 mol/L Na_2HPO_4, 0.002 mol/L KCl, 0.15 mol/L NaCl) pH 7.2 at 37 ^oC and stirred at 150 rpm for 4 h. The extract was centrifuged at 8000 x g for 30 min and the supernatant was collected. The protein content of the mycelial extract was measured by Bradford's method (Bradford*,* 1976).

4.2.5 Immunization and antibody production

Female New Zealand white rabbit weighing 1 - 1.5 kg was immunized with 1 mg (protein) of mycelial antigen of *A.ochraceus* CFR 221 in 0.5 ml PBS (pH 7.2) emulsified with 0.5 ml Freund's complete adjuvant (FCA). Immunization of rabbit was by intramuscular injections at multiple sites. Subsequent booster injections of mycelial antigen (0.5 mg protein) in Freund's incomplete adjuvant (FICA) were given at weekly intervals. The trial bleeding and final bleeding were done after 7 days of 3^{rd} and 4^{th} booster dose respectively. The serum was separated and the immunoglobulin (IgG) fraction was isolated by precipitation in saturated ammonium sulphate (66%) and dialyzed exhaustively against PBS (pH 7.2) as described by Tijssen (1985) and purified IgG was stored at –20 ^oC.

4.2.6 ELISA

An indirect non-competitive ELISA was standardized using optimized reagent concentration obtained by checkerboard titration. Microtitre plate was coated with 100 µl of antigen/sample per well in PBS (pH 7.2) and incubated overnight at 4^oC. Appropriate experimental controls comprising wells without

antigen and antigen coated plates treated with pre-immune serum or without antibody were maintained. The antigen coated plate was washed thrice with PBS containing 0.05% (w/v) Tween–20 (PBS-T) and blocked with skimmed (defatted) milk (1% w/v) for 1 h at 37 ^{o}C. Anti-*A.ochraceus* antibody (100 µl) diluted 1:1000 in PBS was added into each well and incubated at 37 ^{o}C for 1 h. The plate was washed thrice with PBS-T and incubated with anti-rabbit IgG HRP conjugate (100 µl/well) diluted 1:5000 in PBS for 30 min at 37 ^{o}C. The plate was washed thrice with PBS-T and incubated with 150 µl TMB-H_2O_2 substrate (0.01 % TMB in 0.1 mol/litre sodium acetate buffer pH 5.0, containing 0.005% H_2O_2) per well for 20 min. The reaction was stopped by adding 10% aqueous sulphuric acid (50 µl) and absorbance read at 450 nm in ELISA plate reader (Model spectramax 340, Molecular devices, USA).

4.2.7 Antibody specificity

Antibody specificity was determined by following the protocol of Tsai and Yu (1997). The mycelial extract was prepared as described earlier from different fungal cultures listed in Table 4.1. Microtiter plates were coated with mycelial extracts (100 µl) of each test cultures (50 µg/ml) and the mycelial extract of *A.ochraceus* CFR 221 culture (50 µg/ml) used for raising antibody. The assay protocol for ELISA (Section 4.2.6) was followed**.** The relative activity of antibody against each test strain was defined as the ELISA reading of test strain divided by the ELISA reading of *A.ochraceus* CFR 221.

4.2.8 Food extracts

Extracts of food were prepared according to Notermans and Heuvelman, (1985) with some modification. Food samples were diluted 10 fold in PBS and homogenized with mortar and pezzle. The homogenates were centrifuged at 10, 000 x g for 10 min and supernatant were collected.

4.2.9 Influence of food matrix on fungal biomass estimation

A known quantity of freeze-dried mycelia was homogenized in PBS with mortar and pezzle, centrifuged at 10,000 x g and supernatants were collected. To determine the matrix effect on ELISA response the extracts prepared from the food samples was mixed with the serially diluted mycelial extracts (1:1, v/v). The treated samples (100 µl) were analyzed by ELISA method as described earlier (Section 4.2.6). The food extract without mycelial extract served as control. The standard graph was prepared for each commodity by plotting ELISA reading against fungal biomass per ml of food extract.

4.2.10 Artificially infected food commodities

Food and feed samples (50 g) were powdered to pass through a 20 BSM sieve (700 µm) and sterilized in 250 ml conical flasks. The flasks were inoculated with spore suspension (A_{440} 0.6) of *A.ochraceus* CFR 221 at 1% level and moisture was adjusted to 20% and 30% with sterile distilled water. The control consisted of uninoculated sterilized food samples. All the samples were incubated at 28 ± 2^{o}C and at different intervals of time flasks were withdrawn and stored at –20^{o}C. The samples were analyzed for fungal biomass by ELISA. Ochratoxin A content was also analyzed.

4.2.11 Estimation of fungal biomass

The food samples were diluted 20 fold in PBS, homogenized with mortar and pezzle, centrifuged at 10000 x g and supernatants were collected. The extracts were analyzed by ELISA (Section 4.2.6). Simultaneously known quantity of food sample was dried at 80 ^{o}C for determination of moisture. The equation derived through regression analysis of the standard graph obtained for each food extract was used for the estimation of the fungal biomass in the samples. Using the ELISA estimate and moisture content of the food sample the fungal biomass per g of food sample (dry weight) was calculated.

4.2.12 Ochratoxin A analysis

Ochratoxin A was analyzed by following AOAC procedure (AOAC 1997). The toxin in the sample (10 g) was extracted with acidified chloroform (1:5) and purified over alkaline diatomaceous earth chromatographic column. Quantification of ochratoxin A was by TLC and densitometry as described in section 3.2.3.4. All sample extracts along with ochratoxin A standard were spotted on TLC plate and developed in benzene-methanol-acetic acid (18:1:1) solvent system. The TLC chromatogram was scanned in Camag Scanner (CAMAG, Switzerland) in the fluorescence/reflectance mode set at 360 nm with K400 filter using CAMAG CATS V.4.software. The ochratoxin content in the sample were determined by comparison of ochratoxin A standard R_f and the corresponding fluorescence intensity (peak area) of the samples as described in section 3.2.2.3.4.4.

4.2.13 Statistical analysis:

The data obtained for different sets of growth and ochratoxin A production experiments were analyzed by ANOVA (analysis of variance) using Microsoft Excel version 97.

4.2.14 Detection of fungal spore in food commodities

A short enrichment technique was followed for detection of *A.ochraceus* spores in poultry feed sample. Sterilized poultry feed sample was inoculated with *A.ochraceus* spore suspension (10^1 – 10^3 cfu/g). Samples were diluted 20 fold in potato dextrose broth prepared in PBS and incubated for 12 h at 28^oC± 2°C. The treated samples were processed for estimation of fungal biomass as described in section 4.2.11. The poultry feed sample inoculated with spores and without enrichment in potato dextrose broth served as control.

RESULTS AND DISCUSSION

4.3 RESULTS

4.3.1 Antibody Specificity

The specificity measured in terms of relative ELISA activity of anti-*A.ochraceus* antibody with various test cultures are shown in Table 4.2. The antibody was relatively specific to *A.ochraceus* and exhibited 93 -120% cross reaction with *A.ochraceus* species. Cross reaction with the other closely related species belonging to *A.ochraceus* group such as *A.sulphureus*, *A.melleus*, *A.auricomus and A.ostianus* were in the range of 40 - 57%. The antibody had less than 10% cross-reaction with *Fusarium* and *Phoma* species and 10 - 17% cross reaction with *Penicillium, A.niger* and *A.flavus.*

4.3.2 Estimation of fungal biomass

The ELISA response for *A.ochraceus* fungal biomass prepared in PBS is shown in Fig. 4.1. The ELISA response was sensitive to fungal biomass at 5 µg/ml, which was significantly higher ($P < 0.05$) than the absorbance obtained in control well (PBS with no fungal biomass). The ELISA response correlated positively with fungal biomass with coefficient of correlation of 0.91 for 5 –160 µg fungal biomass /ml of food extract. The food extract has profound influence on the ELISA responses while determining the fungal biomass. The effect of matrix (food extract) while quantifying fungal biomass by ELISA is therefore plotted for each commodity. The fungal biomass correlated well with the ELISA reading and was linear in the range of 10 -160 µg per ml food extract in all the commodities with regression coefficient (R^2) of 0.989 (standard error = 0.0320), 0.989 (standard error = 0.0212), and 0.986 (standard error = 0.0313)

Table 4.2 Relative ELISA activity of anti-*A ochraceus* antibody with various molds.

Test Culture [a]	RA[b] (at 450nm)
Aspergillus ochraceus CFR 221 [c]	1.00
Aspergillus ochraceus MTCC 1877	0.93
Aspergillus ochraceus MTCC 1810	1.23
Aspergillus melleus CFR 226	0.43
Aspergillus sclerotiorum CFR 227	0.48
Aspergillus ostianus CFR 228	0.41
Aspergillus auricomus CFR 229	0.57
Aspergillus sulphureus CFR 230	0.49
Aspergillus niger CFR 1398	0.12
Aspergillus oryzae NCIM 665	0.17
Pencillium verrucossum MTCC 2007	0.12
Penicillium viridicatum MTCC 1758	0.11
Phoma CFR 231	0.03
Fusarium NCIM 3329	0.05

[a] ELISA was performed with 50 μg/ml of each culture.

[b] Relative ELISA activity (RA) = ELISA reading of tested culture/ELISA reading of antibody inducing strain.

[c] Antibody inducing strain.

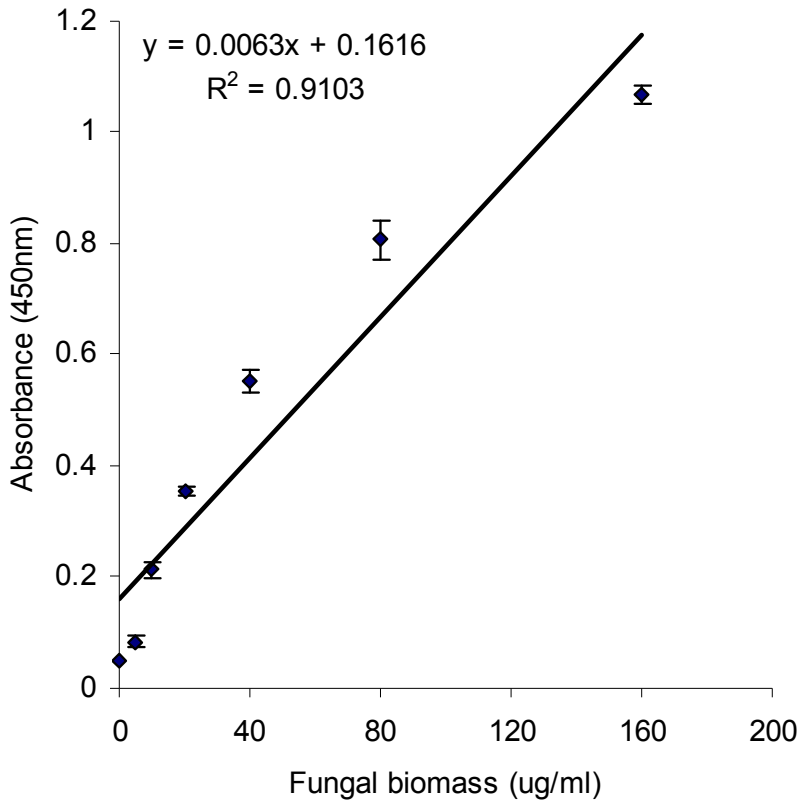

Fig. 4.1 Dose response of Indirect non-competitive ELISA for *A.ochraceus* fungal biomass.

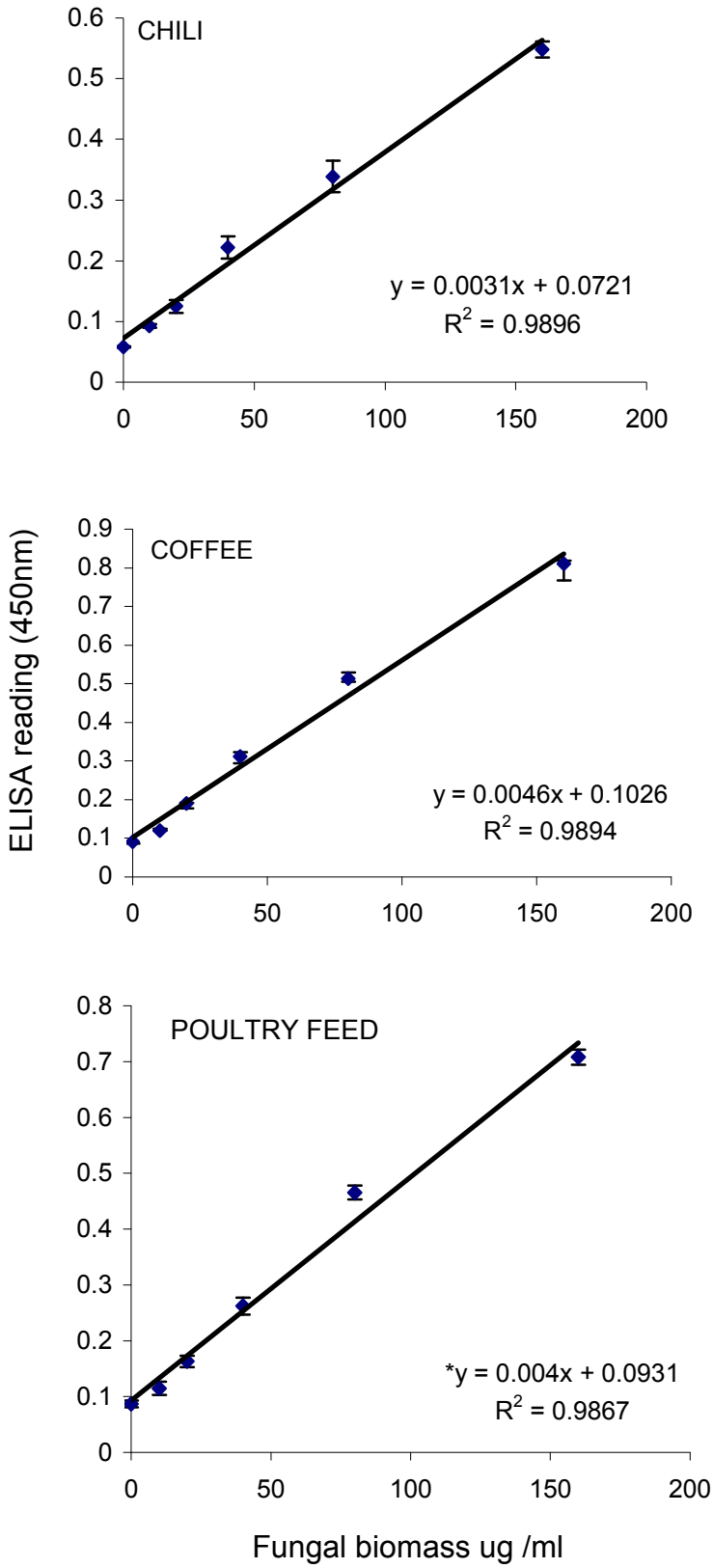

Fig 4.2 Standard graph used for determination of fungal biomass in food commodities. (* Regression equation and Regression coefficient).

for coffee, chili and poultry feed respectively. The standard graph obtained by plotting ELISA absorbance versus fungal biomass per ml of food extracts are shown in Fig 4.2. The maximum ELISA reading obtained in control wells (uninoculated food extract) were in the range of 0.059 (chili extract) - 0.12 (coffee extract), which were significantly lower ($P < 0.05$) than samples treated with 10 µg fungal biomass per ml of food extract. The standard graph generated for each commodity was subsequently used for monitoring the fungal biomass. The following equations were derived by regression-analysis fit to estimate fungal biomass $Y_{coffee} = 0.0046 X + 0.1026$, $Y_{chili} = 0.0031 X + 0.0721$ and $Y_{poultry\ feed} = 0.004 X + 0.0931$ in coffee, chili and poultry feed respectively, where y is the ELISA absorbance and X the biomass correlation coefficient.

4.3.3 Monitoring of *A. ochraceus* fungal growth in food commodities

4.3.3.1 Fungal biomass

The variations in fungal colonization were influenced by the substrate, moisture and incubation time. From an initial biomass of about 0.55 ± 0.01 mg /g, the fungus grew and colonized up to 9.8 ± 0.53, 6.8 ± 0.32 and 5.1 ± 0.27 mg/g in poultry feed, coffee and chili respectively at 20% moisture (Fig 4.3a). Similarly from an initial fungal biomass of about 0.53 ± 0.03 mg/g substrate the *A.ochraceus* grew and colonized up to 11.8 ± 0.29, 11.3 ± 0.34 and 6.3 ± 0.05 mg/g substrate after 20 days of incubation in poultry feed, coffee and chili respectively at moisture of 30% (Fig. 4.3b). The ANOVA for fungal growth (Table 4.3) show that the significant single factors affecting *A.ochraceus* growth were time ($P < 0.0001$) and substrate ($P < 0.001$).

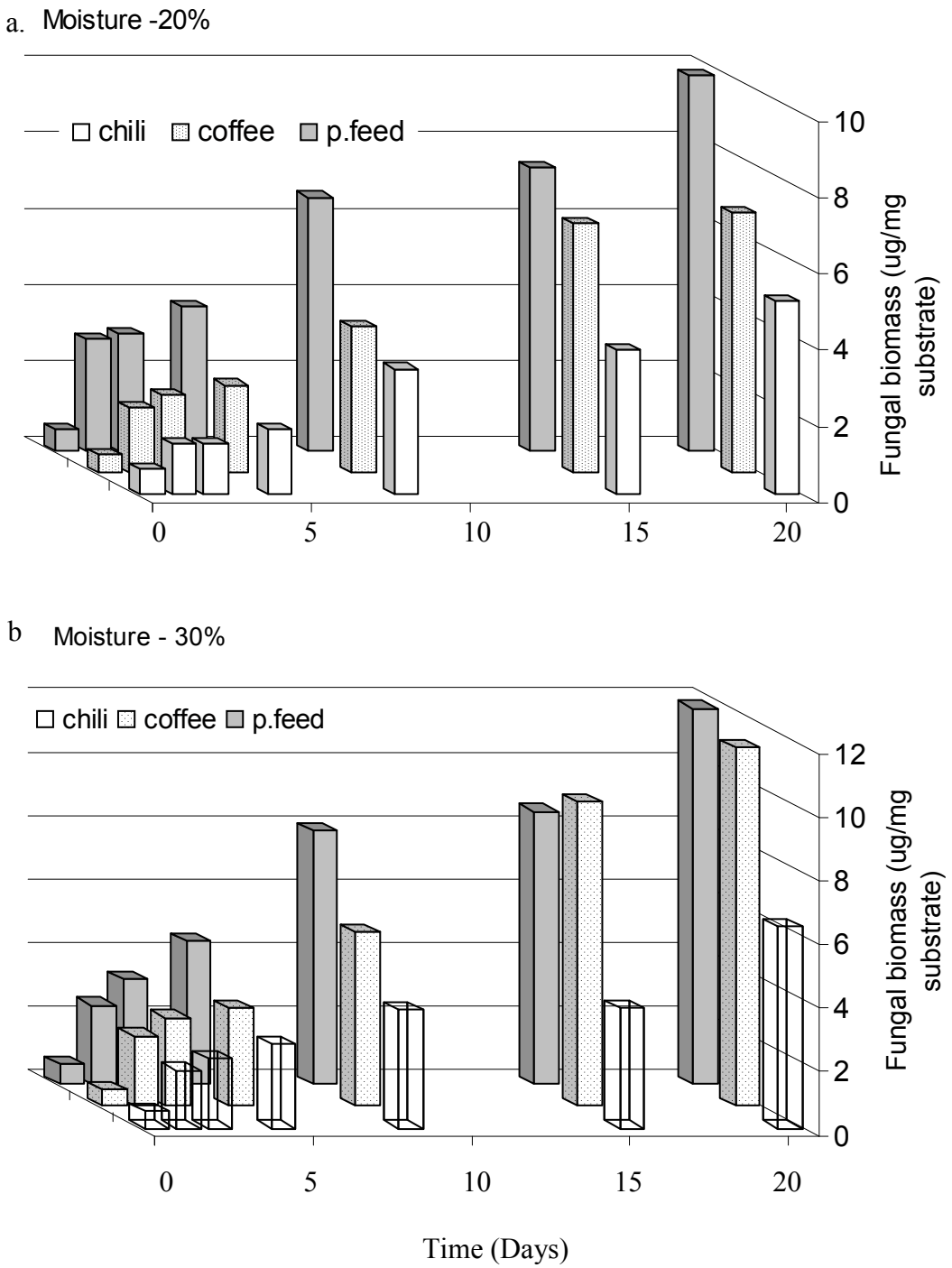

Fig 4.3 Influence of substrate, moisture and time on *A.ochraceus* colonization.

Moisture content a-20%, b-30%.

Table 4.3 ANOVA for *A. ochraceus* growth (fungal biomass).

Source of Variation	Degree of freedom	Mean Square	F	P-value
Time	6	148.62	65.24	< 0.0001
Substrate	2	59.88	7.06	< 0.001
Moisture	1	28.43	3.10	0.08 ^{ns}
Time-substrate	12	6.16	8.37	< 0.0001
Moisture-time	6	3.30	1.66	0.13^{ns}
Moisture-substrate	2	3.58	0.42	0.60 ^{ns}

* *ns –not significant*

The two factor interactions of time and substrate had a significant effect (P <0.0001), among the different two factor interaction analysis.

4.3.3.2 Viable count

The influence of moisture on viable count of *A.ochraceus* was not apparent. Comparatively higher increase in viable count (2 - 3 $\log_{10}$ cfu/g) was observed after 2 days of incubation in poultry feed than other substrates. Maximum viable count (~9 $\log_{10}$ cfu/g) was observed after 8 days of incubation in all the commodities studied. The viable counts tend to decrease at the end of 20 days of incubation in all the food commodities (Fig. 4.4 a, b, c). The ANOVA for fungal viable count (Table 4.4) show that the significant single factors affecting *A.ochraceus* viable count were time (P <0.0001) and substrate (P < 0.05). The two factor interactions of time and substrate had a significant effect (P < 0.0001), among the different two factor interaction analysis.

4.3.4 Ochratoxin A production in food commodities

The substrate moisture had a marked influence on time of ochratoxin A elaboration by the fungus. Detectable amount of ochratoxin A was produced after 2 days of incubation in all the food commodities at 30% moisture, whereas ochratoxin A was detected after 4 – 8 days of incubation at 20% moisture (Fig 4.5 a, b, c). Maximum ochratoxin A was produced in poultry feed with an ochratoxin A content of 120 ± 0.5 µg/g followed by ochratoxin A content of 24 ± 0.2 µg/g and 0.45 ± 0.02 µg/g in coffee and chili respectively at 30% moisture after 20 days of incubation (Table 4.6).

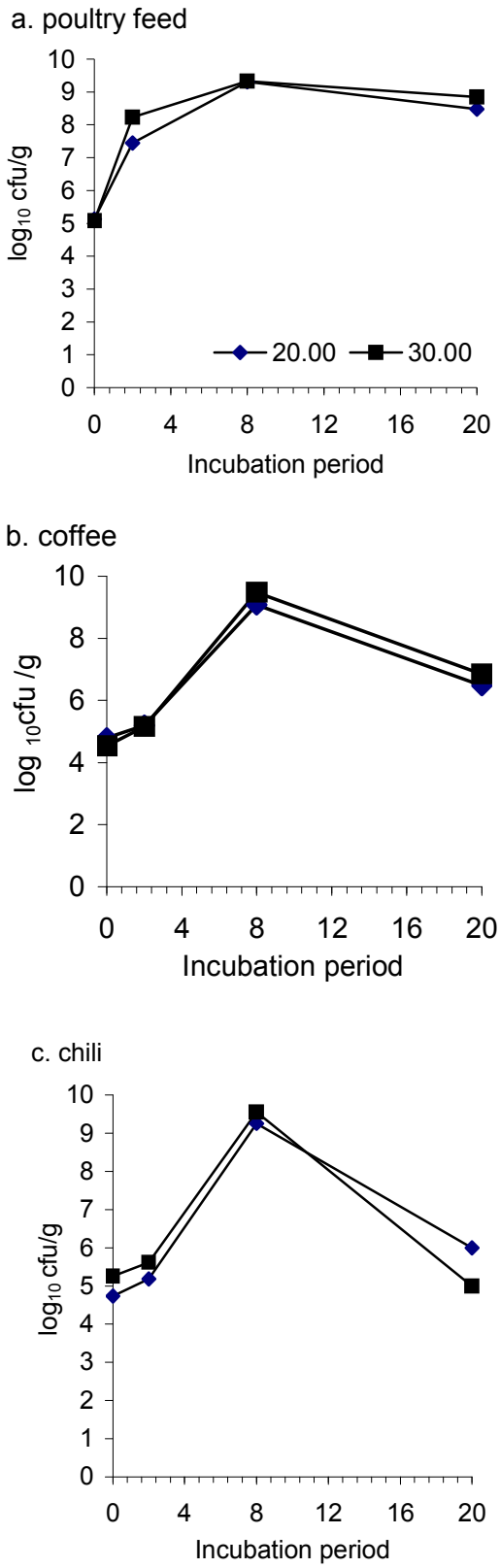

Fig 4.4 Influence of substrate, moisture and time on growth (viable count) of *A.ochraceus*. Substrate: a-poultry feed, b- coffee, c-chili.

Table 4.4 ANOVA for *A. ochraceus* growth (viable count).

