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.