Source of Variation	Degree of Freedom	Mean square	F	P-value
Substrate	2	13.92	4.51	< 0.05
Moisture	1	1.08	0.31	0.57^{ns}
Time	3	61.03	72.19	< 0.0001
Moisture-substrate	2	0.04	0.01	0.98^{ns}
Time-substrate	6	4.19	56.45	< 0.0001
Moisture-time	3	0.082	0.09	0.96^{ns}

* *ns –not significant*

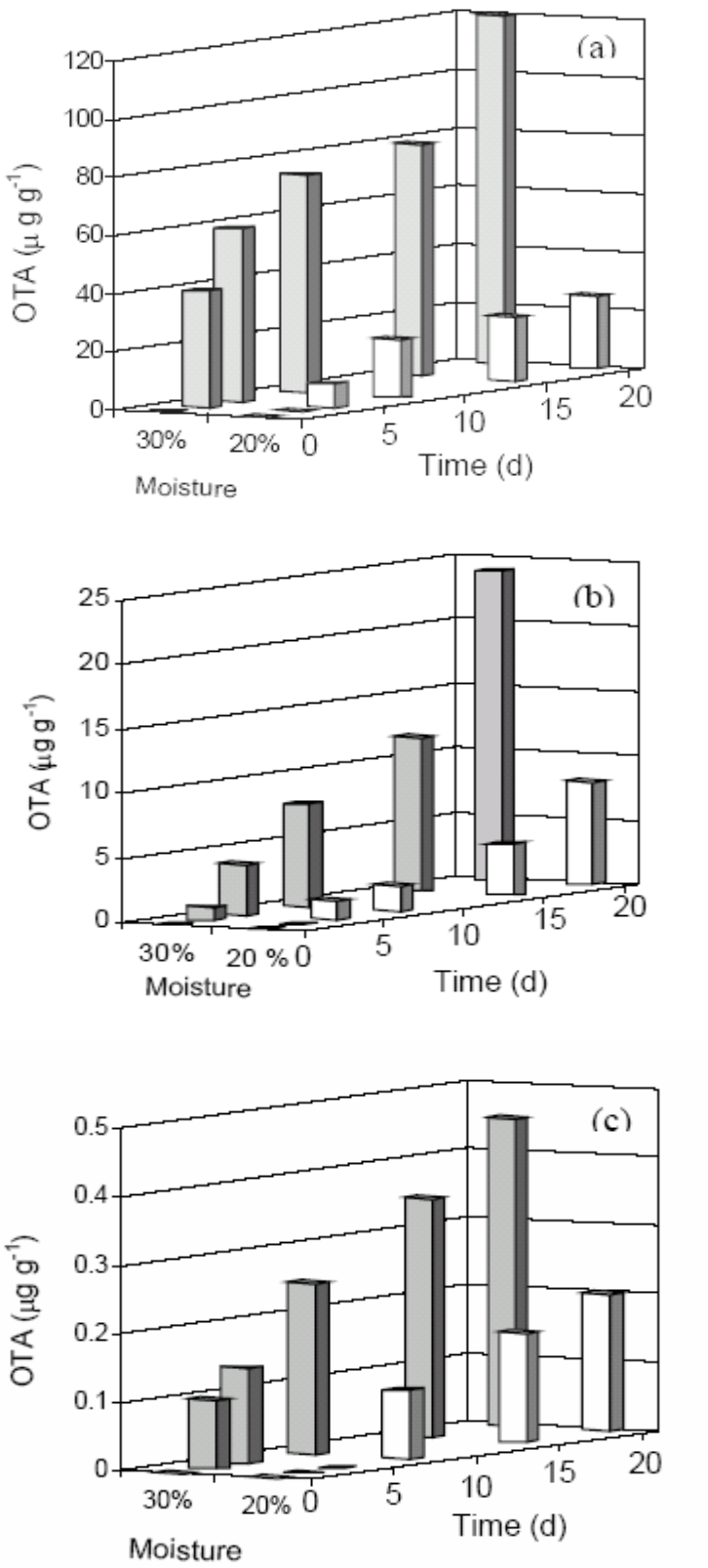

Fig 4.5 Influence of substrate, moisture and time on ochratoxin A production by *A.ochraceus*. Substrate: a-poultry feed, b- coffee, c-chili.

Table 4.5 ANOVA for ochratoxin A production by *A. ochraceus*.

Source of Variation	Degree of Freedom	Mean square	F	P-value
Substrate	2	9963.03	20.13	< 0.0001
Moisture	1	6255.80	9.15	< 0.005
Time	5	1305.09	1.81	0.12^{ns}
Moisture-substrate	2	4522.99	16.00	< 0.0001
Time-substrate	10	722.33	1.93	0.06^{ns}
Moisture-time	5	437.31	0.68	0.64^{ns}

ns –not significant

Table 4.6 Influence of moisture and substrate on *A. ochraceus* growth and ochratoxin A production.

Substrate	Moisture	Fungal growth		Ochratoxin A (ppm)
		Fungal biomass (μg/mg substrate)	Viable count (log_{10} cfu/g)	
Chili	20	5.1 ± 0.27	5.84 ± 0.16	0.20 ± 0.05
	30	6.3 ± 0.05	5.40 ± 0.40	0.45 ± 0.02
Coffee	20	6.8 ± 0.32	6.36 ± 0.11	8 ± 0.70
	30	11.3 ± 0.34	7.05 ± 0.20	24 ± 0.20
Poultry feed	20	9.8 ± 0.53	8.24 ± 0.24	25 ± 0.80
	30	11.8 ±0.29	8.87 ± 0.44	120 ± 0.50

Similarly at 20% moisture maximum ochratoxin A elaboration was observed in poultry feed with an OTA content of 25 ± 0.8, followed by 8 ± 0.7 and 0.2 ± 0.05 µg/g in coffee and chili respectively. The ANOVA for ochratoxin A production (Table 4.5) show that the significant single factor affecting ochratoxin A production by *A.ochraceus* was substrate ($P < 0.0001$) and moisture ($P < 0.005$). The two factor interactions of substrate and moisture had significant effect ($P < 0.0001$), among the two factor interactions.

4.3.5 Detection of fungal spores in poultry feed

The ELISA showed a detection limit of $\geq 10^5$ spores/g for *A.ochraceus* spores. Enrichment in potato dextrose broth increased the detection limit to $\geq 10^3$ spores/g. The biomass was estimated at 0.56 ± 0.13 µg fungal biomass per mg of substrate (Table 4.7) in poultry feed sample spiked with 10^3 spores/ g and incubated at 28 ± 2 ^{o}C for 12 h in potato dextrose broth.

4.4 DISCUSSION

The immunoassay developed to monitor the fungal biomass in different substrates is sensitive to low level of mold colonization. The method was sensitive to fungal infestation of ≥0.2 µg fungal biomass per mg of substrate. The ELISA method can detect the fungal colonization well before the visible mold growth. The visible mold growth for different substrates studied varied from 2 days in poultry feed and coffee to 5 days in chili. In the present study there was relatively lower rate of increase in fungal biomass compared to increase in viable count (Fig. 4.3 & 4.4). This may be due to lower sensitivity of antibodies towards spore and inherent limitations of viable count analysis.

Table 4.7 Determination of fungal biomass by ELISA in poultry feed inoculated with *A.ochraceus* spores

Fungal load (spores /g)	Fungal biomass (µg/ mg substrate)	
	Control	Treated
10^3	ND	0.56 ± 0.13
10^2	ND	ND

* ND - not detected

Treated: Enrichment of spores in PDB (12h).

The viable count depicts degree of sporulation rather than the actual fungal growth (Jarvis, *et al*., 1983). The viable count may come from both spores and hyphal fragments. Antibodies raised against mycelial antigens are reported to be less sensitive to fungal spore (Tsai and Yu, 1999). The differential sensitivity may be due to melanin deposition during conidial maturation (Bartnicki-Garcia, 1968), The antibody sensitivity could be increased by physical rupturing of spore coat or germination of spore. In the present study a short enrichment of fungal spore in potato dextrose broth was evaluated to increase the sensitivity of antibody towards germinated spores. The fungal spores of $\geq 10^3$ cfu/g could be detected after 12 h enrichment in potato dextrose broth (Table 4.7).

Most of the antibodies raised against mycelial antigens are genus-specific (Li, *et al*., 2000). The antibodies raised against mycelial antigen in the current study were relatively specific to *A.ochraceus*. Lu and coworkers (1995) reported an immunoassay for determination of *A.ochraceus* in wheat based on antibodies raised against exoantigen. They reported that the immunoassay is specific to *A.ochraceus* with 0 - 2.5% cross-reaction with other fungal cultures and sensitive to exoantigen concentration at 0.19 µg per ml. They stated that the quantity of fungal biomass will be much higher than the exoantigen concentration and hence the sensitivity in terms of fungal biomass will be much higher than 0.19 µg per ml. As they have not reported actual fungal biomass, no direct comparison can be made with our method. Although exoantigens are good source for raising species-specific antibodies, their quantity per unit fungal biomass is reported to vary with type of media and age of culture and hence immunoassays using antibodies raised against

exoantigen has limitation in use for accurate estimation of fungal biomass in foods (Li, *et al*., 2000).

Notermans *et al.* (1986) reported genus specific immunoassay based on antibodies raised against mycelial antigens with sensitivity ranging from 6 - 108 ng per ml for different fungi in analysis involving fruit juices. The present method is less sensitive than that reported by Notermans *et al.* (1986). Similarly, Tsai and Yu (1999) reported an immunoassay with lesser sensitivity (1 µg/ml) in analysis involving solid food substrates such as corn, rice, wheat, and peanut. The higher sensitivity reported by Notermans *et al.* (1986) may be due to liquid substrate used (fruit juices). In analysis involving solid substrate, the fungus is intimately bound to solid matrix and hence not available for direct analysis (Harris and Kell, 1985).

Ergosterol and glucoseamine estimation have been used as tool for estimations of *A.ochraceus* growth in food commodities, but have their own limitation. Nout *et al.* (1987) reported the limitations of using ergosterol measurement as a chemical index for the quantification of fungal biomass in solid-substrate fermentations. They demonstrated that ergosterol content varies significantly from 2 to 24 µg/mg of fungal biomass when grown under different synthetic media and varied from 60 to 90 µg/mg of fungal biomass when grown on solid substrates. Saxena *et al.* (2001) used ergosterol measurement as index of *A.ochraceus* biomass in rice and reported a decrease or constant ergosterol content after a period of 10 days although growth (increase in viable counts) of the fungus was apparent. Hagglabom (1982) reported an increase of 2 - 3 fold of *A.ochraceus* biomass as expressed in glucoseamine content during 30 days of incubation. He reported

a linear relationship between glucoseamine content and fungal biomass in the range of 1 – 10 mg dry weight of mycelium and hence cannot be used for quantitative estimation of low levels (<1 mg) of fungal biomass in solid media.

Unlike ergosterol measurement the immunological detection of water extractable mold antigens has been shown to correlate well with fungal biomass under different growth conditions. Notermans *et al.* (1986) reported that the mold antigen correlates with the fungal biomass when grown in different synthetic media and natural food substrates such as fruit juice under different temperature, water activity and submerged/surface growth condition.

The influence of substrate and moisture on fungal colonization monitored by ELISA and ochratoxin A elaboration in our investigation is summarized in Table 4.6. Madhyastha *et al.* (1990) reported similar influence of substrate on *A.ochraceus* and *P.verrucossum* colonization and ochratoxin A production in different cereals and oilseeds. In the present study poultry feed supported highest ochratoxin elaboration and fungal growth, measured in terms of biomass and viable count. Rati (1975) reported comparatively higher influence of poultry feed extract on fungal spore germination, among the different food extracts analyzed. She reported 80 -100% germination of fungal spore within 6 to 8 h of incubation in poultry feed extract. The results suggests that poultry feed has factors which stimulate fungal growth and toxin elaboration and perhaps, this may be the reason for natural occurrence of high level of mycotoxin contamination observed in poultry feed, than other food commodities (Table 3.6).

The growth responses of individual fungal species to different environmental factors as determined from experiments conducted on artificial

synthetic medium has been shown to vary in different natural substrates (Magan and Lacey, 1988). In the present study the immunoassay was applied for studying the influence of substrate, moisture and time on *A.ochraceus* colonization in three food commodities representing feed, beverage and spice. The ELISA method gives a direct measure of fungal biomass for comparing the fungal growth responses in different natural substrates under different culture conditions. The relative specificity of antibody towards *A. ochraceus* antigen and detection of fungal spores by enrichment shows that the method can be adopted for monitoring of ochratoxigenic fungi in food commodities. The ELISA method can be adapted for studying specific fungal colonization in different food systems.

CHAPTER 4B

DOT- BINDING IMMUNOASSAY FOR DETECTION OF *A.OCHRACEUS* IN FOODS

CHAPTER 4B

DOT- BINDING IMMUNOASSAY FOR DETECTION OF *A.OCHRACEUS* IN FOODS

RESULTS AND DISCUSSION

4.5 INTRODUCTION

Detection and monitoring of food borne pathogens in food commodities in field and storage conditions is important for ensuring food products free from microbial hazard. Most of microplate immunoassays employed for detecting molds are optimized at controlled temperature requiring laboratory set up, thus makes it difficult to adopt the method in field conditions. The cost of solid phase used for microplate ELISA is a limitation for application of immunoassays for monitoring of mycotoxigenic fungi in developing countries. Hence there is a need for immunoassays with cheaper solid phase to overcome the limitation posed by microplate immunoassays. In this regard various synthetic membranes available can be used as solid matrix for immunoassays. Nitrocellulose membrane is relatively cheaper than microplate and can be adopted according to the needs of sample size and assay format. Nitrocellulose membrane (NC) has been widely used in various immunoblotting techniques for characterization of fungal antigen and antigen-antibody interactions (Hearn, *et al*., 1990; Lu, *et al*., 1995). Various dipstick immunoassays, which use NC as solid phase, are available for detection of mycotoxins (Saeger & Peteghem, 1996; Abouzied & Pestka, 1994). Commercial available membranes are also increasingly used for online flow through immunoassays for detection of microbial pathogens and their metabolites (Saeger and Peteghem, 1999). Dot-binding immunoassay obviates the need for sophisticated instruments and can be used for visual detection of the food spoilage organism with great potential for application at the field. Dot-binding immunoassay has great potential application in detection of fungal infestation in field condition. Moreover the color generated can be

analyzed densitometrically and stored for comparison. In the present study the work was carried out to develop dot-binding immunoassay for detection of *A.ochraceus* in food.

4.6 MATERIALS AND METHODS

4.6.1 Culture

The list of fungal cultures used in the present study is presented in Table 4.8. Fungal cultures were grown on PDA slants and maintained at 4 ± 2^{o}C.

4.6.2 Chemicals

Anti-*A.ochraceus* antibody was from previous study (chapter 4A). BCIP-NBT substrate and anti-rabbit IgY ALP conjugate were procured from Bangalore, Genei, India. Anti-rabbit IgG HRP enzyme conjugate and TMB was procured from M/s Sigma, USA. All other chemicals were of analytical grade.

4.6.3 Fungal extracts

Fungal extracts were prepared as described in section 4.2.4. Fungal extracts were used for determination of antibody specificity and sensitivity.

4.6.4 Food extracts

Extracts of food were prepared according to Notermans *et al.* (1985). Food samples were homogenized with double volume of Tris buffer saline (0.02 mol/L Tris, 0.15 mol/L NaCl, pH 7.2) in mortar and pezzle. The homogenates were centrifuged at 10,000 x g for 10 min and supernatant were processed for dot-binding ELISA.

4.6.5 Indirect non-competitive dot- binding ELISA

Mycelial extract/sample extract (2 µl), prepared from known concentrations of freeze-dried mycelium/food extracts was spotted equidistantly on nitrocellulose strips (30 mm x 5 mm, L x B). The strips were air dried and

blocked with 3% gelatin for 1h at room temperature (28 ± 2 ^{o}C). Excess gelatin was washed thrice with Tris buffer saline with 0.05% tween-20 (TBST) for 10 min each. The nitrocellulose strips were incubated with anti-*A.ochraceus* antibody diluted 1:100 with TBS (pH 7.2) for 1h at R.T. The strips were washed thrice with TBS-T for 10 min each. The strips were further processed separately for visual and spectrophotometric detection as illustrated in Fig. 4.6.

4.6.6 Visual detection

For visual detection of fungi the strips processed as described in section 4.6.5 were incubated with anti-rabbit IgG-Alkaline phosphatase enzyme conjugate diluted 1:1000 in TBS (pH 9.5) at R.T. for 1 h. The strips were washed thrice with TBS-T (pH 9.5) of 10 min each. The strips were incubated with BCIP-NBT substrate system at R.T under dark for 30 min. The nitrocellulose strips were observed for appearance of purple color and stored for further analysis. Nitrocellulose strips treated with no antigen or sample served as control.

4.6.7 Color measurement

The color on the NC paper was measured using colorimeter (Minolta chroma meter CR-321, Minolta, Japan) using software Jaypak version 2.0. The color was expressed as the difference (ΔE^{ab}) between the color of a white membrane as a reference membrane and the dot color intensity of the test membrane.

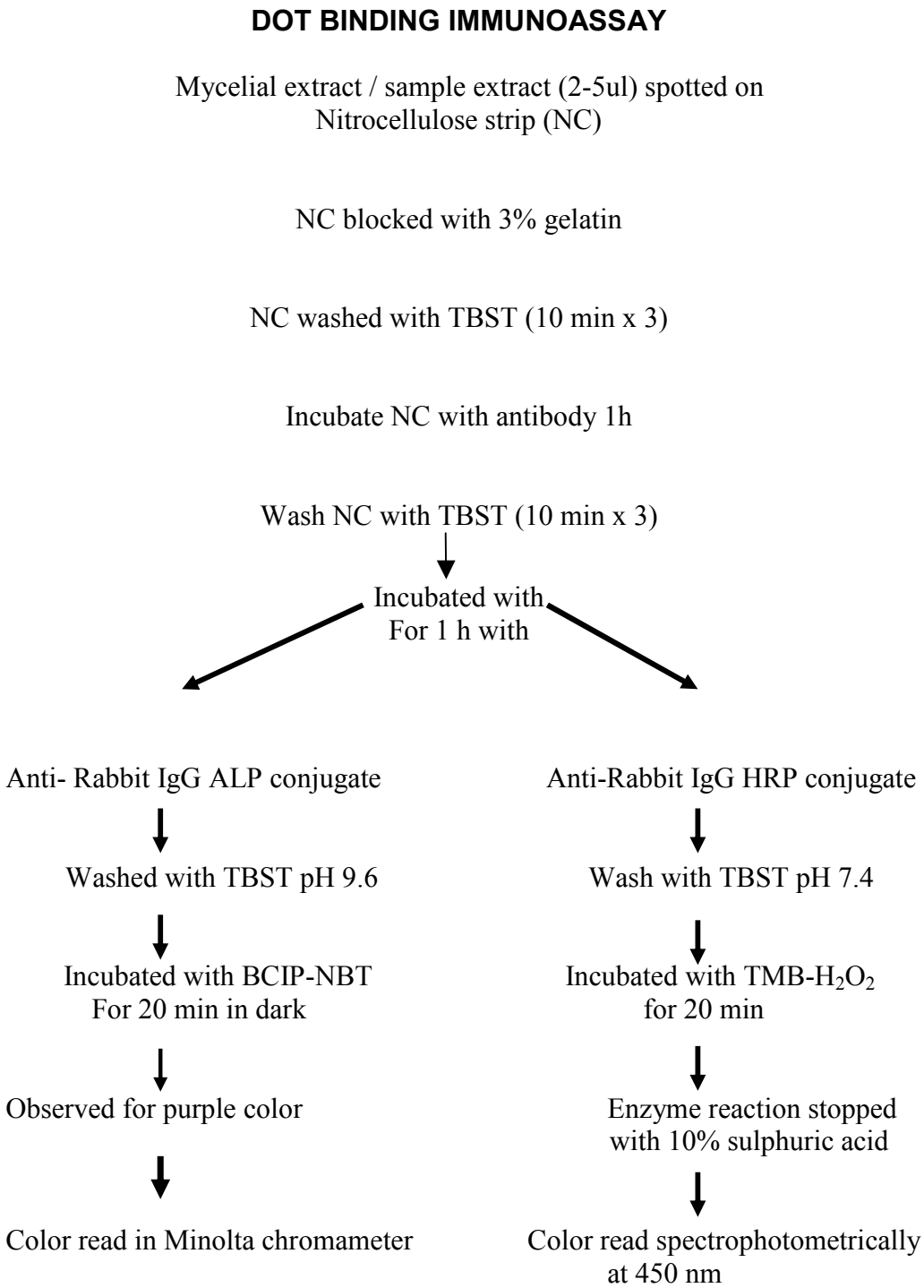

Fig. 4.6 Flow chart showing steps involved in DOT-ELISA

4.6.8 Spectrophotometric color measurement

The anti-*A.ochraceus* antibody treated NC (Section 4.6.5) were washed with TBS-T (pH 7.2). The NC strips were incubated with anti – IgG HRP conjugate diluted 1:1000 in TBS for 1 h at R.T. The strips were washed with TBS-T and cut apart into 5 x 5 mm strips around the antigen/sample spot. Each individual bits were transferred into labeled glass vials. Each vial was treated with 150 µl of TMB-H_2O_2 substrate (0.01 % TMB in 0.1 mol/l sodium acetate buffer pH 5.0, containing 0.005% H_2O_2) for 30 min. The color reaction was stopped with 50 µl of 10% aq sulphuric acid. The contents of individual vials were made up to 1 ml with distilled water and absorbance was measured at 450 nm in a spectrophotometer using negative control as blank.

4.6.9 Indirect competitive dot-binding ELISA

An indirect competitive ELISA was evaluated by the dot-binding immunoassay. The protocol was similar to that described in section 4.6.5 except *A.ochraceus* mycelial extract was used as competitor for the antibody. The strips were loaded with 10 µg (5 mg/ml, 2 µl) of *A.ochraceus* mycelial extract. The strips were air dried and blocked with 3% gelatin for 1 h at room temperature (28 $\pm$ 2 ^{o}C). Excess gelatin was washed thrice with Tris buffer saline with 0.05% tween-20 (TBS-T) for 10 min each. The strips were cut apart into 5 x 5 mm strips around the sample spot and transferred in to separate individual vials. The nitrocellulose strips were incubated with known quantity of mycelial extracts (50 µl) and anti-*A.ochraceus* antibody (100 µl) diluted 1:50 with TBS (pH 7.2) for 1 h at R.T. The strips incubated with no competitor (mycelial extracts) served as control. The strips were washed

thrice with TBS-T for 10 min each and processed further by the immunoassay for spectrophotometric determination (Section 4.6.8). A graph was plotted for inhibition of absorbance for different competitor concentration of *A.ochraceus* mycelial antigen.

4.6.10 Antibody specificity

A known quantity of fungal biomass was taken and mycelial extracts were prepared as described in section 4.2.4 and adjusted with buffer to give a concentration of 10 mg/ml. The extracts (5 µl, 50 µg) were spotted on nitrocellulose strips and processed by ELISA as described previously. The color developed was measured by Minolta chromameter (Section 4.6.7). The relative activity of antibody against each test strain was defined as the ELISA reading of test strain divided by the ELISA reading obtained for *A. ochraceus* CFR 221.

4.6.11 Sensitivity determination

The minimum quantity of fungal biomass that could be detected by the dot-binding ELISA was determined. A known quantity of *A.ochraceus* CFR 221 mycelial extracts was serially diluted. The extracts (2 µl) were spotted on nitrocellulose strips and processed by the dot-binding immunoassay (Section 4.6.5 & 4.6.7).

4.6.12 Influence of food matrix on dot-binding immunoassay.

A known quantity of freeze-dried mycelia was homogenized in PBS with mortar and pezzle, centrifuged at 10000 x g and supernatants were collected. To determine the matrix effect on ELISA response the extracts prepared from

the food samples was mixed with the serially diluted mycelial extracts (1:1, v/v). The treated samples were analyzed by the ELISA method as described in section 4.6.5 & 4.6.8. Graph was prepared for each commodity by plotting ELISA reading against fungal biomass per ml of food extract.

4.6.13 Artificially infected food commodities

Food and feed samples inoculated with *A.ochraceus* spore suspension (Section 4.2.10), adjusted to 20% and 30% moisture and incubated for different time intervals were used in the present study.

4.6.14 Detection of *A.ochraceus* in food

The food extracts were prepared from artificially infected food and feed samples as described in section 4.2.4. The food extracts were processed by the dot-binding immunoassay for detection of *A.ochraceus* as described in section 4.6.5 & 4.6.6.

4.6.15 Statistical analysis

The color reading values obtained for different concentration of *A.ochraceus* biomass in the dot-binding immunoassay was subjected to linear regression analysis using Microsoft Excel version 97. The correlation between fungal biomass and ELISA reading was determined by linear regression analysis. The variance between the immunoassay reading for different dose responses were analyzed by paired T-test using Microsoft Excel program (version 97).

RESULTS AND DISCUSSION

4.7 RESULTS

4.7.1 Visual detection of *A. ochraceus*

The control strips (negative control) did not show any color reaction indicating that background coloration due to non-specific reaction was absent. The color observed for different fungal extract and various concentrations of *A.ochraceus* mycelial extracts are shown in Fig. 4.7 and Fig. 4.8 respectively. *Phoma* and *Penicillium* species showed less color reaction with the anti-*A.ochraceus* antibody.

4.7.2 Colorimetric measurement

The color measurement could differentiate differences in the color. The color value obtained for different fungal extracts and various concentrations of *A.ochraceus* mycelial extracts are shown in Table 4.8 and Table 4.9 respectively. The negative control strip, which did not have any perceptible color, showed a reading of 15 ± 2 units.

4.7.2.1 Antibody specificity

The antibody was relatively specific to *A.ochraceus* mycelial extracts in the immunoassay. Among *Aspergillus* species also higher color reaction was shown towards *A.ochraceus* cultures thus showing relative antibody specificity towards *A. ochraceus* species. The dot-binding ELISA responses for all the test cultures tested as shown in Fig. 4.7 are shown in Table 4.8. The antibody showed 85 - 100% cross reaction with *A.ochraceus* mycelial extracts.

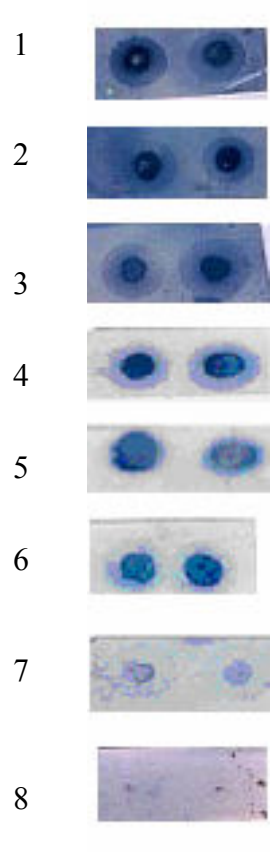

Fig. 4.7 Cross reaction of anti-*A.ochraceus* antibody

Test cultures (50 µg / spot)

1 *Aspergillus ochraceus CFR* 221
2 *Aspergillus ochraceus* MTCC 1877
3 *Aspergillus ochraceus* MTCC 1810
4 *Aspergillus niger*
5 *Aspergillus oryzae*
6 *Fusarium NCIM* 3329
7 *Penicillium verrucossum* MTCC 2007
8 *Phoma* CFR 231

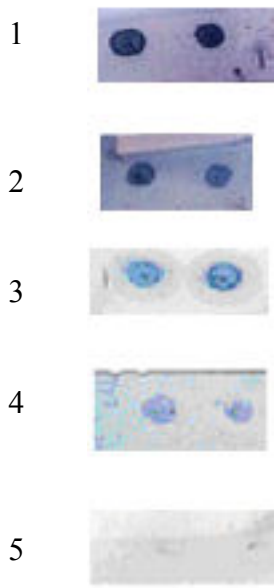

Fig. 4.8 Dose response of dot-binding ELISA against *A.ochraceus* biomass.

Mycelial concentration (µg/spot)

1 -10

2 - 5

3 - 2

4 - 1

5 - 0 (Control)

Table 4.8 Cross reaction of anti-*A.ochraceus* antibody

Culture	Color intensity* ($\delta E^{ab}=\sqrt{\delta l^2+\delta a^2+\delta b^2}$)	Relative activity**
Aspergillus ochraceus CFR 221	65.92 ± 11.97	1.00
Aspergillus ochraceus MTCC 1877	60.41 ± 3.89	0.91
Aspergillus ochraceus MTCC 1810	56.23 ± 0.13	0.85
Aspergillus niger CFR 1398	48.84 ± 8.47	0.74
Aspergillus oryzae NCIM 665	38.43 ± 14.46	0.72
Fusarium NCIM 3329	31.53 ± 9.26	0.43
Penicillium verrucossum MTCC 2007	27.48 ± 2.26	0.41
Phoma F11 CFR 231	20.23 ± 4.10	0.30

* Dot–binding ELISA response for 50 µg of test culture.

**

Relative activity = color reading of test culture/ color reading of strains used for raising antibody.

Table 4.9 ELISA response for different concentration of *A.ochraceus*

Concentration of fungal biomass (µg/spot)	Color intensity ($\delta E^{ab} = \sqrt{\delta l^2 + \delta a^2 + \delta b^2}$)
0	15.12 ± 0.22
1	29.85 ± 8.50
2	35.87 ± 6.18
5	45.98 ± 12.69
10	51.51 ± 1.90

The antibody showed 72 - 74% cross-reaction with other *Aspergillus* species and 30 - 42% cross-reaction with *Pencillium, Fusarium* and *Phoma* strains tested.

4.7.2.2 Antibody sensitivity

The minimum quantity of fungal biomass to give color reaction was observed at 0.5 mg mycelial extract per ml i.e the dot binding immunoassay was sensitive to 1µg (2 µl, 0.5 mg/ml) of fungal biomass. The regression analysis of response for different concentration of fungal biomass had a positive correlation with a coefficient of regression of 0.78 (standard error = 7.56). The dose response curve is presented in Fig. 4.9. The negative control strips showed a reading of 15 ± 2, which was significantly different ($P < 0.05$) from strips loaded with 5 mg fungal biomass per ml (10 µg/spot).

4.7.3 Spectrophotometric measurement

The sensitivity of the assay was similar to that of visual detection limit in the dot-binding immunoassay. The absorbance for negative control was significantly different ($P < 0.05$) from mycelial extract spiked at 0.5 mg/ml of PBS in the dot-binding immunoassay. The regression analysis of dot- binding ELISA response for different concentration of fungal biomass (Fig. 4.10a) had a positive correlation with a coefficient of regression of 0.91 (standard error = 0.02).

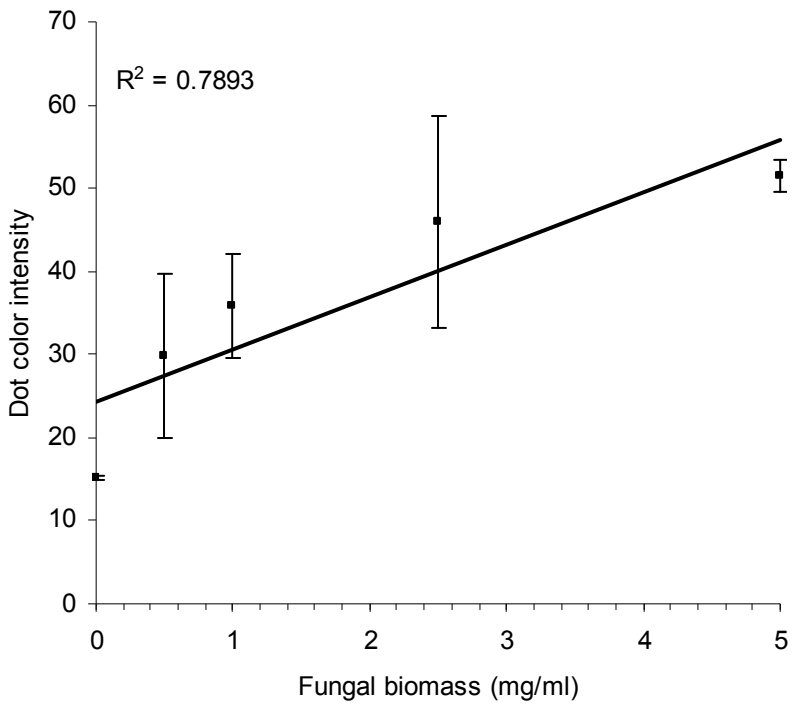

Fig. 4.9 Dose response curve for fungal biomass in the dot-binding immunoassay

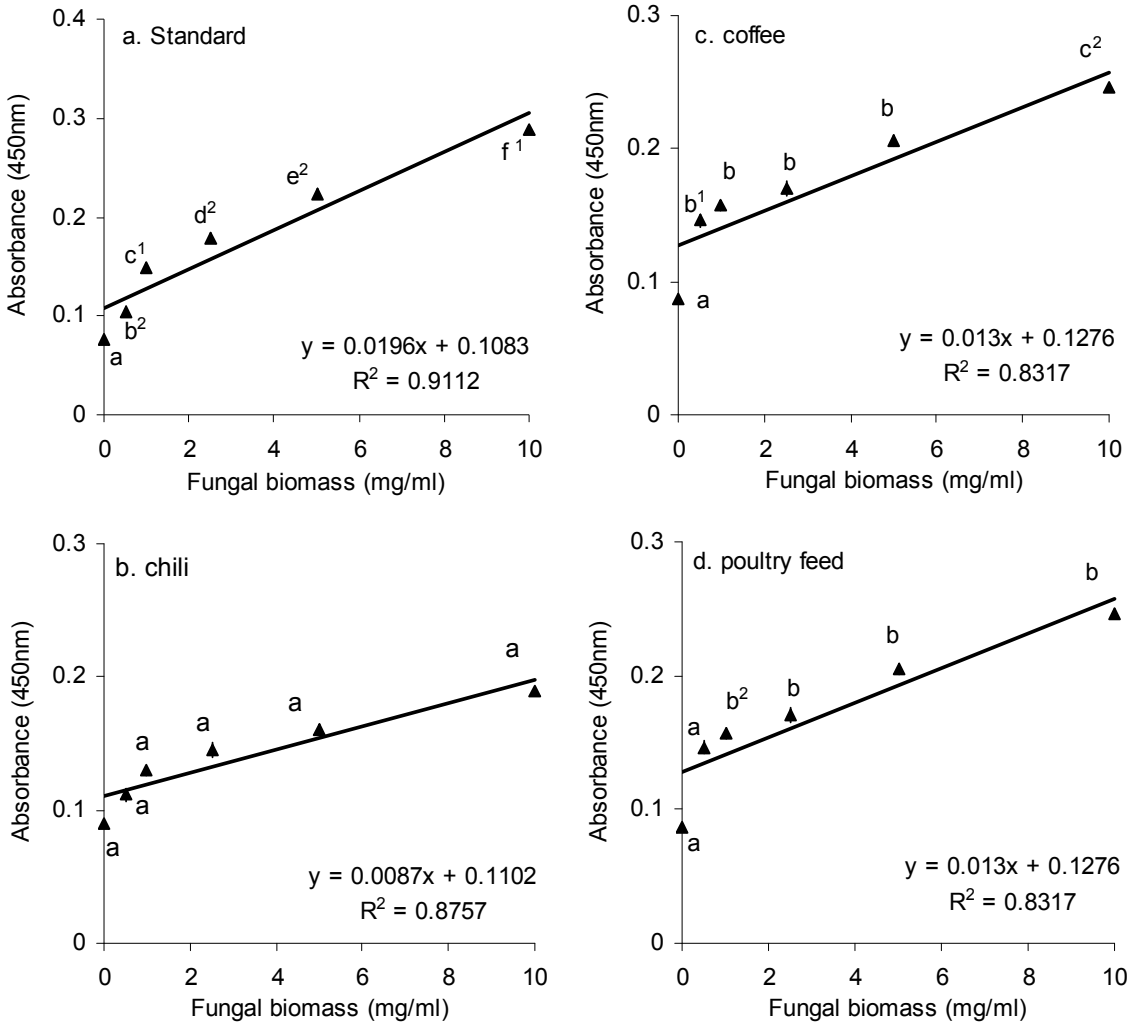

Fig. 4.10 Influence of food matrix on dose response curve for *A. ochraceus* biomass as determined in non-competitive dot–binding immunoassay.

For each plotted value (n=3) and means in any one of the ELISA response followed by different letter are significantly different (1=P<0.005, 2= P<0.05) from the ELISA responses for the fungal biomass prepared from lower dilution.

4.7.4 Influence of food matrix on dot-binding immunoassay

The influence of food matrix was observed in both of the dot-binding immunoassay involving different enzyme-substrate systems; ALP-BCIP-NBT and HRP-TMB-H_2O_2. The absorbance for control strips was significantly different ($P < 0.05$) from sample extract spiked with 0.5 mg fungal biomass per ml of in coffee (Fig. 4.10c), whereas in chili extracts and poultry feed extract (Fig. 4.10d) significant difference ($P < 0.05$) from control well was recorded in extracts spiked with 2.5 and 1 mg fungal biomass per ml respectively. The coefficient of correlation for fungal biomass and ELISA responses was decreased due to interference from food and feed extract. The coefficient of correlation for ELISA responses (HRP-TMB-H_2O_2 enzyme-substrate system) for different concentration of fungal biomass in chili, coffee and poultry feed (Fig 4.10b, Fig 4.10c, Fig 4.10d) was 0.87 (standard error = 0.01), 0.83 (standard error = 0.02) and 0.83 (standard error = 0.02) respectively.

Profound interference in color development was observed for BCIP-NBT substrate system in all the food commodities tested. Masked color development was observed at ≥ 2.5 mg mycelial extract per ml in poultry feed and chili sample, whereas in coffee samples the masked color development was observed at a concentration of ≥5 mg/ml (Fig. 4.11). The results indicate that the dot-ELISA could be used for detecting fungal biomass at a level of >10 mg/ml in all the food commodities studied. The variations among duplicates makes it difficult to distinguish fungal biomass at the concentrations tested, which makes it unreliable for quantification of fungal biomass in food systems.

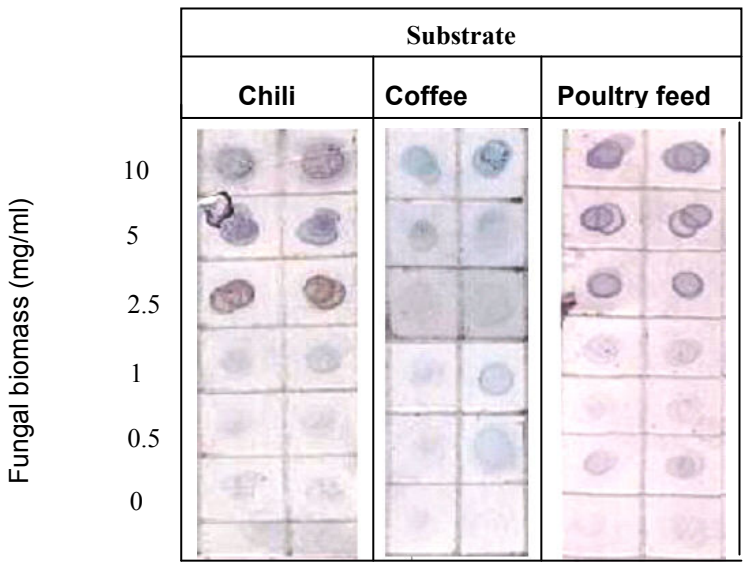

Fig. 4.11 Influence of food matrix on color response in dot-binding immunoassay

4.7.5 Detection of *A.ochraceus* in food commodities

The color as shown by the spiking study the food matrix interfered in the color development in the dot-binding immunoassay. The color observed for food and feed samples from different time intervals of growth is shown in Fig. 4.12. The biomass estimations from microplate immunoassay (Fig. 4.3, chapter 4A) show that dot-binding ELISA could detect fungal infestation at ≥ 5ug fungal biomass/mg of substrate in poultry feed and chili sample. The detection limit in coffee was lower at ≥10 µg/mg substrate because of comparatively higher matrix interference in coffee.

4.7.6 Sensitivity of indirect competitive dot-binding immunoassay

The indirect competitive dot-binding immunoassay was sensitive to 0.1 mg/ml ($P < 0.05$) of competitor *A.ochraceus* mycelial antigen. The inhibition curve obtained in the competitive dot-binding ELISA is presented in Fig. 4.13. The sensitivity in terms of mycelial antigen giving 50% inhibition (IC_{50}) was estimated to occur at 1 mg/ml of competitor concentration.

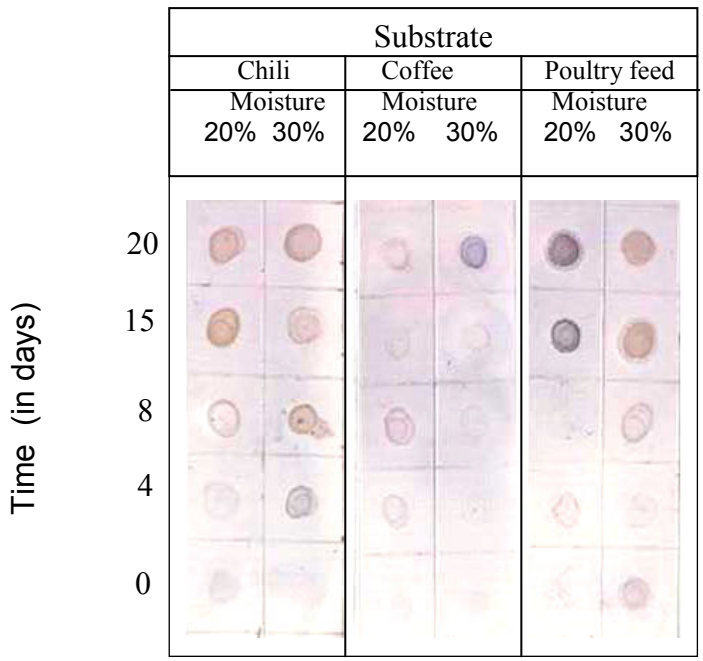

Fig. 4.12 Detection of *A.ochraceus* in food commodities by the dot-binding immunoassay

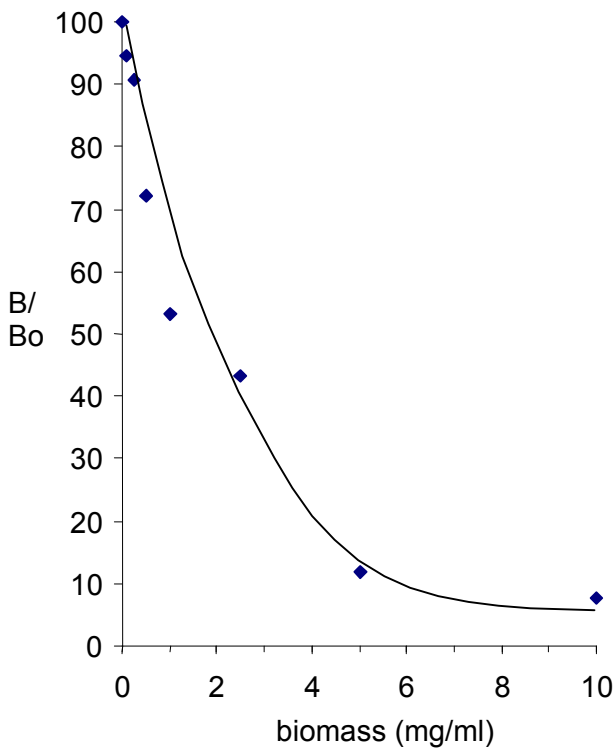

Fig. 4.13 Inhibition curve obtained for *A.ochraceus* fungal biomass in indirect competitive dot-binding ELISA.

4.8 Discussion

The dot-binding immunoassay was similar to microplate ELISA in terms of cross-reaction and influence of food matrix. The antibody specificity in terms of relative ELISA activity for different fungal cultures as determined by indirect non-competitive microplate ELISA (Section 4A, Table 4.2) and dot-binding ELISA (Table 4.8) were comparable. With dot-binding assay relatively higher cross-reaction was observed for fungal cultures other than *A.ochraceus* group. The variation in the antibody specificity may be due to higher biomass used as coating antigen in the dot-binding immunoassay (50 µg/assay in dot-binding immunoassay v/s 5 µg/assay in microplate ELISA,) and different enzyme substrate system (HRP-TMB-H_2O_2 v/s ALP-BCIP-NBT) used in both the ELISA protocols.

The interference from the food matrix drastically influenced the sensitivity of visual detection in food matrices in the dot-binding ELISA for visual assessment. The sensitivity was reduced from 0.5 mg/ml to 2.5 mg/ml in chili and poultry feed samples and 10 mg/ml in coffee samples. The variation within the samples varied widely in the visual detection in the dot-binding ELISA (Table 4.9.). The high variability and lower correlation coefficient (Fig. 4.9) suggest that the dot-binding immunoassay cannot be used for quantitative estimation of fungal biomass. The higher variations in color reading were observed in strips with non-uniform color development due to variations in sample application on nitrocellulose membrane (Fig. 4.7 & 4.8 and Table 4.8 & 4.9). This variation may be minimized by using sample applicator, where in the sample may be applied as thin film on nitrocellulose membrane by the instrument (Saeger and Peteghem, 1996).

The dot-binding immunoassay optimized for spectrophotometric measurement of *A.ochraceus* was also influenced by different food matrices (Fig. 4.10). The minimum detection limit was raised from 0.5 mg/ml to 1mg/ml in poultry feed and 2.5 mg/ml in chili sample. The lower correlation coefficient and variability within duplicates make the assay less dependable for quantitative estimation of fungal biomass.

The sensitivity of a solid support immunoassay for a specific antigen is determined by the ability of antigens and antibodies to remain bound to the matrix under the assay conditions (Schneider, *et al*., 1991). Coffee extract had a maximum interference leading to decrease in detection limit from 0.5 mg/ml to 10 mg/ml (Fig. 4.11) for dot-binding immunoassay optimized for visual detection, whereas no such influence on detection limit was observed in dot-binding immunoassay optimized for spectrophotometric measurement. This may be due to the use of different enzyme-substrate systems in the two dot-binding immunoassay protocols. The color in the AIP-BCIP–NBT enzyme substrate system is due to insoluble precipitate formed on nitrocellulose membrane and hence may be affected by interference due to food matrix in the antibody-binding site on the nitrocellulose membrane. The color in HRP-TMB-H_2O_2 enzyme substrate system will be in soluble form and hence may be less affected by the changes on the antibody-binding site on the nitrocellulose membrane for color reaction. The binding of proteins (antigens and antibodies) on nitrocellulose membrane occurs through a hydrophobic, electrostatic, or ionic interactions. The pH used for the enzyme substrate systems is different in the both the protocols, which may also have an influence on antigen-antibody binding.

Both the dot-binding immunoassays can be used for the detection of fungal infestation in food commodities. The detection limit for dot-binding immunoassays were in the range of 5 – 20 µg fungal biomass/mg of substrate for ALP-BCIP-NBT substrate system and 1 - 2 ug fungal biomass /mg of substrate for HRP-TMB-H_2O_2 enzyme substrate system, which is 5 -100 fold less sensitive than microplate ELISA (0.2 µg/mg). Further study is needed to reduce the influence of matrix by sample cleanup and alternative extraction protocols. The sensitivity of the dot-binding immunoassay can be considerably improved by prior enrichment of samples for increasing the fungal biomass.

In this study all the immunoassay protocols were standardized at room temperature (28 ± 2^{o}C), which makes it adoptable for field conditions. To increase the sensitivity of dot-binding assay the competitive dot-binding immunoassay for the detection of fungal biomass was evaluated. The lower detection limit (0.1 mg/ml) in competitive dot-binding immunoassay is sensitive enough to detect 0.2 µg fungal biomass per mg of substrate. The sensitivity of competitive dot-binding immunoassay is similar to that observed for microplate ELISA (Fig. 4.1, Chapter 4A). Competitive dot-binding immunoassay can be used as an alternative solid matrix for the immunoassay. In the present study the influence of food matrix on competitive dot-binding immunoassay could not be evaluated. Further work is needed in this direction to adopt the competitive dot-binding immunoassay for application in food system.

CHAPTER 5.0

POLYMERASE CHAIN REACTION METHOD FOR DETECTION OF OCHRATOXIGENIC FUNGI

CHAPTER 6.0

IMMUNOLOGICAL METHOD FOR DETERMINATION OF OCHRATOXIN A

CHAPTER 6.0

IMMUNOLOGICAL METHOD FOR DETERMINATION OF OCHRATOXIN A

RESULTS AND DISCUSSION

6.1 INTRODUCTION

Mycotoxin contamination is one of the important health hazards to humans and animals caused by mold colonization. Unlike bacterial contamination, the toxic metabolites of the fungus are of main concern rather than the fungus itself. Hence many agencies have imposed the regulation on the mycotoxin rather than the fungal contamination. Health risks caused by ingestion of mycotoxin contaminated food may range from feed refusal in animals to death of livestock and humans, and hence their presence in food is regulated. Owing to enormous health risks especially the carcinogenic nature of some mycotoxins, including ochratoxin the various international agencies have stipulated stringent regulation that can be implemented using the available analytical methods. For ochratoxin the permissible tolerable weekly intake has been arrived at 112 ng/kg bw based on the lowest observed effect level (LOEL) of deterioration of renal function. The European Union in their regulation (No.466/2001) have set maximum limit at 5, 3 and 10 ppb for cereals, cereal products and dried vine fruits respectively

To ensure food safety the raw materials and food products need to be monitored for presence of hazardous microorganisms and their toxic metabolites. In this regard immunological methods are advantageous over chemical methods, as the extraction and sample purification procedures using minimum organic chemicals are simple which do not require sophisticated costly instrumentation. At the same time large number of samples can be analyzed. Moreover, the immunochemical methods are now being used for efficient sample purification steps for quantification by sophisticated instrument. The review of literature on immunoassays for mycotoxin reveals

the potential use of immunoassays in *in vitro* and *in vivo* metabolism studies of mycotoxin, immuncytochemistry and analysis of mycotoxins in foods (Chu, 1984; Pestka, 1988). The use of antibodies as prophylactic method against aflatoxicosis has been reported by Van and Langone (1980). The development of accurate immunoassays for detection of mycotoxins is dependent on efficient method for antibody production. In this regard egg yolk antibodies has the potential to be used for large-scale production of antibodies against mycotoxins. In this chapter the study relating to production of antibodies in rabbit and hen egg has been evaluated and development of immunological method for determination of ochratoxin A in food is presented..

6.2 MATERIALS AND METHODS

6.2.1 Chemicals

Ochratoxin A standard (OTA), ochratoxin A-BSA conjugate (OTA-BSA), bovine serum albumin (BSA), 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDPC) and polyethylene glycol 8000 (PEG 8000) were procured from sigma Aldrich USA. Disodium hydrogen phosphate dihydrate (Na_2HPO_4 $2H_2O$), sodium dihydrogen orthophosphate dihydrate (NaH_2PO_4 $2H_2O$), benzene, acetic acid and hydrochloric acid (HCl) were of analytical grade and procured from S.D.Fine chemicals, India. Silica gel–G was procured from E-Merck, Mumbai, India.

6.2.2 Ochratoxin A production and purification

A.ochraceus was grown on Yeast extract sucrose growth supplemented with 1% glutamic acid in 250 ml flasks for 7days. After incubation the cultural filtrate was separated from the mycelium and acidified to pH 4 with 1N HCl. Ochratoxins were extracted into chloroform by liquid-liquid partitioning. Ochratoxin A was purified further by TLC. The quantity and purity of ochratoxin A was determined by spectrophotometric method as described in section 3.2.3.3.

6.2.3 Ochratoxin-BSA conjugation

The ochratoxin A was conjugated to BSA by water-soluble carbodiimide method (Chu, 1976). BSA in 4ml 0.1N NaCl (10 mg/ml) was reacted with 20mg OTA in 0.5ml ethanol and 3ml 0.1M sodium phosphate buffer in presence of 150mg of EDPC. The reaction mixture was shaken for 24 h in dark and was subjected to exhaustive dialysis against 0.1 M PBS for 72 h.

The conjugation was confirmed by TLC technique using chloroform:acetone (9:1) solvent system, wherein the conjugate being heavier/insoluble in developing solvent remains at the origin. The molar ratio of conjugation was calculated spectrophotometrically by reading absorbance at 333 nm and 280 nm for determination of quantity of ochratoxin A and BSA respectively.

6.2.4 Immunization and antibody production

6.2.4.1 Rabbit antibody (IgG)

Female New Zealand white rabbit weighing 1-1.5 kg was immunized with 1mg of OTA-BSA conjugate (Sigma) in 0.5ml PBS pH 7.2 emulsified with 0.5 ml Freund's complete adjuvant (FCA). Immunization of rabbit was by subcutaneous injections at multiple sites on the dorsal side. Subsequent Booster injections with Freund's incomplete adjuvant (FICA) were given at monthly intervals. The trail bleeding (5 ml) was made 7 days after first booster injection and final bleeding (20ml) was made after fourth booster dose through marginal ear vein bleeding The serum was separated from the blood by low speed centrifugation (4000 x g) and the immunoglobulin (IgG) fraction was isolated by ammonium sulphate precipitation using 66% saturated ammonium sulphate and dialyzed exhaustively against 10 mM PBS (pH 7.2) as described in Tijjsen (1985). The antibody solutions were distributed in vials and stored at –20 ^{o}C.

6.2.4.2 Hen egg yolk antibody (IgY)

Hens 27 weeks old, weighing 1-1.5 kg were used in immunization experiment. Hens were immunized with OTA-BSA (1mg, Sigma) dissolved in 0.5 ml PBS (pH 7.2) emulsified with 0.5 ml Freund's complete adjuvant. Immunization of

hens was by intramuscular route wherein injections were given at multiple sites into the pectoral muscles. Subsequent booster injections were given at monthly intervals. The eggs were collected after 7 days of first booster injection. The egg yolk antibodies were isolated and purified by the method of Clarke *et al.* (1993). The egg yolk was separated from the albumin and diluted with 4 volumes of 0.1 mM PBS (pH 7.2) and 1 volume of chloroform. The mixture was well shaken and centrifuged at 10,000 x g for 30 min at 4^0C. The supernatant was collected and IgY was precipitated twice with 14% (w/v) PEG 8000. The IgY was reconstituted in 0.1mM of PBS (pH 7.2) with 0.005% sodium azide. The total protein content of the antibody preparation was measured by Bradford method (1976).The antibody preparations (10mg/ml) were distributed in sterile vials and stored at –20^{o}C.

6.2.5 Determination of antibody activity

The antibody activity was confirmed by ouchterlony double diffusion assay. Agarose gel (1%) was prepared in PBS (10 mM, pH 7.2) containing 0.005% sodium azide and poured into petriplate. Wells (Ca 5 mm) were cut equidistantly on the agarose gel. The ochratoxin A conjugate (1 mg/ml, 25 µl)) prepared in PBS (10 mM, pH 7.2) was dispensed into central well and antibody preparation from different harvest adjusted to identical protein concentration were dispensed into the peripheral wells. The agarose plate dispensed with antigen and antibody preparations were incubated at 37^{o}C under humid condition. The plates were observed for antigen-antibody precipitation band after 8 – 24 h of incubation.

6.2.6 ELISA

Two forms of ELISA were used in the present study An indirect non-competitive ELISA was used for optimization of ELISA reagents by using checkerboard titration. The optimized reagent concentrations from indirect non-competitive ELISA were used in developing indirect competitive ELISA for the mycotoxin analysis in food samples.

6.2.6.1 Indirect non-competitive ELISA

All the assay protocols were optimized at room temperature. The Microplates were coated with 0.25 – 1 µg/ml of OTA-BSA conjugate prepared in 0.1 M PBS (pH 7.2) at 100 µl/well and incubated overnight. The plate was washed thrice with PBS containing 0.05 % Tween-20 (PBS-T). Skimmed milk powder, casein and BSA at 1 - 5% concentration were evaluated as blocking agents to get a low background absorbance. The microplate was incubated with blocking agents prepared in PBS at 150 µl/well. The plates were incubated with 100 µl/well of anti-ochratoxin A antibody prepared in PBS diluted appropriately as determined by checkerboard titration. Wells without antigen and antigen-coated plates treated with no antibody or pre immune serum served as controls. The neutralization of BSA specific antibody by preincubation of antibody prepared in PBS containing 0.1 - 5% BSA for 1h was evaluated. The antibody preparation in PBS served as control. The plates were washed thrice with PBS-T. The Microplate was coated with 100 µl anti-Hen IgY HRP conjugate and anti-rabbit IgG HRP conjugate per well for wells treated with antibodies raised in hen egg and rabbit respectively. The microplates treated with secondary antibody enzyme conjugate were incubated for 30 min at R.T. The microplates were washed six times with

PBS-T. The microplates were incubated with 150 µl TMB-H_2O_2 substrate per well for 30 min. The plates were read at 450 nm in ELISA reader (Spectramax 340, Molecular Devices, USA).

6.2.6.2 Indirect competitive ELISA

The optimized reagent concentrations from indirect non-competitive ELISA were used for developing indirect competitive ELISA protocol. All conditions were optimized at room temperature. The Microplates were coated with 0.5 µg/ml OTA-BSA conjugate (100µl) per well prepared in 0.1 M PBS pH 7.2 and incubated overnight. The plate was washed thrice with PBS containing 0.05 % Tween-20. The Microplate was blocked with 1% skimmed milk for 1h. The plates were incubated with 100 µl anti-ochratoxin A antibody per well. For antibody raised against the ochratoxin A-BSA conjugate the antibody was diluted in PBS containing 1% BSA and pre incubated for 1 h at R.T to neutralize the antibody specific to BSA. To each well 100 µl of sample containing compounds tested for cross reactivity / standard ochratoxin A in PBS / unknown sample in 55% aqueous methanol was added into each well and incubated for 2 h at R.T. The plates were washed thrice with PBS-T. Microplate was coated with 100 µl of 1:5000 diluted anti IgY HRP conjugate and 1:2000 diluted anti-IgG HRP conjugate per well prepared in PBS for wells treated with antibodies raised in hen egg and rabbit respectively. The microplates treated with secondary antibody enzyme conjugate were incubated for 30 min at R.T. The Microplates were washed six times with PBS-T. The microplates were incubated with 150 µl TMB-H_2O_2 substrate per well (0.01 % TMB in 0.1 mol/L sodium acetate buffer pH 5.0, containing

0.005% H_2O_2) and incubated for 30 min. The plates were read at 450 nm in ELISA reader (Spectramax 340, Molecular Devices, USA). The antibody cross reactivity, sensitivity and ochratoxin estimations were determined by indirect competitive ELISA.

6.2.7 Comparison of antibody quality

The antibody preparation from each bleeding and IgY preparation from two eggs per week collected after booster dose were collected. The antibody preparations were analyzed by indirect non-competitive ELISA at optimized reagent concentration and ELISA absorbance was compared with a reference absorbance. The absorbance for the optimized reagent concentration for indirect non-competitive ELISA concentration was taken as reference absorbance. The quality and quantity of antibody from rabbit and hen egg was compared.

6.2.8 Sensitivity and specificity determination

The standard inhibition curve for different concentrations for ochratoxin A was determined by indirect competitive ELISA. The cross reactivity of the antibody was determined for OTB, aflatoxin B_1 and phenylalanine by using these metabolites as competitors in the indirect competitive ELISA developed for OTA. The competing ochratoxin A concentration giving an inhibition of 50% of maximum absorbance was used for determination of specificity of the antibody for other metabolites tested.

6.2.9 Preparation of reference OTA contaminated poultry feed samples

The poultry feed samples were procured from local markets and ground to pass through 20 BSM (British standard Mesh) sieve. The feed samples were spiked with ochratoxin A at desired concentrations as check sample. The samples were extracted and analyzed for ochratoxin A by HPLC and ELISA.

6.2.10 HPLC analysis

The ochratoxin A content in the samples were extracted according to standard AOAC procedure (AOAC, 1997) as described in section 3.2.3.4. The sample extract /Ochratoxin A standard were derivatized by methyl ester formation using BF_3 as described in section 3.2.2.4. The derivatized sample extract/standard were reconstituted in known quantities of HPLC mobile phase comprising methanol: water: acetic acid (70:70:2). The sample extract/ochratoxin A standard (10 µl) were injected into C-18 column (Schimadzu, RP-18) equipped with fluorescence detector (RF–10, Schimadzu, Japan) set at 333 nm excitation wavelength and emission wavelength of 460 nm. The ochratoxin content in the sample was quantified by comparing the peak area and retention time of the ochratoxin A standard and sample. The retention time of OTA standard were compared with that of sample. The peak area given by sample and known quantity of OTA standard was determined and OTA was quantified using the following formula

$$\text{Toxin Concentration (µg/g)} = \frac{X}{Y} \times \frac{S}{V} \times \frac{D}{G}$$

Where X = Peak Area given by sample

V = Volume of sample spotted.

Y = Peak Area given by standard

S = Quantity of standard injected (ng)

D = Sample dilution (ml)

G = Corresponding weight of the sample equivalent to the volume of extract taken for analysis.

6.2.11 Sample extraction for ELISA

The samples (10 g) extracted with 55% aq methanol (1:5, w/v) in a wrist action shaker for 30min. The extracts were filtered through Whatmann No.1 filter paper. The extracts (100 µl) were analyzed for ochratoxin A content by indirect competitive ELISA as described in section 6.2.6.2.

6.2.12 Comparison of OTA estimation by ELISA and HPLC

The toxin spiked feed samples were analyzed for recovery of ochratoxin by ELISA. The intra-assay coefficient of variation (CV%) was determined from replicate analyses on single plate. The feed samples procured from market were analyzed for ochratoxin A content by HPLC and ELISA.

RESULTS AND DISCUSSION

6.3 RESULTS

6.3.1 Ochratoxin –BSA conjugation

The ochratoxin-BSA conjugate prepared in our laboratory had a molar ratio of 2:1 as determined spectrophotometrically. The ochratoxin-BSA conjugate was used as coating antigen in ELISA.

6.3.2 Determination of antibody activity

The formation of antibody-antigen precipitin band for antibody preparation after booster dose in the ouchterlony double diffusion assay revealed that antibodies were elicited in host against the immunogen in both egg yolk antibody (Fig. 6.1a) and rabbit serum preparation (Fig. 6.1b).

6.3.3 comparison of antibody quality

6.3.3.1 Anti-OTA egg yolk antibodies

It is estimated that egg contains about 160 mg of egg yolk protein (Clarke, *et al*, 1996). The recovery of IgY was 83 ± 19 mg IgY/egg (n=11, Fig. 6.2). Thus on an average it was estimated that 51.87% of yolk protein can be precipitated as IgY antibodies from hen egg yolk by the method . There was no significant drop in antibody titer after booster dose. The absorbance for 1:1000 diluted egg yolk antibody preparations from eggs collected after booster dose were 0.810 ± 0.037 at a coating antigen concentration at 0.5 µg/ml.

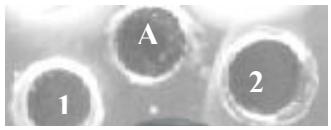

Fig. 6.1a Anti-OTA IgY

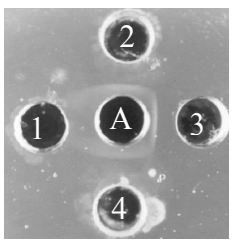

Fig. 6.1b Anti-OTA IgG

Fig. 6.1 Immunodiffusion reactions of antibody raised against OTA A – OTA-BSA conjugate, 1 - Pre immune antibody preparation, 2, 3, 4 - antibody harvested after booster injections.

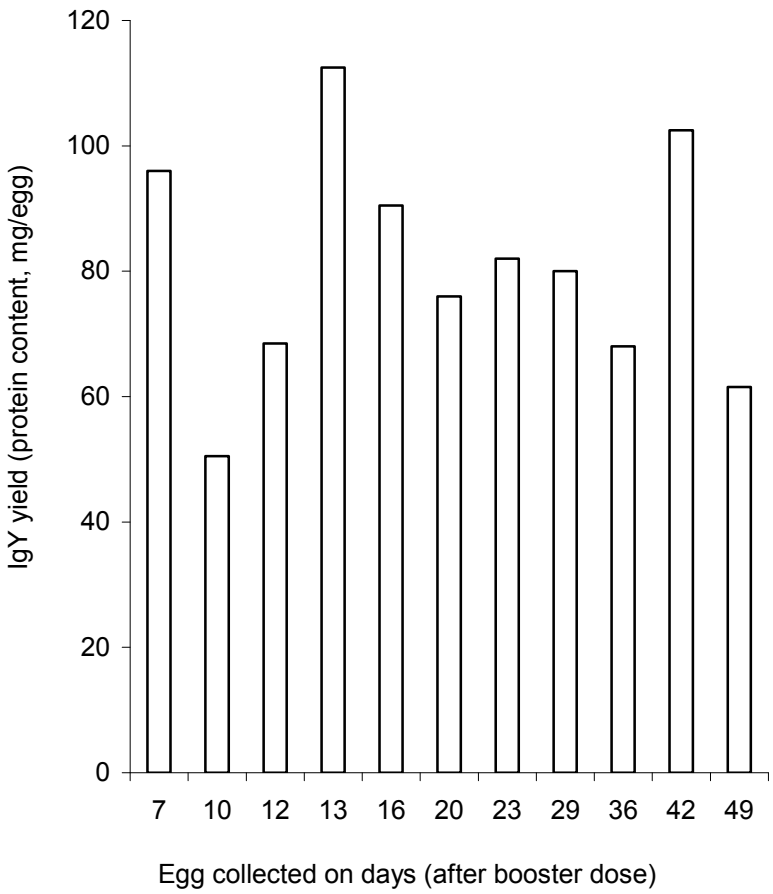

Fig. 6.2 Recovery of IgY antibody

6.3.3.2 Anti-OTA rabbit serum

The yield of IgG in serum was estimated to be 10 mg per ml of serum. There was no significant drop in antibody titer in antibody preparations from different bleeding after booster dose. The absorbance for 1:10000 diluted rabbit anti-OTA antibody preparations from different bleeding after booster dose were 0.65 ± 0.029 at a coating antigen concentration of 0.5 µg/well.

6.3.4 Optimization of ELISA protocol

Ochratoxin–BSA conjugate was used to immunize the animals for the production of antibody against mycotoxin and was used as coating antigen in the ELISA. The antibody against carrier protein (BSA) interferes in ELISA protocol for ochratoxin. In order to neutralize the BSA specific antibodies the antibody preparations were made in PBS containing BSA and preincubated for 1h prior to using the same in the assay. Antibody preparation in BSA at 1% neutralized the BSA specific antibodies. Among the different blocking agent tested skimmed milk at 1% concentration gave a lower background binding (Fig. 6.3). Antibody preparation from rabbit serum and hen egg yolk gave a linear response curve for ochratoxin A-BSA coating antigen at antibody dilution of 1:10000 (Fig. 6.4a) and 1:1000 (Fig. 6.4b) respectively.

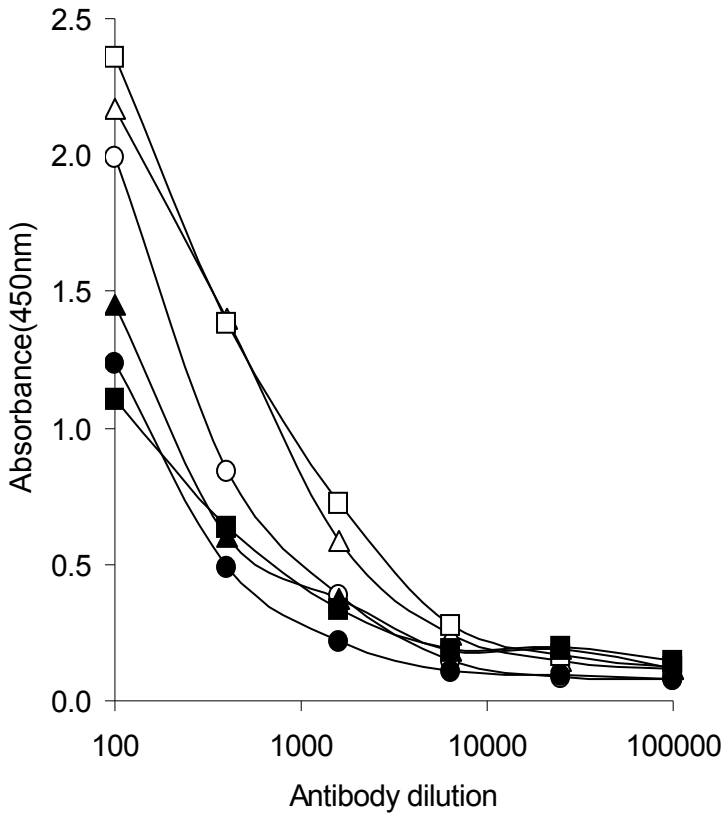

Fig. 6.3 Influence of blocking agent and neutralization of BSA specific antibody on background inhibition and titer of antibody. (□) Antibody preparation in PBS with 1% casein as blocking agent (■) Antibody preparation in 1% BSA in PBS with 1% casein as blocking agent (○) Antibody preparation in PBS with 1% skimmed milk as blocking agent (●) Antibody preparation in 1% BSA in PBS with 1% skimmed milk as blocking agent (△) Antibody preparation in PBS with 1% BSA as blocking agent (▲) Antibody preparation in 1% BSA in PBS with 1% BSA as blocking agent

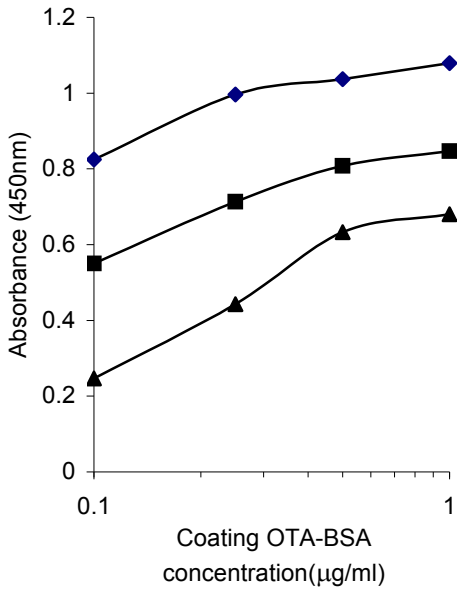

Fig. 6.4a Rabbit antiserum

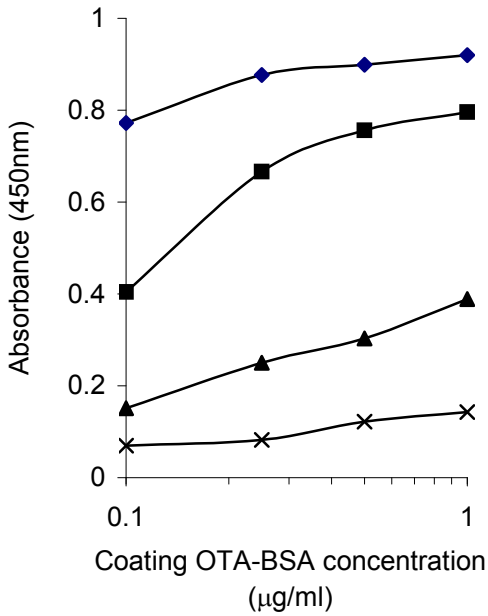

Fig. 6.4b Egg yolk antibodies

Fig. 6.4 Influence of antibody dilution and coating concentration on indirect non-competitive ELISA. **Fig. 6.4a**. Rabbit Anti-OTA antibody. **Fig. 6.4b**. Hen egg yolk anti-OTA antibody. Antibody dilution: (◆) 1:100, (■) 1:1000, (▲) 1:10000, (X) 1:100000.

6.3.5 Sensitivity and specificity

6.3.5.1 Rabbit anti-OTA serum

The inhibition curve was sensitive to OTA competitor concentration of 0.1 ng/ml, which was significantly lower ($P < 0.05$) than the well with no OTA as competitor. The assay had a working range of 0.1 - 100 ng/ml of OTA. The amount of competing OTA to produce 50% of maximum absorbance was estimated to occur at 15 ng/ml (Fig. 6.5) and this value was used for the determination of antibody specificity. The 50% inhibition for OTB was estimated to occur at 200 ng/ml and 50% inhibition appeared to occur at >1000 ng/ml for aflatoxin B_1 and phenylalanine. The 50% inhibition concentration for afaltoxin B_1 and phenylalanine was estimated from the slope of the respective inhibition curve. The antibody had 8.3%, 0.8% and 0.5% cross reaction with OTB, aflatoxin B_1 and phenylalanine respectively (Table 6.1).

6.3.5.2 Anti-OTA egg yolk antibodies

The inhibition curve was sensitive to OTA competitor concentration of 0.1ng/ml, which was significantly lower ($P < 0.05$) than the well with zero OTA concentration. The working range the assay was 0.1 – 300 ng/ml of OTA. The amount of competing OTA to produce 50% of maximum absorbance was estimated to occur at 40 ng/ml (Fig. 6.6) and this value was used for the determination of antibody specificity. The 50% inhibition for OTB was estimated to occur at 180 ng/ml and 50% inhibition appeared to occur at >1000ng/ml for aflatoxin B_1 and phenylalanine. The antibody had 19.05%, 2.7% and 2.17% cross reaction with OTB, aflatoxin B_1 and phenylalanine respectively (Table 6.2).

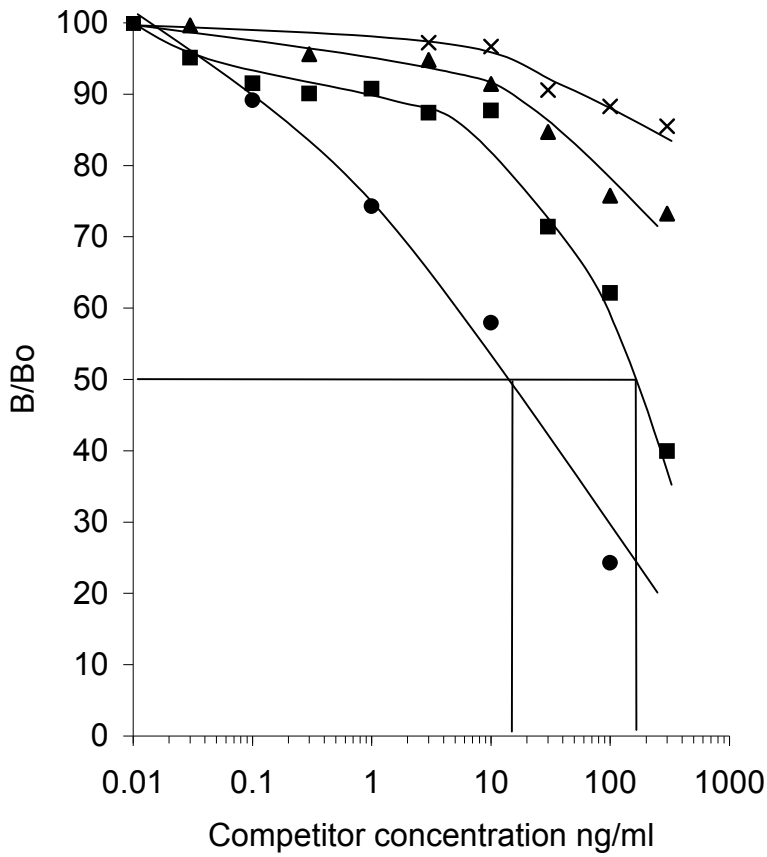

Fig. 6.5 Cross reactivity of rabbit anti-OTA antibody to (•) OTA, OTB (■), Aflatoxin B_1 (▲), and Phenylalanine (X)

Table 6.1 Anti-OTA rabbit antiserum specificity and cross reactivity

Competitor	Slope of inhibition curve	IC_{50} (ng)	Cross reactivity (%)
OTA	-0.22	15	100
OTB	-0.17	180	8.33
Aflatoxin B_1	-0.03	1697	0.88
Phenylalanine	-0.02	2744	0.55

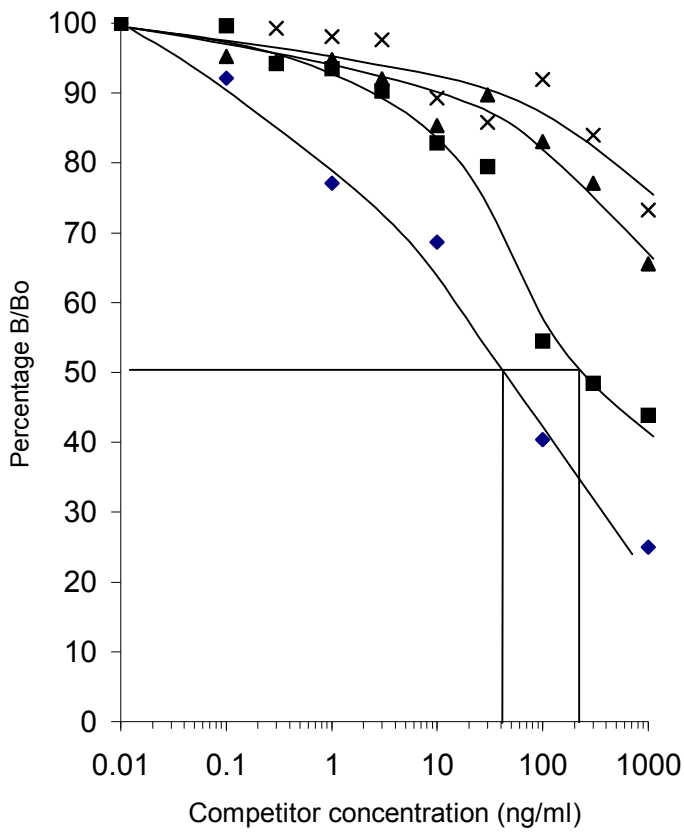

Fig. 6.6 Cross reactivity of anti-OTA hen egg yolk antibody to OTA (•), OTB (■), Aflatoxin B_1 (▲), and Phenylalanine (X)

Table 6.2 Anti-OTA hen egg yolk antibodies specificity and cross reactivity

Competitor	Slope of inhibition curve	IC_{50} (ng)	Cross reactivity (%)
OTA	-0.06	40	100
OTB	-0.05	210	19.05
Aflatoxin B_1	-0.03	1480	2.70
Phenylalanine	-0.03	1844	2.17

6.3.6 Effect of poultry feed extract on the ELISA

It was found in the initial experiments that the poultry extract interfered in the immunoassay. The inhibition curves were plotted for ochratoxin A prepared in poultry feed extracts (Fig. 6.7 and Fig. 6.8) and these curves were used for estimation of ochratoxin A in feed samples.

6.3.7 Recovery of OTA from artificially contaminated feed samples

The recovery of toxin from poultry feed samples spiked with 5 - 500 µg/kg OTA ranged from 83 to 125% when anti-OTA rabbit antibody was used in the ELISA. The intra-assay coefficient of variation for feed samples spiked with OTA at 5ppb, 20 ppb, 100 ppb, 200ppb and 500ppb ranged from 6.9 to 15.7% (Table 6.3)

Similarly the recovery from poultry feed samples spiked with 5 - 500 µg/kg OTA ranged from 67 to110% when anti-OTA egg yolk antibody was used in the ELISA. The intra-assay coefficient of variation for feed samples spiked with OTA at 5, 20, 100, 200 and 500ppb ranged from 6.3 to 29% (Table 6.4).

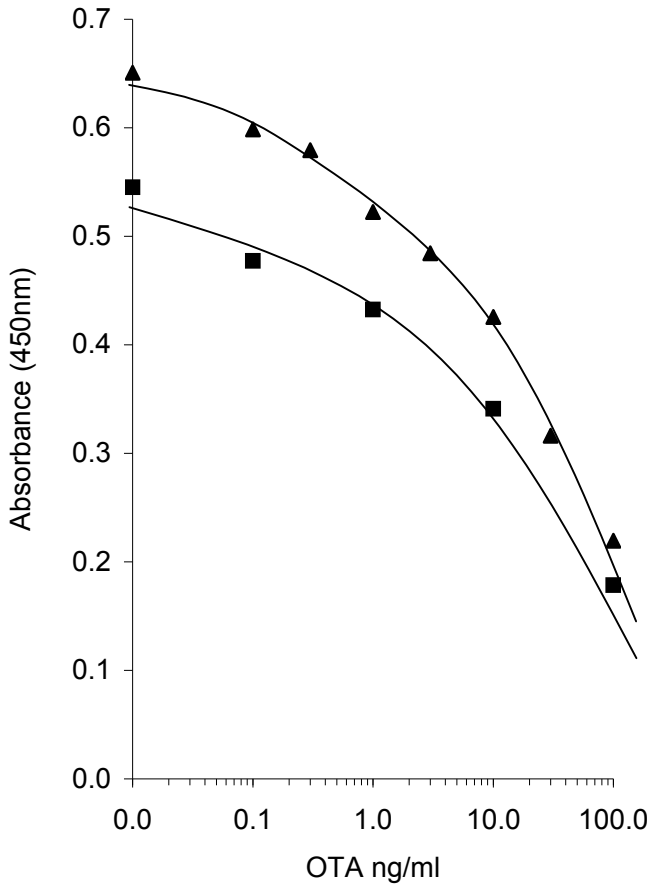

Fig. 6.7 Influence of poultry feed extract on determination of ochratoxin A by rabbit anti-OTA serum. OTA standards in phosphate buffer (▲) and poultry feed extract (■)

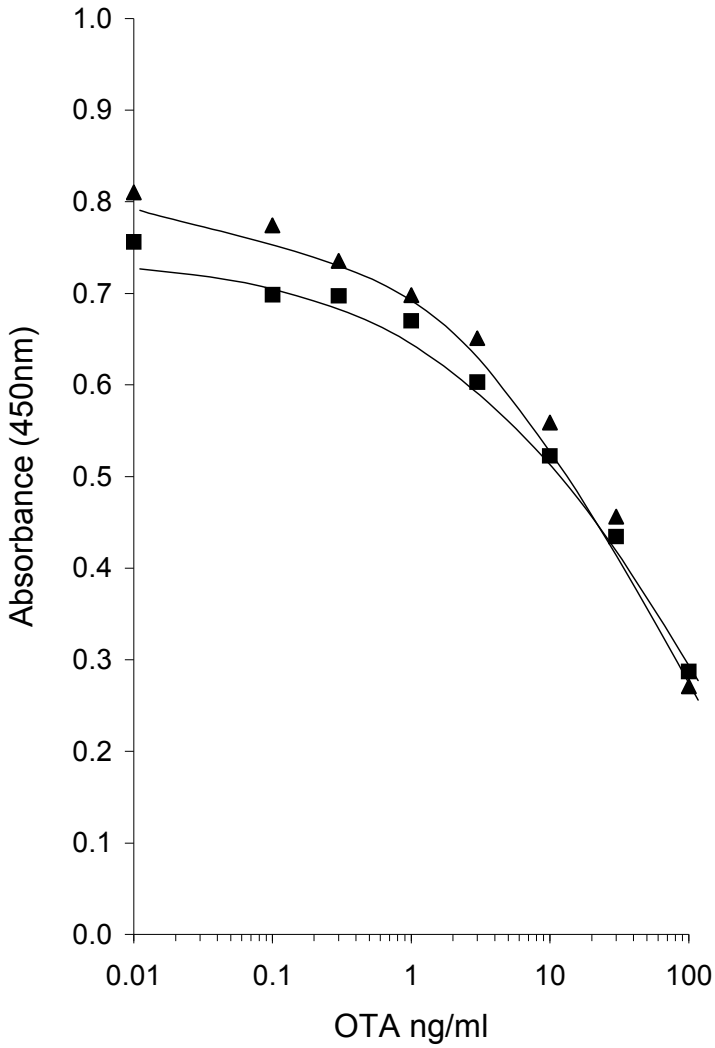

Fig. 6.8 Influence of poultry feed extract on determination of ochratoxin A by anti-OTA hen egg yolk antibodies. OTA standards in phosphate buffer (▲) and poultry feed extract (■)

Table 6.3 Recovery of ochratoxin A from artificially contaminated poultry feed samples as determined by indirect competitive ELISA using anti-OTA rabbit antiserum.

OTA spiked (µg/kg)	OTA estimated (µg /kg)	Recovery (%)	CV%
5	6.25 ± 0.75	125.00	12
20	23.75 ± 1.7	112.50	7
100	96.50 ± 12	96.50	12.43
200	225.12 ± 35.5	112.50	15.76
500	416.66 ± 28.7	83.32	6.88

Table 6.4 Recovery of ochratoxin A from artificially contaminated poultry feed samples as determined by indirect competitive ELISA using anti-OTA- hen egg yolk antibodies

OTA spiked (µg /kg)	OTA estimated (µg /kg)	Recovery (%)	CV%
5	3.37 ± 1.22	67.48	29
20	17.85 ± 2	89.25	11.2
100	117 ± 18	117.00	15.38
200	227.5 ± 14.42	113.75	6.33
500	550 ± 70	110.00	12.72

6.3.8 Comparison of ELISA with HPLC estimations for ochratoxin A.

Ochratoxin A in poultry feed samples was analyzed by indirect competitive assay and HPLC method, and the results for both the immunoassays is presented in Table 6.5. There was good agreement between HPLC and ELISA estimations in all the nine samples of poultry feed tested. The OTA contamination was detected by HPLC in only one sample at 200 ppb and this correlated well with a coefficient of variation of ~9% in both immunoassays developed using rabbit and egg yolk antibodies. The analysis revealed that false positive in ELISA was less than 11% (< 1/9).

6.4 DICUSSION

Optimization of ELISA protocol at room conditions was conducted in order to facilitate the adoption of the ELISA for determination of ochratoxin A at field condition. The assay involved a simple extraction method involving methanol, a solvent compatible with immunoassay. The inhibition curve for ochratoxin standards in poultry feed extracts was used to estimate ochratoxins in samples to overcome interference due to food matrix. Immunization host (rabbit/hen) dependent antibody specificity and sensitivity was observed towards the structural analogs (variation in IC_{50} for structural analogs). Egg yolk antibody preparation showed ~22% cross reaction with ochratoxin B which is comparatively less than that reported by Clarke *et al.* (1993). They reported egg yolk antibody preparation with 100% cross-reaction towards ochratoxin B. Rabbit anti-OTA preparation showed comparatively less cross-reaction towards ochratoxin B (8.33%) and other structural analogs than egg yolk antibody preparation.

Table 6.5 Comparison of analysis of ochratoxin A in naturally contaminated poultry feed samples by indirect competitive-ELISA using anti-OTA IgG and egg yolk anti-OTA antibody with HPLC analysis

Sample*	**OTA estimated (µg/kg ± SD)**					
	HPLC		ELISA			
			Anti-OTA rabbit serum		Anti-OTA hen egg yolk antibodies	
	Mean (n=3)	CV %	Mean (n=3)	CV %	Mean (n=3)	CV %
1	Nil	-	Nil	-	Nil	-
2	200 ± 8	4	200 ± 18	9	227 ± 22	9.69

* 9 samples of poultry feed

It can be seen that the antibody has the following efficiency of inhibition to the metabolites viz., OTA > OTB > aflatoxin B_1 > phenylalanine. The sensitivity of the antibody preparation at 15 and 40ng/ml for rabbit anti-OTA serum and egg yolk anti-OTA antibody respectively are comparable to the published literature (Clarke, *et al*., 1993: Chu, 1976). Clarke *et al* (1993) reported an immunoassay developed using egg yolk antibodies raised against ochratoxin–BSA conjugate which was prepared by mixed anhydride method. The method optimized by them to isolate IgY preparation was followed in the current study. The yield of IgY (83 mg/egg) obtained in the current study was similar to that reported by them which was 95 mg/egg. The sensitivity of the assay reported by them was ~20 ng/ml with a detection limit of 50ppb. The detection limit in the present study was 5ppb. The detection limit is lower in the current study due to the sensitivity of the antibody to 0.1 ng/ml of OTA competitor concentration as compared to ~ 1ng/ml reported by them. In order to minimize the background they used ochratoxin conjugated to oval albumin as the coating antigen. In the present study neutralizing the antibody specific to carrier protein minimized the background binding to carrier protein.

Chu (1976) evaluated ochratoxin conjugated to different carrier protein as immunogen. They reported that ochratoxin conjugated to BSA was the best immunogen preparation for immunoassay. They reported an IC_{50} of 23 ng for ochratoxin A by a radioimmunoassay. Thirumala-Devi *et al.* (2000) reported an immunoassay for determination of ochratoxin in chilli using antibodies raised in rabbit against ochratoxin–BSA conjugate as immunogen. They reported antibody preparation with high titer (1: 100000) and IC_{50} of 5 ng/ml in the indirect competitive ELISA. Candlish *et al.* (1988) reported

immunoassay for determination of ochratoxin in wheat based on monoclonal antibody with a detection limit of 5 ppb. Gyongyosi-Horvath *et al.* (1996) reported monoclonal antibody based direct competitive ELISA with a detection limit of 42 picogram/ml. The variations in sensitivity and detection limit are influenced by various factors such as host, assay protocol, enzyme substrate system etc (Chu, 1976; Schneider and Hammock, 1992).

One of the limitations of polyclonal antibodies is that it can be produced only in limited amounts. The results from the current study reveals that egg yolk antibody can be used for large-scale production of antibodies comparable to that produced in rabbits. Although the IC_{50} values for egg yolk anti-OTA antibody were higher than that for rabbit anti-OTA antibody, the recoveries of ochratoxin A spiked poultry feed samples were similar except at 5ppb wherein the egg yolk antibody gave a lower recovery (67.48%). There were no significant variations in antibody titer in antibody purified from eggs from different days after booster dose. The egg yolk antibodies are advantageous over antibodies raised in rabbits as the antibody harvesting is non invasive as compared to repeated bleeding required for collection of sera from rabbits. Poultry feed is a complex food system rich in various nutrients, which makes an ideal food system to study the interference of matrix in the immunoassay. In the current assay of the nine samples analyzed only one sample of poultry feed was found to be contaminated with ochratoxin A at 200 ppb. The immunoassay estimations of the toxin correlated well with the HPLC analysis with a intra assay coefficient of variation of ~9%. None of the samples were false positive. Results suggest that the ELISA procedure developed is dependable and can be used for the determination of ochratoxin A in foods

CHAPTER 7.0

A PREDICTIVE MICROBIOLOGICAL STUDY – INTERRELATIONSHIPS OF TEMPERATURE, MOISTURE AND INOCULUM LEVEL ON BEHAVIOR OF TOXIGENIC *A.OCHRACEUS*

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A PREDICTIVE MICROBIOLOGICAL STUDY – INTERRELATIONSHIPS OF TEMPERATURE, MOISTURE AND INOCULUM LEVEL ON BEHAVIOR OF TOXIGENIC *A.OCHRACEUS*

RESULTS AND DISCUSSION

7.1 INTRODUCTION

Mycotoxin contamination is an important health concern associated with the fungal invasion in food. Mold infestation and toxin elaboration result by a complex interaction of fungus with various biotic and abiotic factors in the storage environment. Understanding the behavior of the fungus in fields and storage environments serve as an important tool for implementing control strategies. Predictive microbiology is an important tool for food technologist to understand the behavior of the microorganism in food systems under different environmental variables. In this regard two types of models are in use

1. Kinetic model
2. Probability model.

In Kinetic models the response variable is expressed in time-based units such as time taken for a particular response. The probability models predict the likelihood of some event such as spore germination or a detectable amount of toxin formed within a given period of time. Polynomial or response surface model is one type of kinetic models, which has also been used for constructing probability model. Response surface methodology is a promising approach useful for modeling and studying microbial behavior in which a response of interest is influenced by several variables. In this technique a linear model is constructed, which has the form of a polynomial function in the modeled parameters. Multiple linear regression is used to determine the best-fit values for the parameters. The polynomial regression has the general form.

$$Y= a + b_1X_1+b_2 X_2+. \ldots\ldots+b_jX_i+\ldots+b_nX_1^2+\ldots bjXi2+b_1X_1X_2+\ldots bzXiXj$$

Where a, $b_{1, 2}\ldots_{z}$ are parameters to be estimated and $X_{1, 2\ldots i, j}$ are variables.

A.ochraceus has been identified as the principal organism responsible for ochratoxin A contamination in tropical countries and ochratoxin A contamination has been frequently reported in cereals (Rao, *et al*., 1979, Janardhan, *et al*., 1999). Temperature and moisture are important abiotic factors that influence mold growth and toxin elaboration in foods. Number of reports on influence of these factors on fungal growth and toxin elaboration is available, but the studies on the influence of initial load of fungi on mold colonization and mycotoxin elaboration are scanty. Studying the influence of fungal load, temperature and moisture on behavior of the mold growth and ochratoxin A elaboration at different storage temperature and substrate moisture in natural food substrates would give information for devising control strategies. *A.ochraceus* is a common storage fungus that has been reported to grow at temperature between 8^{o}C and 37^{o}C with an optimum ranging from 24 to 31^{o}C. The fungus can produce ochratoxin A within a temperature range of 15-37^{o}C, with an optimum ranging from 28 to 31^{o}C. The fungus is known to produce ochratoxin in substrates with minimum a_w of 0.79, with an optimum ranging from 0.95 to 0.99 (ICMSF, 1996). In the present chapter the influence of inoculum, temperature and moisture on *A.ochraceus* viable count and biomass, ochratoxin A elaboration and their interrelationships in maize were studied using the predictive microbiological approach.

7.2 MATERIALS AND METHODS

7.2.1 Culture

A.ochraceus CFR 221, an isolate from maize, maintained in culture collection at CFTRI, Mysore was used in the current study. The fungus was subcultured periodically at monthly intervals on PDA and maintained at 4 ^{0}C.

7.2.2 Anti-*A.ochraceus* antibody

The antibody raised against *A.ochraceus* CFR 221 mycelial antigen obtained from the previous studies was used in the study (Section 4.2.5).

7.2.3 Maize

Maize was procured from local market and tested for presence of any ochratoxin contamination. The toxin free maize samples (50 g) were dispensed into 500 ml conical flasks and autoclaved at 121 ^{0}C, 15 p.s.i for 15 min.

7.2.4 Experimental Design

A three factorial central composite rotatory design (CCRD) was used to study the influence of three variables viz temperature, moisture, and inoculum at five levels of each variable. The samples were analyzed after 4 weeks of storage. The experimental design used in the study is shown in Table 7.1. The five coded levels of each variable at –1.6, -1, 0, +1 and +1.6 for temperature range of 20 - 35^{o}C, substrate moisture range of 10 - 30%, and inoculum range of 3 - 7 $\log_{10}$ spores/g were used.

Table 7.1 Three factorial central composite rotatary experimental design used for studying the influence of temperature, moisture and inoculum level on *A.ochraceus* growth and ochratoxin A production in maize.

	Coded variables		
Sl. No.	Temperature (X1)	Moisture (X2)	Inoculum level (X3)
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	-1.682	0	0
10	+1.682	0	0
11	0	-1.682	0
12	0	+1.682	0
13	0	0	-1.682
14	0	0	+1.682
15	0	0	0
16	0	0	0
17	0	0	0
18	0	0	0
19	0	0	0
20	0	0	0
21	0	0	0

7.2.5 Inoculum

The spore suspension from the fungus, *A.ochraceus* CFR 221 grown on PDA for 7 days was used in the study. The spore suspension was counted in haemocytometer and appropriate dilutions of this suspension were inoculated into maize samples to achieve the desired level of inoculum.

7.2.6 Treatment

The different sets of culture flasks containing maize samples (50 g) were autoclaved and labeled. The required quantities of spore suspension were added into each flask. The moisture was adjusted with sterile distilled water. The flasks were incubated in B.O.D (Dalal and Co, Mumbai) incubator maintained at required temperature. The flasks were mixed aseptically at weekly intervals to prevent caking. The experiment was terminated after 4 weeks of incubation. The contents of individual flasks were analyzed for viable count, fungal biomass and ochratoxin content.

7.2.7 Enumeration of viable counts

The maize sample (11 g) was mixed with saline (99 ml) and serially diluted. The appropriate aliquots of dilutions (0.1 ml) were inoculated onto prepoured sterile PDA plates in triplicate by spread plate technique. The inoculated plates were incubated at 28 ± 2^{o}C for 3 – 5 days and the characteristic colonies were counted, calculated and expressed as mean $\log_{10}$ cfu/g.

7.2.8 Biomass determination

Biomass was estimated by indirect non-competitive enzyme linked immunosorbent assay described earlier (Section 4.3.2). The maize extract was mixed with serially diluted mycelial extract prepared from known quantity of mycelium (dry wt) and analyzed by ELISA. The standard graph was prepared by plotting the fungal biomass/ml in maize extract versus ELISA absorbance. A linear relationship was obtained between ELISA response and fungal biomass/ml of maize extract in the range of 10-160 µg. The equation derived by regression analysis fit was used for estimation of fungal biomass in the maize samples. The standard graph used for estimation of fungal biomass in maize is shown in Fig. 7.1. The following equation derived by regression analysis fit of the standard graph was used to estimate the fungal biomass in maize.

$Y = X * 0.0051 + 0.078$

Where Y is the ELISA absorbance

X the fungal biomass correlation coefficient.

7.2.9 Estimation of Ochratoxin A

Ochratoxin A in maize samples were extracted by following standard AOAC procedure (AOAC, 1997) as described in section 3.2.3.4. Ochratoxin A in maize was quantified by TLC and densitometry as described in section 3.2.2.4.

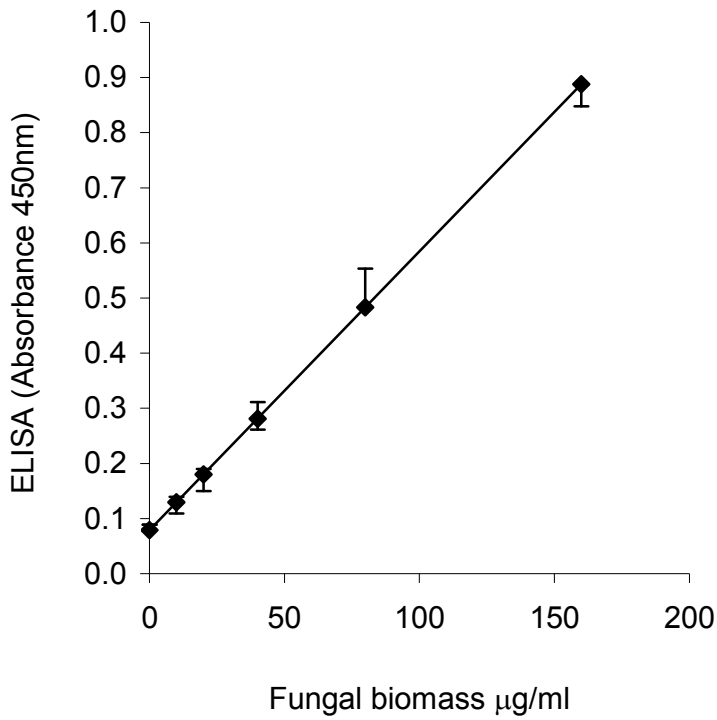

Fig. 7.1 Standard graph used for determination of *A.ochraceus* biomass in maize.

7.2.10 Modeling of *A.ochraceus* growth and ochratoxin A production

The data obtained for different set of experiment variables for growth and ochratoxin production were analyzed by multiple linear regression (MLR) using the Microsoft Excel program (version, 1997). The regression coefficients obtained by MLR was used for predicting the fungal growth (viable count and biomass) and ochratoxin A content for different combination of parameters within the range of experimental domain using the polynomial regression equation represented by

$$Y = \beta_0 + \sum_{i=1}^{4} \beta i Xi + \sum_{i=1} \beta ii Xi^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta ij Xi Xj$$

Where β_0 is the constant coefficient, β_i is the linear coefficient, βii is the quadratic coefficient and βij is the second order interaction coefficient.

7.2.11 Model validation

The predicted values obtained by using the response surface models were compared with the experimental values. The bias factor and accuracy factor were calculated according to Ross (1996) and Baranyi, *et al*. (1999). The bias factor was calculated using the formula

$$\text{Bias factor} = 10^{\left\{\text{Mean log}\left[\, Y_{predicted} / Y_{observed} \right]\right\}}$$

$$\text{Accuracy factor} = 10^{\left\{\sqrt{\text{Mean}\left[\, \log (Y_{predicted} / Y_{observed})^2 \right]}\right\}}$$

The root mean square error was calculated using the following formula

$$\text{RMSE} = \sqrt{\frac{\sum \left| (\text{Predicted} - \text{Observed})^2 \right|}{n}}$$

Where n is the number of comparisons

RESULTS AND DISCUSSION

7.3 RESULTS

7.3.1 Influence of variables on fungal behavior

7.3.1.1 Growth study

7.3.1.1.1 Viable count

The viable counts obtained for the different sets of experimental variables are shown in Table 7.2. Maximum viable count (10.3 $\log_{10}$ cfu/g) was observed at substrate moisture of 30% and incubation temperature of 28^{o}C. At lowest and highest temperature of 20^{0}C and 35^{0}C marginal increase in viable count was observed. At temperature range of 28 - 32^{o}C comparatively higher viable count was observed at >15% substrate moisture than that at sub optimal temperature (<28^{0}C) and above optimum temperature (>32 ^{0}C). At constant substrate moisture (20%) and temperature (28 ^{0}C), the substrate inoculated with lower inoculum level (3 $\log_{10}$ spores/g) had a higher viable count with an increase of 5 log units than that inoculated with higher inoculum level (7 $\log_{10}$ spores/g), wherein only an increase of 2 log units was observed. Similar behavior was observed at substrate moisture of 15% with inoculum level of 4 and 6 $\log_{10}$ spores/g .At inoculum level of 4 $\log_{10}$ spores/g an increase in viable count of 0.36 and 1.26 log units was observed at temperature of 23^{o}C and 32^{o}C respectively. Where as at inoculum level of 6 $\log_{10}$ spores/g and substrate moisture of 15% a decrease in viable count was observed at both temperatures. This could be due to competition at higher initial inoculum level.

Table 7.2 Observed and predicted *A.ochraceus* viable count in maize**.**

Temperature	Moisture (%)	Inoculum level Log_{10} cfu/g	observed	Predicted
23	15	4	4.30	5.18
32	15	4	5.26	6.42
23	25	4	4.90	5.96
32	25	4	9.30	8.83
23	15	6	4.90	6.38
32	15	6	5.40	5.46
23	25	6	8.70	8.70
32	25	6	9.32	9.40
20	20	5	5.36	3.74
35	20	5	5.26	5.36
28	10	5	6.85	5.59
28	30	5	10.30	10.50
28	20	*3*	8.48	7.69
28	20	7	9.51	9.23
28	20	5	9.32	9.30
28	20	5	9.41	9.30
28	20	5	9.15	9.30
28	20	5	9.54	9.30
28	20	5	9.53	9.30
28	20	5	9.41	9.30
28	20	5	9.30	9.30

7.3.1.1.2 Fungal biomass

The fungal biomass obtained for the different sets of experiments is shown in Table 7.3. Maximum fungal biomass (13.3 mg/g of substrate) was observed at substrate moisture of 30% and incubation temperature of 28^{o}C. At temperature range of 28 - 32^{o}C comparatively higher fungal biomass than that at sub optimal temperature (<28^{0}C) and above optimum temperature (35^{o}C) was observed. To determine the influence of inoculum on biomass the ratio of biomass after incubation period of 30 days and initial inoculum at different combination of variable was calculated and compared. At constant substrate moisture (20%) and temperature (28^{o}C), the substrate inoculated with lower inoculum level (3 $\log_{10}$ spores/g) had a higher fungal biomass at 2.1 mg/g of substrate per $\log_{10}$ spores than that inoculated with higher inoculum level (7 $\log_{10}$ spores/g), wherein only biomass of 1.13 mg/g of substrate per $\log_{10}$ inoculum was observed. Similar behavior was observed at inoculum level of 4 and 6 $\log_{10}$ spores/g and substrate moisture of 15% and 25%. At inoculum level of 4 $\log_{10}$ spores/g and moisture of 15% the biomass was estimated at 0.80 and 0.89 mg/g substrate per $\log_{10}$ inoculum at temperature of 23^{0}C and 32^{0}C respectively. The biomass for initial inoculum level of 6 $\log_{10}$ spores/g and substrate moisture of 15% was estimated at 0.72 and 0.83 mg/g substrate per $\log_{10}$ inoculum. At substrate moisture of 25% the influence of inoculum level was more pronounced than that observed for 15% substrate moisture. The biomass for experiments carried out at 23^{0}C and 32^{0}C was estimated at 1.51 and 2.37 mg/g substrate per $\log_{10}$ inoculum respectively for substrate inoculated with 4 $\log_{10}$ spores/g, which is comparatively higher than that estimated for substrate inoculated with 6$\log_{10}$ spores/g, where in biomass

of 1.15 and 1.78 mg/g substrate per $\log_{10}$ inoculum respectively was recorded. Similar behavior was observed in viable counts, which may be due to competition at higher level of inoculum. In the experimental design at inoculum level of 5 $\log_{10}$ spores/g and substrate moisture of 20% the biomass at lowest temperature of 20^0C was 1.3 fold less than that at highest temperature of 35^oC. Similarly in the experiment carried out at lowest and highest moisture level within the experimental domain 2.5 fold higher biomass was recorded in substrate with 30% compared to 10% substrate inoculated with 5 $\log_{10}$ spores/g inoculum at incubation temperature of 28^oC.

7.3.1.2 Ochratoxin A elaboration

The ochratoxin A production estimated for the different sets of experiments is presented in Table 7.4. Maximum ochratoxin A elaboration of 30 ppm was observed at substrate moisture of 30% and incubation temperature of 28^oC. At temperature range of 28 - 32^0C, comparatively higher ochratoxin A elaboration than that at sub optimal temperature (<28^0C) and above optimum temperature (>32^oC) was observed. To determine the influence of inoculum on ochratoxin A production the ratio of OTA after 30 days of incubation and initial inoculum at different combination of variable was calculated and compared. At constant substrate moisture (20%) and temperature (28^0C), the substrate inoculated with lower inoculum level (3 $\log_{10}$ spores/g) supported higher ochratoxin A production at 2.5 ppm/ $\log_{10}$ inoculum than that inoculated with higher inoculum level (7 $\log_{10}$ spores/g), wherein only OTA content at 1.14 ppm/ $\log_{10}$ inoculum was estimated. Similar behavior was observed at inoculum level of 4 and 6 $\log_{10}$ spores/g and substrate moisture of 15% and

25%. At inoculum level of 4 $\log_{10}$ spores/g and moisture of 15% the ochratoxin A production was estimated at 0.5 and 0.87 ppm /$\log_{10}$ inoculum was observed at temperature of 23^0C and 32^0C respectively. The ochratoxin A elaboration in experiment carried out at inoculum level of 6 $\log_{10}$ spores/g and substrate moisture of 15% was estimated at 0.33 and 0.66 ppm/$\log_{10}$ inoculum when incubated at 23^0C and 32^0C respectively. The ochratoxin A elaboration for experiments carried out at with initial inoculum of 4 $\log_{10}$ spores/g and substrate moisture of 25% was estimated at 2 and 2.5 ppm/$\log_{10}$ inoculum at 23^0C and 32^0C respectively which is comparatively higher than that estimated for substrate inoculated with 6 $\log_{10}$ spores/g, which was estimated at 1.66 and 2 ppm/ $\log_{10}$ inoculum. Similar behavior was observed in fungal growth studies, which may be due to competition at higher level of inoculum. At substrate moisture of 20% with initial inoculum level of 5 $\log_{10}$ spores/g and incubated at lowest temperature of 20^0C and highest temperature of 35^oC no ochratoxin A production was observed. In the experiment carried out at lowest and highest moisture level with in the experimental domain 15 fold higher ochratoxin A was recorded in substrate with 30% compared to 10% substrate inoculated with 5 $\log_{10}$ spores/g inoculum at incubation temperature of 28^oC.

Table 7.3 Observed and predicted *A.ochraceus* biomass in maize.

Temperature	Moisture (%)	Inoculum level Log_{10} cfu/g	Observed	Predicted
23	15	4	3.24	4.07
32	15	4	3.59	3.96
23	25	4	6.06	6.73
32	25	4	9.49	9.68
23	15	6	4.36	5.03
32	15	6	4.99	5.19
23	25	6	6.92	7.46
32	25	6	10.68	10.67
20	20	5	3.98	2.68
35	20	5	5.24	5.27
28	10	5	5.17	4.56
28	30	5	13.32	13.05
28	20	*3*	6.33	5.72
28	20	7	7.98	7.70
28	20	5	9.38	9.48
28	20	5	9.48	9.48
28	20	5	9.54	9.48
28	20	5	9.98	9.48
28	20	5	9.35	9.48
28	20	5	9.99	9.48
28	20	5	9.17	9.48

Table 7.4 Observed and predicted values of ochratoxin A production by *A.ochraceus* in maize.

Temperature	Moisture (%)	Inoculum level Log_{10} cfu/g	Y	Predicted
23	15	4	2.0	1.52
32	15	4	3.5	2.09
23	25	4	8.0	10.67
32	25	4	10.0	12.25
23	15	6	2.0	1.25
32	15	6	4.0	1.97
23	25	6	10.0	12.15
32	25	6	12.0	13.89
20	20	5	0	-1.73
35	20	5	0	0.18
28	10	5	2.0	4.86
28	30	5	30.0	28.00
28	20	3	7.5	6.51
28	20	7	8.0	7.90
28	20	5	10.8	11.39
28	20	5	10.0	11.39
28	20	5	12.25	11.39
28	20	5	11.5	11.39
28	20	5	10.5	11.39
28	20	5	12.8	11.39
28	20	5	12.5	11.39

7.3.2 Relationship between viable count, fungal biomass and ochratoxin elaboration

The viable count, fungal biomass and ochratoxin A elaboration had a positive correlation among each other (Fig. 7.2). The viable count correlated well with the fungal biomass (R^2 0.79). A positive correlation was observed (R^2 0.57) between viable count and ochratoxin A elaboration in maize. The ochratoxin A elaboration correlated more with biomass (R^2 0.82) than that with viable count.

7.3.3 Modeling of behavior of *A.ochraceus* in maize

A total of 21 experiments were carried out of which the fifteenth experiment performed at the center of the experimental domain was repeated six times in order to determine the residual variance value (Table 7.1). The experimental data were subjected to multivariate analysis and a polynomial quadratic model containing ten coefficients and interaction terms was adopted to describe the relationship of the responses Y. The fungal responses studied were viable count of *A. ochraceus* per gram of maize (Table 7.2), the fungal biomass per gram maize (Table 7.3) and ochratoxin A elaboration (Table 7.4) at the end of four week storage period with the experimental variables. The polynomial quadratic equation used to predict fungal response is as follows

$$Y_{predicted} = A + B * X_t + C * X_m + D * X_i + E (X_t * X_m) + F (X_m * X_i) + G (X_i * X_t) + H (X_t)^2 + I (X_m)^2 + J (X_i)^2$$

Where X_t is the temperature.

X_m is the moisture.

X_i is the initial inoculum.

A, B, C…J is the regression correlation coefficients.

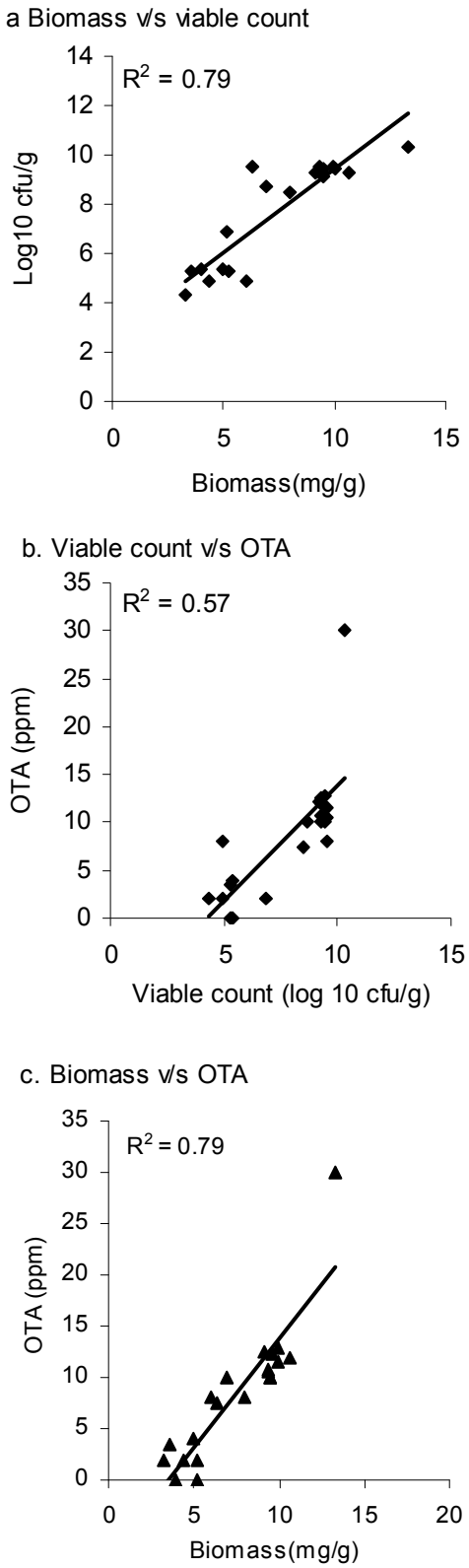

Fig. 7.2 Correlation between fungal biomass, viable count and ochratoxin A production in maize.

7.3.4 Prediction of *A.ochraceus* growth

The observed and predicted responses for viable count and fungal biomass are shown in Table 7.2 and 7.3 respectively. The coefficients of regressions for different factors used for predicting viable count and fungal biomass are shown in Table 7.5 and 7.6 respectively. The coefficient of temperature and inoculum were highest in the polynomial equation derived for predicting the viable count and fungal biomass indicating their combined effect on prediction of viable count and fungal biomass. In the polynomial equation used for predicting the viable count the coefficient of temperature was highest indicating its major effect on the predicted response. In the polynomial equation used for predicting the fungal biomass the coefficient for inoculum was highest indicating its major effect on the predicted response. The coefficients for temperature ($P < 0.005$) and square effects of temperature ($P < 0.005$) were significant in the polynomial equation derived for predicting the viable count. The coefficients for single factor of temperature ($P<0.005$) and inoculum ($P<0.05$) were significant in equation derived for predicting biomass. The coefficients for interaction effects of temperature and moisture ($P < 0.05$), square effects of temperature ($P < 0.005$) and inoculum ($P < 0.05$) were also significant in equation used for predicting fungal biomass. The R^2 value of 0.96 and 0.87 obtained for the polynomial equation representing the experimental samples involving biomass and viable count respectively show the derived model fits well with experimental data.

Table 7.5 Coefficients of multiple linear regressions for effects of variables on *A.ochraceus* viable count in maize.

Factors	*Coefficients*
Intercept	-73.27**
Temperature (X1)	4.96**
Moisture (X2)	-0.14
Inoculum level (X3)	4.32
$(X1)^2$	-0.08**
X1*X2	0.02
$(X2)^2$	-0.01
X2*X3	0.08
$(X3)^2$	-0.21
X3*X1	-0.12
R^2	0.87
Standard error	0.99
Bias factor	1.00
Accuracy factor	1.14
Root mean square error	0.72

** Significant at P <0.005

Table 7.6 Coefficients of multiple linear regressions for effects of variables for *A.ochraceus* biomass in maize.

Factors	Coefficients
Intercept	-79.46**
Temperature (X1)	4.73**
Moisture (X2)	-0.20
Inoculum level (X3)	7.24*
$(X1)^2$	-0.10**
X1*X2	0.03*
$(X2)^2$	-0.01
X2*X3	-0.01
$(X3)^2$	-0.69**
X3*X1	0.01
R^2	0.96
Standard error	0.69
Bias factor	1.00
Accuracy factor	1.12
Root mean square error	0.50

* Significant at P < 0.05

** Significant at P < 0.005

7.3.5 Prediction of ochratoxin A elaboration in maize

The observed and predicted responses for ochratoxin A elaboration has been shown in Table 7.4. The coefficients of regressions for different factors used for predicting ochratoxin A is shown in Table 7.7. The coefficients of temperature and inoculum were highest indicating their combined effect on ochratoxin prediction. The coefficient for single factor of temperature was statistically significant ($P<0.005$). The coefficients for terms of square effects of temperature ($P <0.005$), moisture ($P <0.05$) and inoculum ($P <0.05$) were also significant. The R^2 value of 0.92 obtained for the polynomial equation represents the experimental samples involving ochratoxin A elaboration show the derived model fits well with experimental data.

7.3.6 Model validation

In the present study the prediction obtained for viable population and biomass had a bias of 1.00 (Table 7.5, Table 7.6), indicating a good match between the predicted and observed values. The bias factor obtained for ochratoxin A prediction was 0.97 (Table 7.7). The bias factor answers as to whether the experimental values lie above or below the line of equivalence and if so how much. A bias factor of 1 indicates a perfect match between the experimental and predicted value. A bias factor of above 1 indicates over prediction by the model. The accuracy factor determined for prediction for viable count, biomass and ochratoxin content were 1.14, 1.12 and 1.37 respectively. The accuracy factor averages the minimum distance between each point and line of equivalence as a measure of how close, on average, predictions are to observation.

Table 7.7 Coefficients of multiple linear regressions for effects of variables on ochratoxin A production in maize.

Factor	Coefficients
Intercept	-172.08**
Temperature (X1)	11.74**
Moisture (X2)	-1.32
Inoculum level (X3)	8.81
$(X1)^2$	-0.22**
X1*X2	0.01
$(X2)^2$	0.04*
X2*X3	0.09
$(X3)^2$	-1.05*
X3*X1	0.01
R^2	0.92
Standard error	2.37
Bias factor	0.97
Accuracy factor	1.37
Root mean square error	1.55

* Significant at P < 0.05

** Significant at P < 0.005

The accuracy factor is a measure of average deviation. The RMSE for predictive model obtained for viable count, biomass and ochratoxin A content were 0.72, 0.50, and 1.55 respectively. The RMSE is a measure of goodness-of-fit of the model. Low RMSE indicates a better goodness-of-fit.

7.3.7 Interpretation of response using contour plots

The secondary predictive models for viable count, fungal biomass and ochratoxin A elaboration in maize at different inoculum within the experimental domain is shown in Fig. 7.3, Fig. 7.4 and Fig. 7.5 respectively. The contour plots indicate the variations in viable count, biomass and ochratoxin A prediction with respect to inoculum at different substrate moisture level and storage temperate within the experimental domain. All the negative values predicted were taken as zero in the response surface models. The contour plots indicate that at optimum temperature and higher moisture levels the fungus can grow at wider range of moisture and temperature respectively. Higher viable count and biomass was observed at all the moisture level at temperature range of 26 - 29^0C and as substrate moisture tends to increase the temperature range for fungal growth increased within the experimental domain. Similarly ochratoxin A elaboration occurs at a wide range of moisture at optimum temperature of 26 - 29^0C and favorable temperature range increases with increase in moisture.

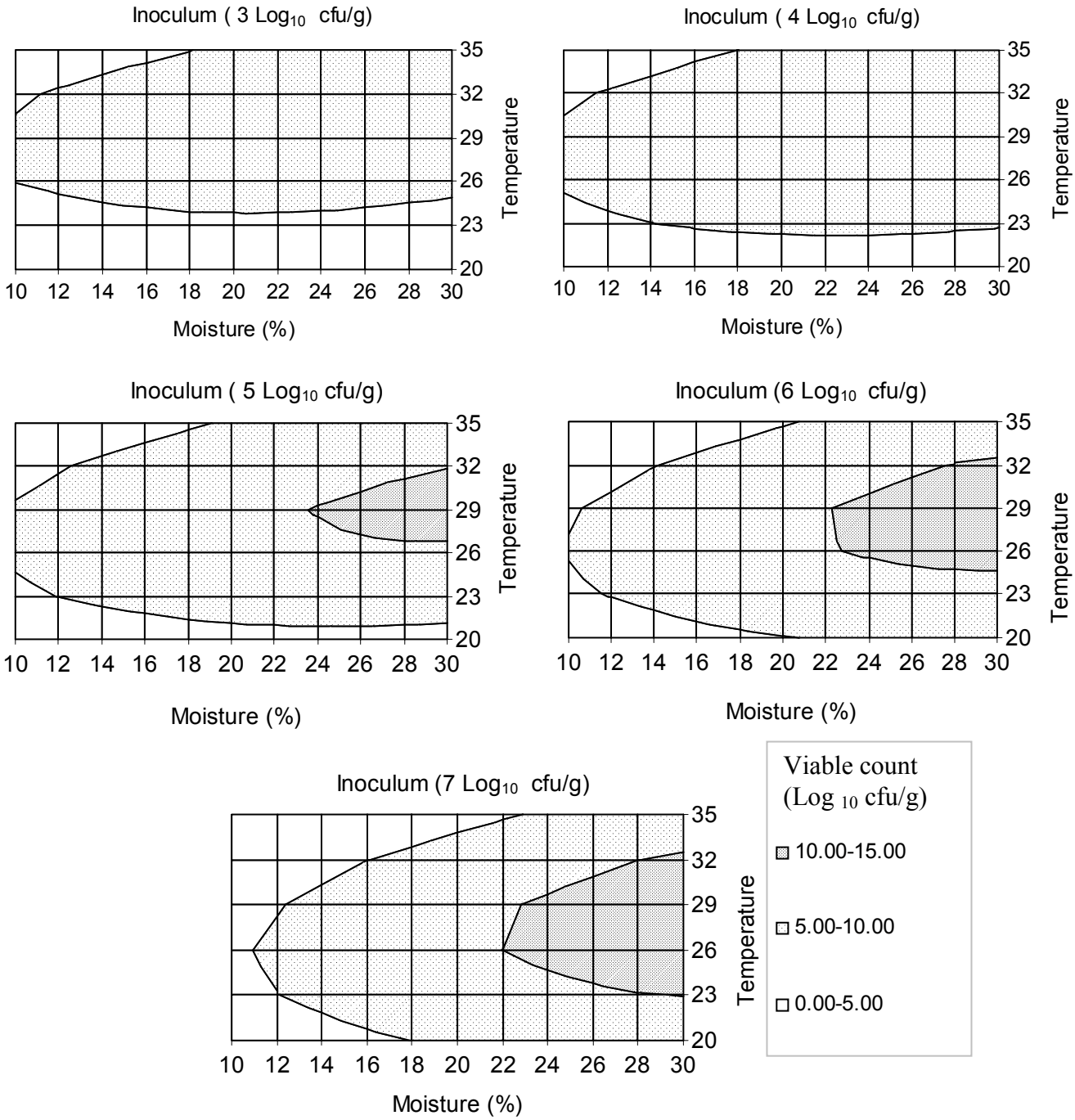

Fig. 7.3 Response surface contour plots showing the influence of temperature, moisture and inoculum on *A. ochraceus* viable count.

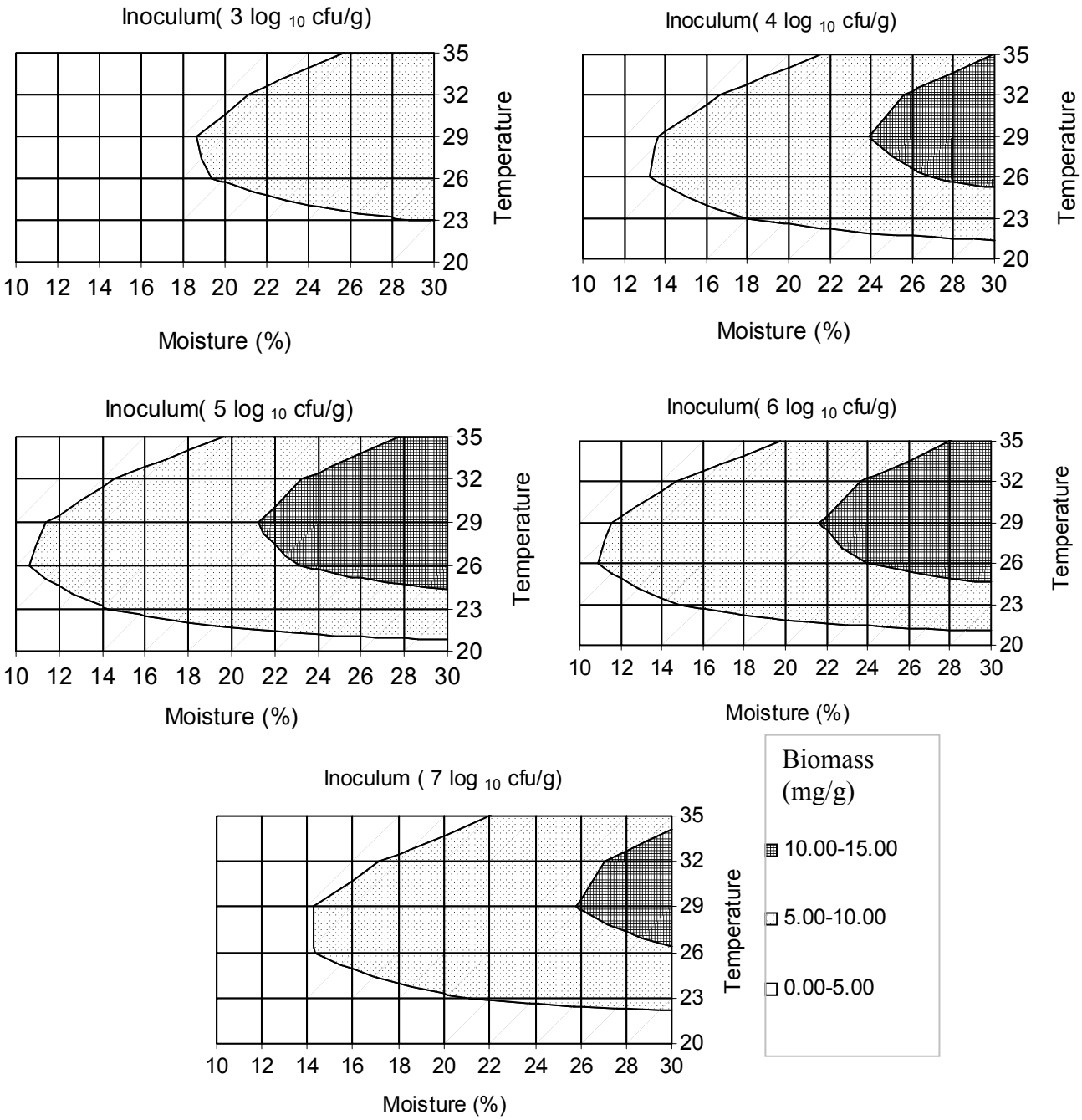

Fig. 7.4 Response surface contour plots showing the influence of temperature, moisture and inoculum on *A.ochraceus* biomass.

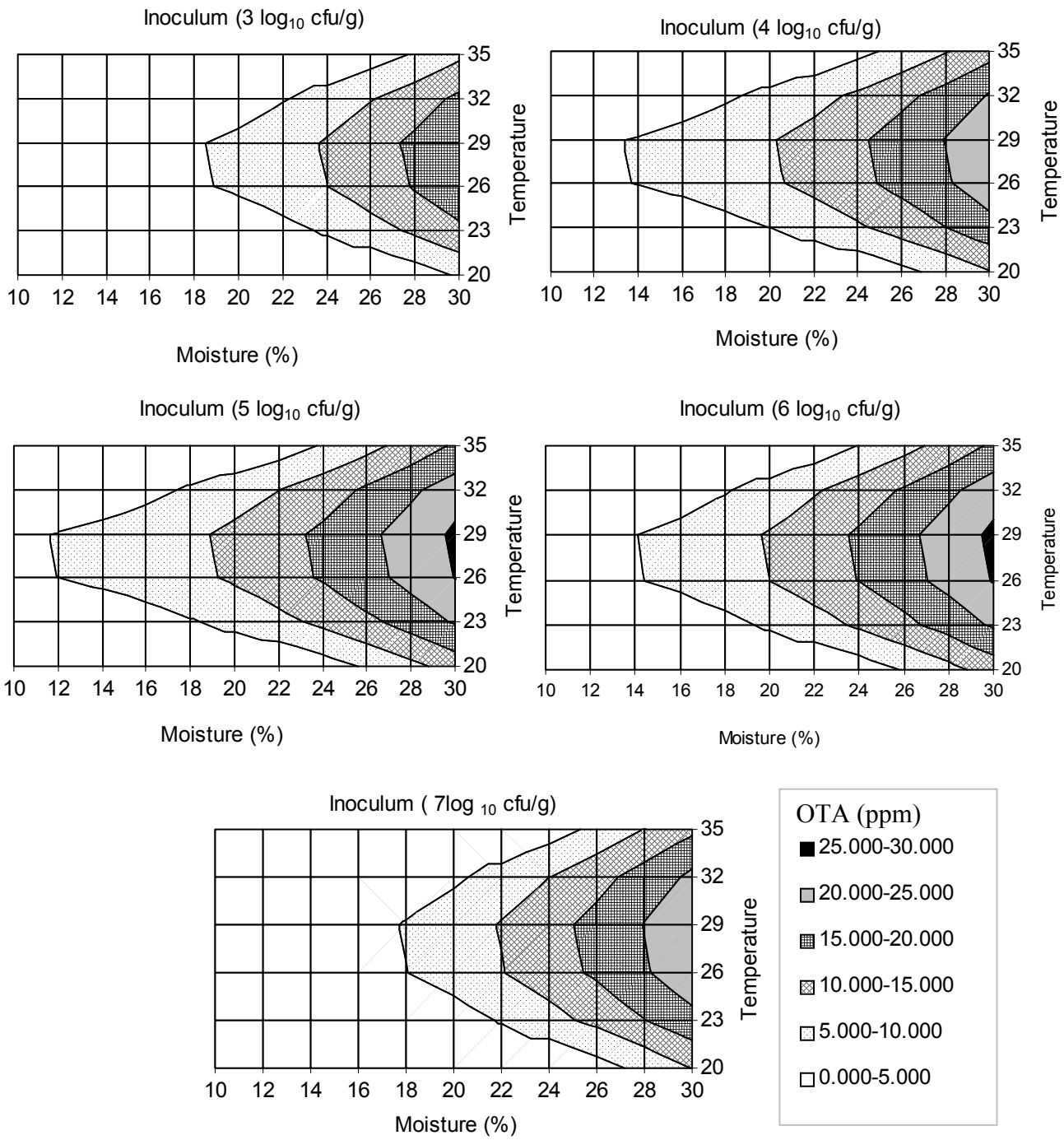

Fig.7.5 Response surface contour plots showing the influence of temperature, moisture and inoculum on ochratoxin A production by *A.ochraceus*.

The contour plots also shows that the viable count is influenced by the initial inoculum level. The fungal biomass and ochratoxin elaboration tend to increase with increase in inoculum from low level (3 $\log_{10}$ spores/g) to intermediate level (≤5 $\log_{10}$ spores/g) and thereafter tend to decrease on further increase in inoculum level from intermediate level (5 $\log_{10}$ spores/g) to higher level (>5 $\log_{10}$ spores/g). The responses of fungal biomass and ochratoxin A elaboration tends to behave similarly and hence fungal biomass measurement gives better indication of mold activity such as mycotoxin elaboration than the viable count measurement.

7.4 DISCUSSION

In the study at constant substrate moisture, significant increase in viable count at higher and nearer to optimum temperature (28 & 32^{o}C) was observed as compared to lower temperature and at all levels of inoculum, without corresponding increase in fungal biomass. This may be due to increase in plate count due to sporulation as indicated by higher plate count at nearer to optimum temperature. The variation in responses for fungal growth as measured by viable count and fungal biomass may also occur due to inherent limitation of viable count estimation and lower responses of antibodies towards fungal spores. The viable count of fungi may come from both hyphal fragments and spores. The viable count analysis varies with sample preparation methods and dilution used for plating (Jarvis, *et al*., 1983). Bacon and coworkers.(1973) studied the influence of temperature and moisture on spore germination, mycelial growth, sporulation and development of sclerotia in *A.ochraceus* grown on poultry feed. They reported that at lower

temperature the fungus produced spores only at the highest water activity, whereas at 30^{o}C the fungus developed conidial heads at all levels of water activity needed for growth. Hence, at condition favorable for sporulation the viable count will be higher than at conditions in which the mold growth occurs predominantly through hyphal extension. Hence, the biomass determination gives a better understanding of fungal growth than viable count for predictive modeling studies. Moreover the immunological method used for determining the fungal biomass can be used for direct comparison with studies performed with substrates varying in composition.

The present work further confirms that temperature and moisture requirements for growth is wider than that required for ochratoxin elaboration. At sub optimal temperature the influence of substrate moisture was pronounced on ochratoxin A elaboration than fungal growth. The available literature shows that the moisture requirement for toxin elaboration is narrower than that required for growth (Lacey, 1994) and moisture requirement is lower at optimum temperature and higher at minimum and maximum temperature for growth (Scott, 1957; Bullerman, *et al.,* 1986). Storage fungi are known to grow and produce mycotoxins in substrate with moisture of >14 % in equilibrium with relative humidity. But in the current study as humidity was not equilibrated the toxin production at low moisture (10%) could not be compared with the published reports. This is because the water activity of the substrate was not monitored. However, the variation in final substrate moisture was observed which is due to mold metabolic activity. It is known that due to the metabolic activity of the fungus the substrate moisture tend to increase with increase in storage period (Sauer, 1988). The

experiments designed with constant water activity would give a better understanding of the fungal behavior. However, in the traditional storage structures, water activity is seldom maintained and interpretation of predictions for fungal behavior in such situation is difficult.

A number of reports are available regarding influence of various single and combination of factors on fungal colonization and toxin elaboration. But most of them are conducted on synthetic medium and not on natural substrates. Pardo *et al.* (2004) reported studies on prediction of *A.ochraceus* growth and toxin elaboration in barley extract based medium and mold inoculated barley grains. They have studied the growth as a function of growth rate, visible mold growth and lag phase behavior prior to visible fungal growth. The reports lack information regarding validation of predicted models, which is useful to determine the confidence of the model in predicting fungal growth and toxin elaboration. The validation of model is necessary to determine the errors of the predictive model so as to determine the over prediction and under prediction at different level of variables.

Pardo and coworkers (2004) reported variation in fungal strain regarding growth rate and toxin elaboration. It is imperative that the growth response and toxin elaboration studied cannot be universally applied / extended for the fungal strain originating from different geographical region and ecological niche. Hence in this regard predictive modeling is useful for studying the variation of fungal strain originating from different source by which the responses of fungal behavior for wide level of variables can be studied with minimum number of experiments. The predictive models are increasingly used to analyze the microbial responses such as microbial

growth, toxin elaboration, storage and shelf life study by food quality management systems.

The inclusion of common mycoflora associated with the substrate / unsterilized substrates in the experimental design is necessary to broaden the information of complex interactions and their influence on the fungal colonization and toxin elaboration in natural condition. Maize substrate used in this study was sterilized to destroy endogenous fungi and spores for easier interpretation of the data. But data generated with controlled experimental conditions would be useful for comparison with studies under natural condition. The interaction of endogenous spores and fungi determines the fungal growth and toxin elaboration in natural condition. Comparison of the model obtained from experimental setup under natural storage systems and controlled condition would be required for better understanding of the complex interaction of other factors on fungal colonization and toxin elaboration. The mathematical model helps in understanding the complex interaction of variable on fungal colonization and OTA elaboration as a function of individual variable and interaction of different variable. In the response surface model mathematical validation proves that the model adequately describes the behavior of *A.ochraceus* in foods. The developed model could be useful for predicting *A.ochraceus* growth and ochratoxin elaboration in foods with a composition similar to the chemical and physical factors studied.

CHAPTER 8.0

CONTROL OF OCHRATOXIGENIC FUNGI AND DEGRADATION OF OCHRATOXIN A BY MICROBIOLOGICAL METHOD

CHAPTER 8A

IDENTIFICATION, ISOLATION, AND APPLICATION OF ANTIFUNGAL METABOLITE

CHAPTER 8A
IDENTIFICATION, ISOLATION, AND APPLICATION OF ANTIFUNGAL METABOLITE

RESULTS AND DISCUSSION

8.1 INTRODUCTION

Mold infestation of food grains may occur during both pre harvest and post harvest condition. The fungal colonization may bring undesirable changes in the physical characteristics such as color, weight loss, caking, etc. The chemical contaminants produced by molds especially the mycotoxin contamination is cause for concern. Hazard posed by fungal infestation depends on the type of fungus, extent of colonization and the type of mold activity, which again depends upon the complex interaction within and between biotic and abiotic factors. Generally, the optimum requirements of important factors such as a_w and temperature is narrower for toxin production than that required for growth. This implies that under conditions adverse to the fungal growth there is less possibility of production of mycotoxins. Number of antifungal chemicals is been used for controlling the fungal infestation, but there is lot of concern regarding their safety. In this direction there are numerous reports on compounds from various microbial and plant origin exhibiting antagonistic activity against fungi. Vicente *et al.* (2003) reviewed various antifungal compounds of microbial origin. There are some reports of bacterial isolates antagonistic towards fungal organisms (Walker, *et al*, 1988) and fungal species (Podile and Prakash, 1996), which are antagonistic to other fungal genera. In the present chapter the work regarding screening, identification and application of an antifungal compound for controlling ochratoxigenic fungi in food is presented.

8.2 MATERIALS AND METHODS

8.2.1 Cultures

Bacterial (10) and fungal (6) isolates, which exhibited inhibitory activity towards other fungal cultures, were screened for antifungal activity towards *A.ochraceus* group and other fungal cultures listed in Table 8.1. All the bacterial isolates were sub cultured on nutrient agar slants. All the fungal isolates were sub cultured periodically on PDA slants and maintained at 4^0C.

8.2.2 Diluent

The diluent used was 0.85% normal saline, which was dispensed in requisite quantities in suitable glass test tubes or flasks and sterilized at 121^0C, 15 p.s.i for 15 min.

8.2.3 Bacteriological media

8.2.3.1 Brain heart infusion broth (BHI)

Composition	g/l
Calf brain, infusion	200.0
Beef infusion	250.0
Protease Peptone	10.0
Dextrose	2.0
Sodium Chloride	5.0
DiSodium Phosphate	2.5
pH	7.4 ± 0.2

Requisite quantity of the medium was dissolved in known volume of distilled water, known amounts were dispensed into suitable test tubes or flasks and were sterilized at 121^0C, 15 p.s.i for 15 min. BHI broth was used to cultivate the bacterial isolates.

8.2.3.2 Potato dextrose agar (PDA)

Composition	g/l
Potato	200g
Dextose	40g
Agar	15g

The Potato infusion was prepared from 200g peeled potato, dextrose and agar was added. The medium was made up to 1L with distilled water, dispensed into suitable glass containers and sterilized at 121^{o}C, 15 p.s.i for 15 min. The PDA agar was used to cultivate and maintain fungal cultures.

8.2.4 Screening of bacterial isolates for antifungal activity

The fungal spore suspension from test culture *A.ochraceus* MTCC 1877 was inoculated on to pre poured PDA plates by spread plate technique. A loopful of 24 h bacterial test culture was point inoculated on the PDA plate inoculated with test culture (spot on lawn technique). The plates were incubated at 28 ± 2^{0}C for 5 – 7 days and observed for any inhibition of fungal growth.

8.2.5 Anti-fungal metabolite preparation

The bacterial cultures showing antifungal activity were studied further for the production of antifungal metabolite in broth culture. The bacterial cultures were inoculated into BHI broth (50 ml) and incubated at 37^{0}C for 48 h. The bacterial cells were separated by centrifugation at 10000rpm for 30 min and supernatant were collected. The supernatant was filter sterilized by membrane filter (0.22 µm). The filtrate was tested for antifungal activity by agar well diffusion assay.

8.2.6 Agar well diffusion assay

The fungal spore suspension from *A.ochraceus* MTCC 1877 was inoculated on pre poured PDA plates by spread plate technique. Agar wells (5 mm, dia) were made on the PDA plates and antifungal metabolite preparation (25 µl) of the test cultures were dispensed separately into each well. The plates were incubated for 5 – 7 days at 28 ± 2 ^{0}C and observed for any zone of inhibition. The antifungal activity was measured in terms of arbitrary units (AU). The volume of culture broth giving a zone of inhibition of 10 mm (dia) was defined as 1 arbitrary unit.

8.2.7 Biochemical characterization of bacterial strains

Native food isolate exhibiting antifungal activity was characterized by biochemical tests. The tests included were as follows:

8.2.7.1 Gram stain

Crystal violet:

Solution A

Crystal violet	2.0 g
Ethyl alcohol	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B were mixed. The prepared stain was filtered and stored in a clean, dry glass stoppered bottle.

Gram's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Saffranin

Saffranin-O	2.5 g
Ethyl alcohol, 95%	100 ml

10 ml of the stock solution was mixed with 90 ml of distilled water to prepare saffranin stain, which is used as counter stain.

The native food isolate was subjected to Gram's staining as following:

1. The heat fixed smear of the isolate was prepared on a clean glass slide.
2. The smear was stained with crystal violet stain for 1 min followed by washing off excess stain with water.
3. Gram's iodine solution was added and allowed to react for 1 min.
4. After washing off the excess iodine, the smear was decolorized with absolute ethanol.
5. Finally the smear was counterstained with saffranin for 30 sec.
6. Smear was washed with water, dried and examined under oil immersion of a compound microscope.
7. Cell morphology and staining characteristics were recorded.

8.2.7.2 Endospore staining

The heat fixed smear of the isolate was stained with malachite green over boiling water bath for 5 min. The smear was washed with water and counterstained with saffranin for 30 sec. Smear was washed with water, dried and examined under oil immersion of a compound microscope. The presence of endospore, position and shape were recorded.

8.2.7.3 Catalase test

A loopful of the 24 h culture was placed on a drop of 3% (v/v) hydrogen peroxide. The effervescence observed due to the release of nascent oxygen is considered as positive for catalase production.

8.2.7.4 Oxidase test

A loopful culture of the 24 h culture was taken in a clean glass rod and rubbed over filter paper soaked in 1% aq tetra methyl p-phenylenediamine dihydrochloride. Appearance of blue color within seconds is considered as positive for oxidase production.

8.2.7.5 Nitrate reduction test

The test culture was grown overnight in nitrate broth 37^0C for 24 h. To the broth few drops of 1% solution of sodium potassium nitrate were added and incubated for 24h. The broth was acidified with few drops of 1N HCl. To the acidified broth 0.5 ml of 0.2% solution of sulphanilamide and 0.5ml of 0.1% N-napthylethylenediamine hydrochloride were added. The appearance of pink color indicates nitrate reduction.

8.2.7.6 Starch hydrolysis

The test culture was inoculated into 1% starch agar and incubated for 24 – 48 h at 37^0C. The agar plate was flooded with Gram's iodine and observed for appearance of clear zone around the colony. The clearance of blue color around the colony against blue background is considered as positive for starch hydrolysis.

8.2.7.7 Casein hydrolysis

The nutrient agar was supplemented with 1% casein and incubated for 24 – 48 h at 37^0C. The presence of clear zone around the test culture is considered positive for casein hydrolysis.

8.2.7.8 Gelatin liquefication

The test culture was inoculated by stab culture into nutrient agar supplemented with 5% gelatin and incubated at room temperature for 7 days. The observation of clearing zones around the culture indicates gelatin digestion.

8.2.7.9 Acid production from sugars

A. Sugar solutions:

Sugar solutions (10%) were individually prepared in distilled water, filter sterilized using 0.22 μm membrane filter and stored in sterile screw capped tubes at 4°C. The individual sugars used were glucose, mannitol, sucrose, xylose, arabinose, trehalose, mannose and maltose.

B. Sugar fermentation basal medium (Composition g/l)

Peptone	10.0
Sodium chloride	5.0
Beef extract	4.0
Bromocresol purple	0.04
pH	7.0 ± 0.2

As per the instructions of manufacturer the requisite amount of dehydrated medium was dissolved in known volume of distilled water. Aliquots of 3 ml each were dispensed into test tubes and sterilized. Respective sugar solutions were added aseptically to each tube to achieve a final concentration of 1%. These

tubes were inoculated with the individual test cultures and incubated at 37°C for a period of 48 h. Change of color from purple to yellow of the indicator dye in the incubated medium is considered positive response.

8.2.7.10 Voges-Proskauer (VP) reaction

A. MR-VP broth (Composition g/l)

Buffered saline	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
pH	6.9 $\pm$ 0.2

The requisite quantity of dehydrated medium was dissolved in known volume of distilled water. Aliquots of 5 ml each of dissolved medium were distributed in test tubes and sterilized. Test cultures were individually inoculated into the broth medium and incubated for 24 h at 37°C.

B. The VP test reagent consists of two solutions

Solution I

α-Naphthol	5.0 g
Absolute alcohol	100 ml

Solution II

Potassium hydroxide	40.0 g
Creatinine	0.50 g
Distilled water	100 ml

To the culture broth, 0.6 ml of solution I and 0.2 ml of solution II were added, mixed well and tubes were kept unplugged, so as to allow the incorporation of atmospheric oxygen. The tubes were observed for the formation of an eosin pink color.

8.2.8 Identification of bacteria

Pure isolates, plated on nutrient agar, were identified based on morphological, microscopic and bio-chemical characteristics using Bergey's Manual of Determinative Bacteriology (1986).

8.2.9 Characterization of antifungal metabolite

8.2.9.1 Effect of temperature on antifungal metabolite

The antifungal metabolite preparation showing initial activity of 2 AU / 25 µl were taken in individual glass vials and exposed to temperature of 60^0C, 80^0C and 100^0C for a period of 2 h in a water bath. The bacterial metabolite (25 µl) treated at different temperature was tested for antifungal activity by agar well diffusion assay as described earlier (Section 8.2.6) using *A.auricomus* CFR 229 as test culture. The antifungal preparation, which was not exposed to higher temperatures served as control.

8.2.9.2 Effect of pH on antifungal activity

The antifungal metabolite preparation showing an initial activity of 2 AU / 25 µl was taken in glass vials and pH was adjusted to 2.5, 4, 7 and 10 with 1 N HCl or 1 N NaOH. The bacterial preparation (25 µl) adjusted to various pH levels was tested for inhibitory activity by agar well diffusion assay as described in section 8.2.6 using *A.auricomus* CFR 229 as test culture.

8.2.9.3 Effect of proteolytic enzymes on antifungal activity

The sensitivity of antifungal metabolites to proteolytic enzymes was tested. The lysozyme and trypsin stock solution were prepared in PBS at pH 6.1 ± 0.2 and pH 8.1 ± 0.2 respectively. The pH of antifungal preparation showing initial activity of 2 AU was adjusted to pH values optimum for each enzyme activity.

The enzyme stock solutions were added to antifungal preparation to give a final enzyme concentration of 1 mg/ml and incubated at 37°C for 2 h. The enzyme treated metabolite preparation (25 µl) was tested for antifungal activity by agar well diffusion assay as described in section 8.2.6 using *A.auricomus* CFR 229 as test culture. Control consisted of enzyme solutions without the antifungal preparation.

8.2.10 Effect of metabolite on various stages of fungal growth.

The antifungal activity of the metabolite was tested at different time period of fungal growth. Spore suspension from *A.auricomus* CFR 229 was inoculated on PDA by spread plate technique and incubated at 28 ± 2 ^{0}C. Antifungal preparation (2 AU) was added into the fungus-seeded plate as described earlier (Section 8.2.6) after 0, 1, 2, 3 and 5 days of incubation. The treated plates were observed for zone of inhibition.

8.2.11 Characterization of antifungal metabolite

8.2.11.1 Molecular mass

The antifungal preparation was dialyzed against distilled water using dialysis membranes with cut off of 2000 Da and 10000 Da (Sigma, USA). The dialyzate (25 µl) was tested for antifungal activity by spot on lawn technique as described earlier in section 8.2.6.

8.2.11.2 Acid precipitation

The antifungal preparation was precipitated with HCl. The precipitate was separated out by centrifugation. The precipitate was dissolved in distilled water and tested for antifungal activity by agar well diffusion assay as described earlier in section 8.2.6.

8.2.11.3 Alcohol precipitation

The antifungal preparation was precipitated with two volumes of isopropanol. The precipitate was separated by centrifugation and dissolved in distilled water and tested for antifungal activity by agar well diffusion assay as described earlier section 8.2.6.

8.2.12 Determination of effective concentration of antifungal metabolite

The *A.ochraceus* CFR 221 spore suspension was inoculated into YES broth (25 ml) and antifungal preparation was incorporated into YES medium at concentration of 1.5, 3, 6, 12 and 25 AU/ml. All the flasks were incubated at 30^0C for 7 days and the mycelial mat was filtered. The fungal biomass was determined by direct weighing of mycelium after drying the fungal mycelium to constant weight at 95^0C. The pH of culture broth was adjusted to 4.0 with 1N HCl and ochratoxin was extracted with chloroform (25 x 3). The ochratoxin A content was determined by TLC and densitometry (Section 3.2.2.4).

8.2.13 Application of antifungal metabolite in food system

The antifungal preparation was tested for inhibition of growth of *A.ochraceus* in maize. Maize grains procured from local market were ground to grits and known quantities were dispensed in to screw cap tubes. The maize samples were autoclaved and inoculated with spore suspension of *A.ochraceus* CFR 221 (10^6 spores) and treated with antifungal metabolite at a concentration of 25 AU/g. The final moisture of the samples were adjusted to 30% with sterile distilled water. The treated samples were incubated at 28 ± 2^0C for 7 days. The samples were analyzed for fungal biomass and ochratoxin content. The maize sample without any antifungal preparation served as control.

8.2.14 Biomass determination

Biomass was estimated by indirect non-competitive enzyme linked immunosorbent assay described earlier (Section 4.3.2). The maize extract was mixed with serially diluted mycelial extract prepared from known quantity of mycelium (dry wt) and analyzed by ELISA. The standard graph was prepared by plotting the fungal biomass in maize extract versus ELISA absorbance. A linear relationship was obtained between ELISA response and fungal biomass by regression analysis fit. The equation derived was used for estimation of fungal biomass in the maize samples. The standard graph used for estimation of fungal biomass in maize is shown in Fig. 7.1. The following equation derived by regression analysis fit of the standard graph was used to estimate the fungal biomass in maize.

$Y = X * 0.0051 + 0.078$

Where Y is the ELISA absorbance

X the fungal biomass correlation coefficient.

RESULTS AND DISCUSSION

8.3 RESULTS

8.3.1 Screening of isolates for antifungal activity

Of the ten bacterial isolates and six fungal isolates screened one bacterial isolate showed inhibitory activity towards *A.ochraceus* MTCC 1877 test culture (Fig. 8.1). The culture produced an extracellular metabolite, it showed inhibitory activity towards all the ochratoxigenic fungi and few other common fungal cultures tested (Table 8.1).

8.3.2 Identification of culture

The biochemical characterstics of the bacterial isolate is presented in Table 8.2. The culture was gram positive, endospore forming bacilli, and had a colony appearance of folded hair structure with rhizoid outgrowths. Based on the morphological and biochemical characterstics the organism was identified as *Bacillus badius*.

8.3.3 Characterization of antifungal metabolite

The antifungal activity of the metabolite was assessed for its stability at range of temperatures, pH and action of proteolytic enzymes and the results is presented in Table 8.3. The effect of temperature, pH and proteolytic enzyme action are also shown in Fig. 8.2, Fig. 8.3 and Fig. 8.4 respectively.

8.3.3.1 Heat stability

The results indicated that the antifungal metabolite was stable at temperature treatment of 80^0C for 2 h but the metabolite was inactivated at boiling temperature.

Fig. 8.1 Inhibition of *Aspergillus ochraceus* MTCC 1877 growth by *Bacillus badius*

Table 8.1 Antifungal activity of *Bacillus badius*

Fungal Isolates	Zone of Inhibition (in mm)
Aspergillus ochraceus	15.0
Aspergillus melleus	14.7
Aspergillus ostianus	19.2
Aspergillus sclerotiorum	20.8
Aspergillus sulphureus	16.5
Aspergillus auricomus	16.2
Aspergillus niger	12.0
Phoma	25.0

Table 8.2 Biochemical characterstics of the bacterial isolate

Test	Result
Gram reaction	+
Endospore	+
Catalase reaction	+
Oxidase reaction	-
Starch hydrolysis	-
Casein hydrolysis	+
Gelatin liquefaction	-
Citrate utilization	-
Nitrate reduction test	-
Voges-Proskauer (VP) reaction	-
Growth in NaCl	
5%	+
10%	-
Growth at	
10^0C	-
30^0C	+
40^0C	+
50^0C	+
Gas production from sugars	
D-Glucose	-
D-Mannitol	-
L-Arabinose	-
D-Xylose	-

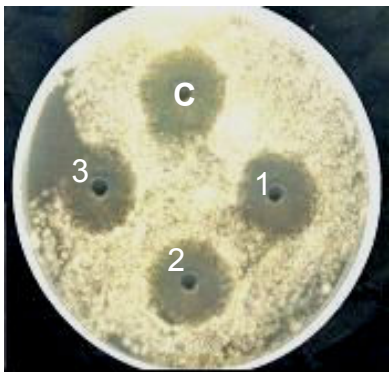

Fig. 8.2 Influence of temperature on the activity of the metabolite

Temperature treatments: C-30^{0}C, 1- 45^{0}C, 2- 60^{0}C, 3- 80^{0}C

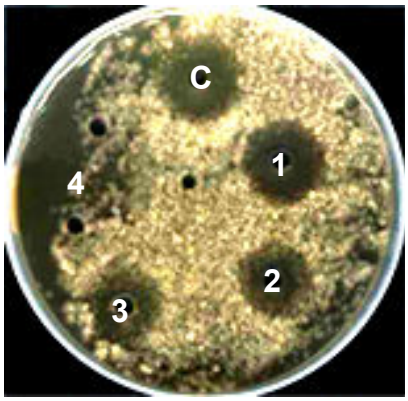

Fig. 8.3 Influence of pH on the activity of the metabolite

pH: C-7.6, 1- 7, 2- 4, 3-2.5, 4-10.

Table 8.3 Effect of pH, temperature and proteolytic enzyme on antifungal activity of metabolite produced *by B.badius* against *A.ochraceus* MTCC 1877.

Factor	Treatment	Zone of inhibition (mm)
pH	2.5	15.00±0.10
	4.0	18.75±0.25
	7.0	18.25±0.20
	10	15.5±0.50
Temperature	Control	21.5±0.5
	45^0C	19.5±0.5
	60^0C	21.0±1.0
	80^0C	20.5±0.5
	100^0C	nil
Enzyme	Control	19.5±1.0
	Trypsin	19.0±1.0
	Lysozyme	18.75±0.2

8.3.3.2 Influence of pH

The antifungal metabolite had a pH of 7.6 and its activity was not affected in the pH range of 4 - 7 and slight decrease in activity was observed at lowest and highest pH of 2.5 and 10.

8.3.3.3 Effect of proteolytic enzymes

The antifungal metabolite preparation was resistant to denaturation by the action of the two proteolytic enzymes tested.

8.3.3.4 Effect of fungal growth on antifungal activity

The antifungal preparation was active in inhibiting the growth of all the ochratoxigenic fungi tested when it was applied before germination of spores. The antifungal metabolite displayed less activity and thus a decreasing trend in inhibitory activity (Fig. 8.5) after germination of spore.

8.3.3.5 Molecular mass of the antifungal metabolite

The approximate molecular weight of the metabolite was tested by dialyzing and testing of dialyzate for antifungal activity. The dialyzate obtained from 2000Da cut off membrane only showed an antifungal activity indicating that the metabolite has a molecular weight between 2000 – 10000 Dalton.

8.3.3.6 Activity determination in culture fractions

The acid and ethanol precipitable fraction of antifungal preparation exhibited inhibitory activity towards the *A.ochraceus* indicating that the antifungal metabolite is compound precipitable by both alcohol and acid precipitation.

Table 8.4 Influence of antifungal metabolite on fungal biomass and ochratoxin A production by *A.ochraceus* CFR 221.

Treatment (AU/ml)	Fungal biomass (Dry weight in mg)	Inhibition (%)	Ochratoxin A (ppm) *	Inhibition (%)
0 (Control)	212	-	0.42	-
1.5	187	12	0.40	6
3	140	34	0.25	42
6	40	82	Nil	100
12	15	93	Nil	100
25	0	100	Nil	100

*Incubation period - 7day

8.3.4 Effect of antifungal metabolite on fungal biomass and ochratoxin A production

The effect of antifungal preparation on the fungal biomass and ochratoxin A elaboration in liquid culture medium is shown in Table 8.4. A dose dependent inhibition of fungal biomass and ochratoxin A elaboration was observed. At a dose of 25 AU/ml complete inhibition of fungal growth was observed. The ochratoxin A elaboration was not detected at a dose of 6 AU/ml.

8.3.5 Application of antifungal preparation in maize

The fungal biomass and ochratoxin A production in treated and control samples of maize is presented in Table 8.5. The inhibition of fungal growth was apparent in treated sample at 25 AU/g as shown in Fig. 8.6. The estimation of fungal biomass revealed 85% inhibition of fungal biomass in treated sample. The ochratoxin was not detected in the treated maize sample.

8.4 DISSCUSSION

The bacterial isolate identified as *Bacillus badius* showed inhibitory activity towards fungal growth in all the ochratoxigenic isolates tested. The bacteria also exhibited antifungal activity against *A.niger* and *Phoma* species among the different common fungal species. The metabolite was produced by the bacteria after log phase of growth after 24 h of growth. It is an extra cellular compound having a molecular weight between 2000 – 10000 Da. The antifungal preparation was water-soluble acid and ethanol precipitable compound. The compound was produced even at the sub optimal temperature for bacterial growth (30^0C).

Table 8.5 Control of fungal colonization and ochratoxin A elaboration in maize by microbial metabolite.

Treatment (AU/g) *	Fungal biomass** (mg/g)	Ochratoxin A (µg/g)
0 (control)	3.56 ± 0.6	2 ± 0.18
25	0.56 ± 0.48	Nil

* Incubation period - 7day

** Estimated by ELISA

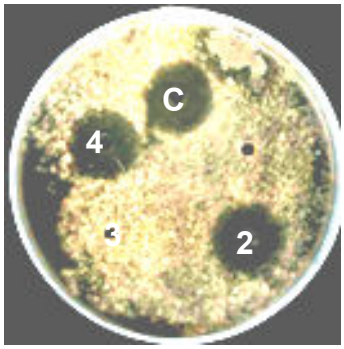

Fig. 8.4 Effect of proteolytic enzymes on the activity of the metabolite

Enzyme treatment: C-untreated control, 1-trypsin, 2-trypsin treated, 3-lysozyme, 4-lysozyme treated metabolite.

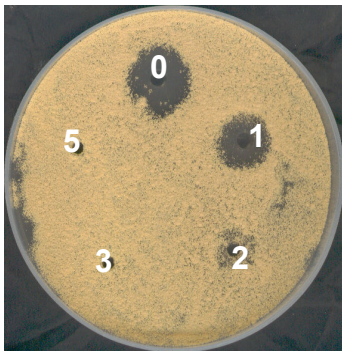

Fig. 8.5 Assessment of the effect of antifungal metabolite on growth of ochratoxigenic fungi.

Numbers on the wells indicate time (days) of addition of the antifungal metabolite.

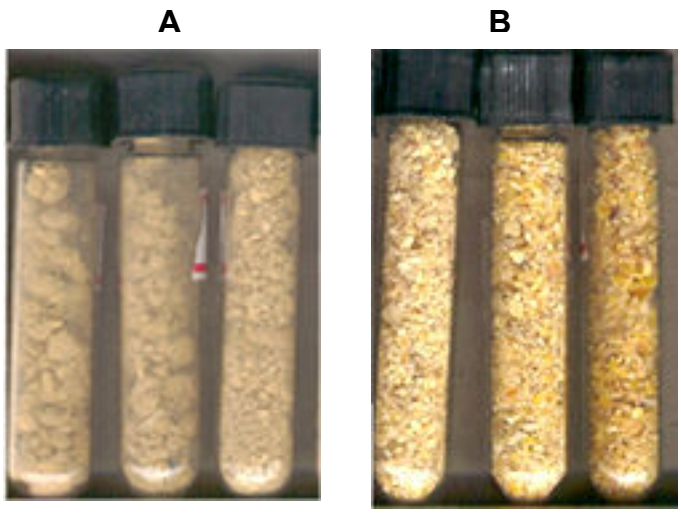

Fig. 8.6 Inhibition of *Aspergillus ochraceus* CFR 221 growth in maize by bacterial metabolite. A - Untreated maize (control), B - Bacterial metabolite treated maize

It is known that *A.ochraceus* has the ability to grow over a wide range of pH ranging from 4 to 9. The antifungal preparation had slightly alkaline pH (7.6) and the inhibitory activity is not due to alkaline pH. The heat stability and pH stability of the antifungal preparation facilitates the application of the antifungal preparation in wide variety of foods with different pH and foods exposed to different storage and processing temperatures. The inhibitory activity of the antifungal metabolite was significant in *A.ochraceus* treated maize and also in pure culture growing in YES broth. In maize samples treated with the antifungal metabolite there was no visible mold growth and sporulation. The treated samples had no detectable amount of ochratoxin A in maize with high moisture of 30% even after 7 days of incubation. Similar inhibition of growth and ochartoxin A production by metabolite was observed against test fungus grown in YES broth. The antifungal compound was effective only when it is applied before germination of spores. This shows that the antifungal metabolite produced by the bacteria affects the germination of spores. Similar literature is available which have reported inhibition of spore germination of certain phytopathogenic fungi by metabolites produced by *Bacillus* species (Walker, *et al*., 1988). Most of the known mycotoxigenic storage fungi belong to class Deuteromycetes in which reproduction occurs predominantly by production of asexual spores. The spores are important source of fungal infestation in foods and are responsible for rapid proliferation in foods. Hence prevention of germination of spores by the application of antifungal metabolite may be used as an effective control strategy for preventing the fungal infestation in foods.

Further work is needed to understand the nature of the compound, its mechanism of action, and its long-term stability. The antifungal compound was

selective and inhibitory towards to all ochratoxigenic fungus tested and few other fungal species tested. Since the antifungal preparation is derived from microbial source it needs to be checked for safety to animal and human before it can be recommended as an effective biocontrol molecule.

CHAPTER 8B
DEGRADATION OF OCHRATOXIN A BY *PSEUDOMONAS* SPECIES

CHAPTER 8B

DEGRADATION OF OCHRATOXIN A BY *PSEUDOMONAS* SPECIES

RESULTS AND DISCUSSION

8.5 INTRODUCTION

The mycotoxin contamination of food commodities render the food unfit for human consumption. The contaminated food commodities are often fed to animal, which not only affects the animal health but also indirectly enters into human food chain. Hence there is need to eliminate or reduce the toxin content in the food and feed stuff. Several chemical, physical and biological methods are reported for inhibiting, decreasing or degradation of mycotoxins. The chemical methods of degradation has its own drawbacks as the chemical residues in the food is of concern and the biochemical changes brought out in the food often alters the composition of food. Arpad and Lasztity (1999) reviewed reports on microbial degradation of mycotoxins. Microorganisms such as Lactic acid bacteria are reported to bind mycotoxins and make it unavailable to induce toxic effects (El-Nezami, *et al*., 1998). Varga *et al.* (2000b) reported degradation of ochratoxin A by *A.niger*. Archana (2000) reported degradation of aflatoxin and ochratoxin A by enzyme preparation isolated from species belonging to the genus *Pseudomonas*.

Microbial assays have been used for quantifying bacterial, fungal and algal toxins. Microbial systems and cell culture assays have also been used for evaluating toxicity of contaminants. The microbial system offers great advantage in studying the interaction of toxic metabolites and *in vivo* toxicity of metabolites.

In the present chapter the study regarding the screening of microbial cultures for degradation of ochratoxin A has been detailed. A bacterial system sensitive to ochratoxin A was identified and used to evaluate the toxicity of *Pseudomonas* biotransformed byproducts of ochratoxin A.

8.6 MATERIALS AND METHODS

8.6.1 Cultures

The cultures screened for degradation of ochratoxin A has been listed in Table 8.6. All the bacterial cultures were maintained in nutrient agar slants at $4 \pm 2\ ^{0}C$.

8.6.2 Chemicals

Ochratoxin A and ochratoxin B standards were procured from sigma Aldrich, USA. Ochratoxin A and B synthesized from culture *A.ochraceus* CFR 221 as described in section 6.2.2 was used for studying the degradation by bacterial cultures. All other chemicals used were analytical grade procured from M/s S.D.Fine chemicals, India.

8.6.3 Bacteriological media

8.6.3.1 Nutrient broth

Composition	g/l
Peptone	5.0
Beef extract	3.0
NaCl	5.0
Distilled water	1L
pH	7.2

Requisite quantity of the medium was dissolved in known volume of distilled water, known amounts were dispensed into suitable test tubes or flasks and were sterilized at $121^{0}C$ for 20 min. Nutrient broth was used to cultivate the bacterial isolates.

8.6.4 Screening of bacterial cultures for ochratoxin A degradation

Bacterial cultures from stock were subcultured on nutrient agar slants and 24 h cultures were used in the study. The nutrient broth in flasks was sterilized and inoculated with the test cultures separately. The flasks were incubated in a rotary shaker for 72 h at RT (26-28 °C) after which the cells were separated from the culture broth by centrifugation at 10,000 rpm at 4°C. The cells were homogenized in phosphate buffer saline (10 mM, pH 7.2). The cell debris was separated by centrifugation at 10,000 rpm at 4°C. The supernatant was used as biocatalyst source. Ochratoxin A in concentration of 200 ng was added to 0.4 ml of culture broth in a 75 mm x 7.5 mm (L x B) test tube (final volume 0.5 ml). The tubes were incubated at 50°C for 6 h. The residual toxin was extracted thrice using 2.5 volume of chloroform. The chloroform layer was resuspended in known volume of chloroform. Known volume of sample along with ochratoxin standard was spotted on TLC silica gel plates and developed in benzene: acetic acid: methanol (18:1:1) solvent system. The plates were air dried and observed for characterstic fluorescence along the ochratoxin standard R_f.

8.6.5 Effect of temperature and time on degradation of ochratoxin A

Ochratoxins A was added to the cell free extract at 1 μg level and the tubes were incubated at 30°C, 40°C and 50°C. Samples were removed at 0, 1 and 6 h time periods and ochratoxin A was extracted thrice with 2.5 volumes of chloroform. Chloroform layer was evaporated to dryness and again resuspended in the known volume of chloroform. TLC analysis was done by spotting known volume of sample along with standard ochratoxin on TLC

plates. These plates were developed in benzene: acetic acid: methanol (18:1:1) solvent system. The air-dried plates were later visualized under UV for characteristic fluorescence along the ochratoxin standard R_f. The ochratoxin A content was estimated by TLC and densitometry as described in section 3.2.2.3.4.

In another experiment the cell free extract (0.4 ml) were incubated with 500 µg of ochratoxin A (0.1 ml) and incubated at 50^0C. The samples were taken after 0, 1 and 6h of incubation. The residual toxin after treatment was analyzed by TLC and estimated by densitometry. The UV spectrum (400–700 nm) after different time intervals of treated toxin was determined in spectrophotometer (UV-10A, Schimadzu, Japan). The absorbance at 333 nm was used to estimate the ochratoxin A content by spectrophotometer as described earlier in section 3.2.3.3.

8.6.6 Effect of concentration of toxin on degradation of ochratoxin A

Ochratoxin A at different concentrations (10 to 500 µg) was added to the culture filtrate (final volume 0.5 ml) and the tubes were incubated at 50°C for 6 h. The residual toxin after treatment was estimated by TLC and densitometry.

8.6.7 Evaluation of toxicity of ochratoxin A degraded products.

A *B.subtilis* isolate which was sensitive to ochratoxin A was used for evaluation of toxicity of ochratoxin A degraded products. Ochratoxin A (500 µg) was treated with cell free extract of *P.fluroscens* for 6 h at 50^oC. The ochratoxin A degraded products were extracted with chloroform (2.5 volumes), evaporated to dryness and redissolved in 100 µl ethanol. The

extract was loaded on Whatmann filter paper disc and mounted on the *B.subtilis* seeded nutrient agar plate. The plate was incubated at 37^{o}C for 24 – 48 h and observed for any zone of inhibition. The disc laid with 100 µl ethanol and 500 µg ochratoxin A served as control.

RESULTS AND DISCUSSION

8.7 RESULTS

8.7.1 Screening of bacterial cultures for degradation of ochratoxin A

Out of ten bacterial isolates screened, the culture filtrates of *Pseudomonas fluroscens* strains A-3a and A-6a-1 showed degradation of ochratoxin A. The results are presented in Table 8.6.

8.7.2 Effect of temperature and time on ochratoxin degradation

The degradation of OTA increased with time and was observed at all temperatures studied (Table 8.7). The biocatalyst showed efficiency both with increase in temperature and period of incubation. The degradation was 50, 75 and 100% at 30, 40 and 50^0C respectively after 6h of incubation. The UV spectrum of 500 µg OTA treated with cell free extract of *P.fluroscens* culture after different intervals of time is shown in Fig. 8.6. Degradation of ochratoxin after different time intervals was observed with corresponding reduction in absorbance at 333 nm in the cell free extract treated OTA. After incubation at 50^oC for 6 h 80% of the added toxin was degraded. Accumulation of compound with lower R_f on TLC was observed after 6 h of incubation (Fig. 8.7).

8.7.3 Effect of ochratoxin A concentration

Cell free extracts from *Pseudomonas fluroscens* A.3a-1 was evaluated for their degrading capabilities at different concentration of ochratoxin A. The results in Fig. 8.8 show that, the isolate possessed the factors capable of degrading higher concentration of ochratoxin A. Cell free extract of cultures of A-3a could degrade 80 - 93% of added OTA.

Table. 8.6 Microbial degradation of ochratoxin A

Pseudomonas isolates	Ochratoxin A (µg)	Isolates showing Degradation
A-3a-1	0.2	+
A-3b-1	0.2	-
A-4a	0.2	-
A-4b	0.2	-
A-6a-1	0.2	+
A-6b-1	0.2	-
Sss4b	0.2	-
A-10a-1	0.2	-
A-10b-1	0.2	-
A-11a	0.2	-

Table 8.7 Effect of time and temperature on degradation of ochratoxin A.

Incubation temperature (°C)	Period of incubation (h)	Residual toxin (μg)	Degradation (%)
30°C	0	1.00	0
	1	1.00	0
	6	0.50	50
40°C	0	1.00	0
	1	0.50	50
	6	0.25	75
50°C	0	1.00	0
	1	0.25	75
	6	0.00	100

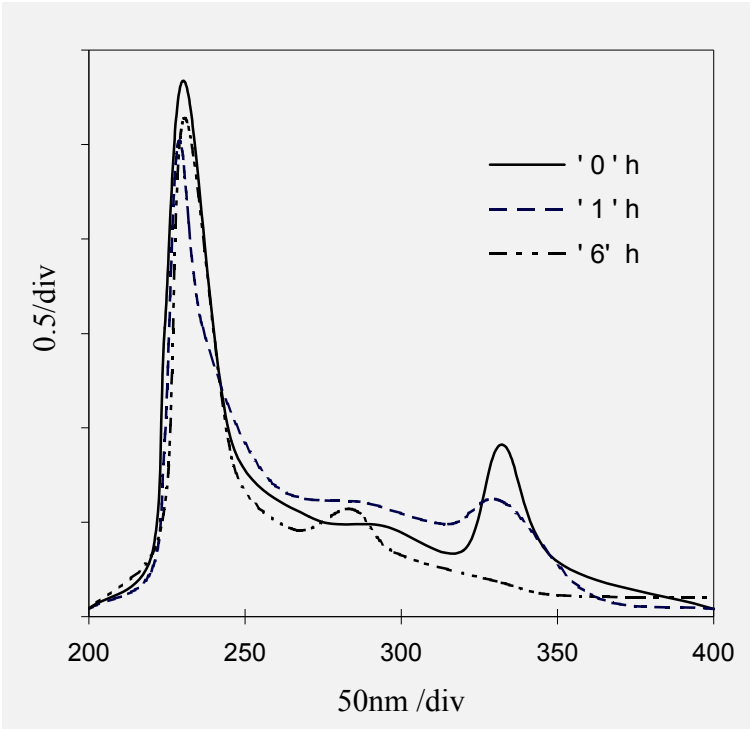

Fig. 8.6 UV spectrum of biocatalyst treated ochratoxin A.

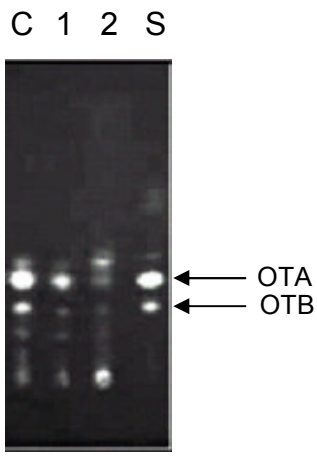

Fig. 8.7 Degradation of ochratoxin A and B as monitored by TLC. Incubation period: C - 0h (Control), 1 - 1h, 2 - 6h, S - standard ochratoxin A.

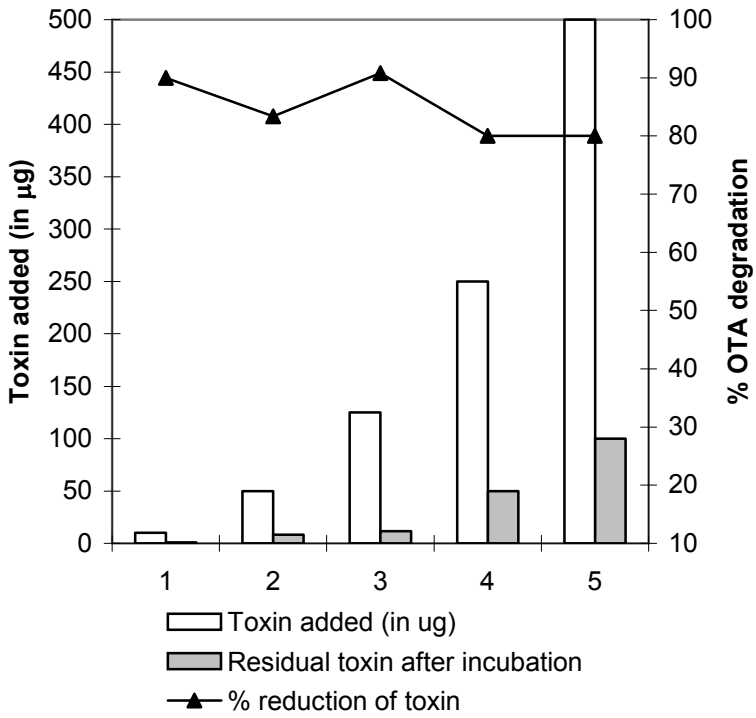

Fig. 8.8 Influence of ochratoxin A concentration on ochratoxin A degradation by *P. fluorescens* A-3a-1.

8.7.4 Toxicity of ochratoxin A degraded products

The agar plate method (Fig.8.9) showed that the degraded products were non-toxic in the prokaryotic bacterial system.

8.8 DISSCUSSION

Samarajeeva and coworkers (1990) reviewed various chemical treatments employed for reduction of aflatoxin. Physical treatment such as milling, baking practiced during processing conditions are known to reduce the toxin content in the foods. Scott (1996) reviewed effects of various processing and detoxification treatments on ochratoxin A. Some of the promising method suggested that could be applied for ochratoxin detoxification is alkaline hydrogen peroxide treatment (Fouler, *et al*., 1994) and ozone treatment (McKenzie *et al*., 1997). However only few of the methods such as degradation of aflatoxin by ammonia and degradation of aflatoxin in oil by sunlight have been accepted for practical use. The chemical methods of detoxification have their own limitation, as they may bring about undesirable chemical changes or alter the chemical composition of foods. Removal of mycotoxins under mild conditions, without using harmful chemicals and without losses in nutritive value and the palatability of detoxified food or feed is required for practical application. In this regard studies have been carried out to reduce/detoxify contaminated foods by microbial fermentation, microbial degradation or binding of mycotoxins by microorganisms, thereby preventing toxic effects (Arpad and lasztity, 1999). Many microbial systems have been reported to degrade ochratoxin A.

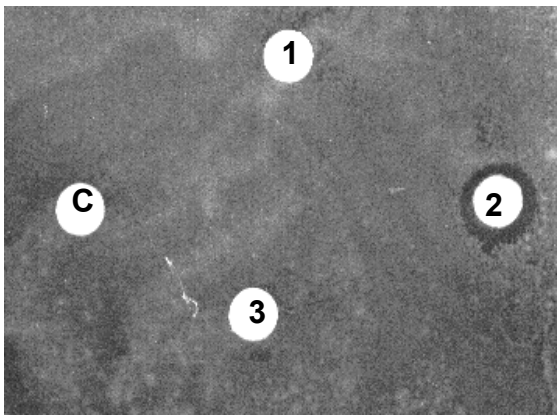

Fig. 8.9 Evaluation of Inhibition of *B. subtilis* CFR 1099 by OTA and OTA degraded products.

C – Control, 1- Ethanol control, 2 -500 µg OTA, 3- OTA degraded products.

Ochratoxin A is known to be degraded to less toxic ochratoxin α in stomach of ruminants by the action of native protozoa present in rumen. Varga and coworkers (2000b) reported complete decomposition of ochratoxin A by an *A.niger* isolate. The microbial degradation of mycotoxins could be used as a promising tool for detoxification of mycotoxins.

The available literature shows that one of the mode of ochratoxin A toxicity is through inhibition of protein synthesis. It acts as an inhibitor of tRNA synthetase and protein synthesis in many microorganism studied viz. *B.subtilis*, *B.stearothermophilus*, *Streptococcus faecalis,* yeast and rat hepatoma cells (Krogh, 1992). Administration of phenylalanine has been reported to prevent the competitive inhibitor effect of ochratoxin A and inhibition of macrophage migration in mice (Creppy *et al*., 1979; Klinkert *et al*., 1981). This indicates that degradation of phenylalanine moiety can prevent the competitive inhibition effect of ochratoxin A in protein synthesis, thereby preventing the toxic effect.

In the present study both ocrhatoxin A and B was degraded by the cell free extracts of *Pseudomonas* species in a time dependent reaction. Complete degradation of ochratoxin was observed after 6 h treatment at 50^{o}C at lower level of OTA (1 µg). Degradation of ochratoxin A was followed with observation of lower Rf compound (Fig. 8.7). Archana (2000) reported enzyme system from *Pseudomonas* species that can degrade aflatoxin and ochratoxin. She reported similar observation of lower R_f non-toxic aflatoxin degraded products. The toxicity evaluation of degraded products is important factor that determines the application of the method for detoxification in foods. The microbial system has been used to study the influence of toxin and toxin

reacted products on aflatoxin toxicity (Shantha, *et al*., 1996). Such microbial assay offers effective mechanism to check the toxicity of the degraded products of mycotoxins. The ochratoxin A degraded by products were found to be non-toxic as analyzed by the *B.subtilis* microbial assay. This shows that the method holds promise for application in detoxification of ochratoxin in foods.

SUMMARY AND CONCLUSION

INTRODUCTION

Fungal infestation of agriculture produce brings about variety of deteriorative changes, which include production of mycotoxins. Monitoring for mycotoxigenic fungi and mycotoxins in raw food commodities provide assurance for further processing of raw materials to finished product with safety aspects built into the system. Prediction of behavior of mycotoxigenic fungi in specific food environment is important to device control stragies in food manufacturing chain from field to consumer. Control of mycotoxigenic fungal infestation and growth is important for preventing mycotoxin contamination. It is well understood that complete elimination of mycotoxin contamination is impractible to achieve, hence regulatory limits are set to limit mycotoxin exposure to humans and animals. It is imperative to develop methods for detoxification of mycotoxin for utilization and safe disposal of contaminated food. In this regard the study focused on assessment of level of ochratoxigenic fungal and ochratoxin contamination in foods. Methods for detection of ochratoxigenic fungi and ochratoxin A were developed. Behavior of ochratoxigenic fungi under the influence of temperature, moisture and fungal load was studied by predictive microbiological approach. Microbiological methods for controlling ochratoxigenic fungi and degradation of ochratoxin A were developed.

The research findings of the study are summarized in the following sections.

1.0 Prevalence of ochratoxigenic fungi and natural occurrence of ochratoxins in foods

- The presence of ochratoxigenic fungi was found to be widespread in the different food commodities representing cereals, oilseeds, beverage, feed and spice with an incidence of ~ 59%.
- Among the ochratoxigenic fungi *A.ochraceus* was common in wide range of food commodities. *A.melleus*, *A.sulphureus*, *A.auricomus*, *A.sclerotiorum* and *A.ostianus* were the other ochratoxigenic fungi isolated from food commodities.
- The isolation of highly potent ochratoxigenic fungi coincided with high toxin contamination in feed commodity. *A.auricomus* which was isolated from feed commodity was found to be potent strain producing high amounts of ochratoxin A. However in a sample of moldy coffee, which contained ochratoxin A (1.25ppm), no ochratoxigenic fungi could be isolated. This indicates the initial abuse of samples by ochratoxigenic strain that did not survive coffee processing conditions.

The incidence of ochratoxigenic fungi with potential to produce high quantity of ochratoxin A reaffirms the need for constant monitoring of food commodities for ochratoxin A contamination and ochratoxigenic fungi.

2.0 Immunological and molecular methods for detection of ochratoxigenic fungi

- Microplate and dot-binding immunoassays were optimized for detection of *A.ochraceus* in foods. Microplate immunoassay developed can be used for estimation of *A.ochraceus* biomass in foods. The ELISA responses

correlated with fungal biomass in the range of 10 - 160 µg/ml of food extract with a correlation coefficient of 0.99 in chili, poultry feed, maize and coffee representing spice, feed, cereal and beverage. The method is sensitive and can detect *A.ochraceus* infestation at ≥0.2 µg biomass per mg substrate.

- The immunological method was applied to monitor and compare *A.ochraceus* growth at substrate moisture of 20 & 30% in different food commodities viz., coffee, poultry feed and chili.
- Dot-binding immunoassays were optimized for detection of *A.ochraceus*. The dot-binding immunoassay optimized can be used for detection of ochratoxigenic fungal infestation at ≥10 µg biomass/mg substrate.
- A PCR method was developed for specific detection of *A.ochraceus* group of fungi. The specificity of PCR was evaluated with common fungal cultures. The PCR method was specific to *A.ochraceus* group of fungi as substantiated with experiments carried out DNA extracted from common foodborne fungal cultures in pure and mixed culture, as well as directly in food commodities. The method could be used for detection of ochratoxigenic fungi in food commodity with limit of detection at ≥ 4 $\log_{10}$ cfu/g.
- Enrichment technique was optimized for increasing the sensitivity of the PCR, by which the detection of ≥ 2 Log_{10} spores /g was possible.

The immunological methods and PCR method can be adopted for rapid monitoring of food commodities for specific fungal colonization. The immunological method developed can be used for direct detection and

quantification of fungal biomass in foods differing in composition. The fungal biomass determination explains the fungal metabolic activity such as toxin elaboration better than the conventional viable count analysis. The immunological and molecular methods when used with appropriate enrichment technique can be applied for monitoring of low levels of fungal spores in food commodities.

3.0 Immunoassays for determination of ochratoxin A

- ELISA protocols were optimized for determination of OTA using antibody elicited against ochratoxin-BSA in rabbit and hen egg yolk. The antibody sensitivity (IC_{50}) to OTA in the immunoassays involving rabbit sera and egg yolk antibody was 15ng/ml and 40ng/ml respectively. Immunoassays were optimized using poultry feed as the food system with a detection limit of 5 ppb for the assay involving both rabbit serum or hen egg yolk antibody preparation. Hen egg yolk antibody preparation was found to be suitable for large-scale antibody production with a yield of 83 $\pm$ 19 mg of IgY/egg.
- ELISA estimations of ochratoxin A in market samples of poultry feed revealed OTA contamination in only one sample (11.1%). ELISA estimations of OTA in poultry feed were compared with HPLC estimations. The ELISA estimations correlated well with HPLC estimations in all the samples tested.

The production of antibodies against haptens in hen egg offers humane way of production of large-scale antibody production. The antibody raised in hen egg yolk can be used for development of immunological method for

determination of ochratoxin A in food commodities with sensitivity and cross reactivity similar to rabbit antisera.

4.0 Interrelations of temperature, moisture and inoculum level on behavior of ochratoxigenic fungi

- A statistical experimental approach using Central Composite Rotatory Design (CCRD) was applied for studying the interactions of temperature, moisture and inoculum on growth and ochratoxin elaboration by *A.ochraceus* strain in maize at 5 levels of each variable.
- Multiple linear regression analysis of the fungal response was used to derive polynomial equation for prediction of *A.ochraceus* growth and ochratoxin A elaboration in maize. Response surface graph were generated for prediction of *A.ochraceus* growth and ochratoxin A elaboration in maize based on the derived polynomial model parameters.
- The predictions as analyzed by bias factor, accuracy factor and root means square error revealed that the predictions within the experimental domain were dependable.

The predictive microbiological approach involving response surface model can be effectively applied for understanding and predicting the behavior of fungi under the influence of specific food environment.

5.0 Control of ochratoxin A contamination

- A potent bacterial isolate, identified as *B.badius,* capable of producing antifungal metabolite against ochratoxigenic *Aspergilli* was isolated. The isolate produced an extracellular metabolite with a molecular weight

between 2000–10000 Daltons, which inhibited the germination of ochratoxigenic *Aspergilli* and few other common mycoflora.

- The antifungal metabolite was effective in wide range of pH and temperature of 2.5 -10 and 25 ^{o}C - 80 ^{o}C respectively. The effectiveness of the metabolite was studied in maize, wherein it exhibited concentration dependent inhibition of growth and OTA elaboration by *A.ochraceus* in broth culture. The metabolite showed 85% inhibition of *A.ochraceus* growth and 100% inhibition of ochratoxin A elaboration in maize compared to an control experiment without the metabolite.
- *P.fluroscens* isolates were identified which exhibited potential to degrade ochratoxin. The cell free extract prepared from *P.fluroscens* culture, was capable of degrading ochratoxin A ranging from 80 -100% of added ochratoxin A in concentration ranging from 0.2 to 500 µg tested under optimum temperature of 50^{o}C, within a period of 6 h.
- A *B.subtilis* isolate was identified which was sensitive to ochratoxin A at ≥ 50µg. The toxicity of the biotransformed toxin metabolites was evaluated for toxicity using the microbial bioassay. The study revealed that cells free extract of the isolates degraded ochratoxin to non-toxic metabolites.

The biocontrol of toxigenic fungal infestation in food commodities needs much attention and research. The *B.badius* isolate identified in the present study has the potential to be explored for biocontrol of ochratoxigenic fungi in food system. Similarly the *P.fluroscens* isolate identified in the present study has potential for application in decontamination of ochratoxin contaminated food.