PURIFICATION AND CHARACTERIZATION OF OCHRATOXINS PRODUCED BY INDIGENOUS *ASPERGILLI OCHRACEUS* UNDER OPTIMIZED CONDITIONS

ASIF AHMED
2007-VA-327

A THESIS SUBMITTED IN THE PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

MASTER OF PHILOSOPHY

IN

MICROBIOLOGY

UNIVERSITY OF VETERINARY & ANIMAL SCIENCES, LAHORE

2019

DEDICATION

Dedicated
To
Holy Prophet Muhammad (Peace Be Upon Him),
My Parents
&
My Supervisor

ACKNOWLEDGEMENTS

I bow my head in utmost gratitude before the most Gracious, the most Merciful and Almighty **ALLAH** without whose will, I could never have accomplished this endearing task, only He gave me the strength and power enough to cope up with all the impediments in the way. I, most modestly, impart my dutiful benefactions to the **Holy Prophet Muhammad** (Peace Be Upon Him) who is persistently a torch of guidance and knowledge for the entire mankind.

I deem it as my utmost pleasure to avail this opportunity to express the heartiest gratitude and deep sense of obligation to my dedicated supervisor **Prof. Dr. Ahmad Anjum, Department of Microbiology UVAS, Lahore** for his valuable suggestions, keen interest, dexterous guidance, enlightened views, constructive criticism, unfailing patience and inspiring attitude during my studies, research project, and writing of this manuscript. Infect his day and night pursuance and sincere efforts made this work to a fruitful conclusion.

I gratefully acknowledge invaluable help rendered by **Prof. Dr. Tahir Yaqub, Chairman, Department of Microbiology UVAS,** Lahore, Pakistan for guiding me at every step of the research work. He gave me time and helped me at every step in an effective development and conductance of research. He was always available when I needed his help. In fact, I do not hesitate to say that without his untiring efforts, it would not have been possible for this work to reach its present effective culmination.

I am honored to express my deepest sense of gratitude and profound indebtedness to **Dr. Muhammad Nasir, Department of Food Science and Human Nutrition, UVAS, Lahore,** for helping me to fulfill my research work. They provided me full moral support and guided me at every step when I required his help.

Here it will be injustice, if I would not avail this opportunity to appreciate **Dr. Mateen Abbas, Quality Operation Laboratory, UVAS, Lahore** for his personal interest and full cooperation during my whole study time. He combines a range of skills and experience, vision and commitment that are unequalled anywhere in the world.

I have no words to thank to all my colleagues and friends for their interest and appreciated advices in my research project, in fact their advices will always serve as a beacon of light throughout the course of my life.

Finally, my unreserved love and thanks to my **Father**, **Mother**, **Sisters** and **Brothers** who have always encouraged me and gave all the love and support they could offer.

Asif Ahmed

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXURES

ABSTRACT
(Only For Ph.D)

MOLECULAR APPROACHES FOR CHARACTERIZATION OF AFLATOXIN PRODUCING *ASPERGILLUS FLAVUS* ISOLATES FROM POULTRY FEED

Aflatoxins are secondary toxic metabolites produced by aspergilli. Aspergillus flavus is one of the major aflatoxins producing specie. Present study was conducted to enumerate mycoflora of poultry feed and aflatoxin production potential of A. flavus. Home mixed and commercial poultry feed (n=20, each) were processed for determination of fungal load and isolation of mycoflora. Isolates were identified by culture and microscopic characters. Thin layer (TL) and high-performance liquid chromatography (HPLC) were used for screening, identification and quantification of aflatoxins produced by A. flavus respectively. A. flavus were confirmed by specie specific polymerase chain reaction (PCR). Isolation frequency of different genera, Aspergillus species and toxigenic A. flavus was calculated. The fungal count in home mixed feed was $2x10^2$ to $1.6x10^4$ CFU/g whereas, in commercial poultry feed from $2x10^1$ to $6x10^3$ CFU/g. Aspergillus was the most prevalent genus in home mixed and commercial feed followed by Mucor. Among aspergilli, the highest percentage was of flavus (95%) followed by A. niger (75%), A. fumigatus (15%) and A. terreus (5%). A total of 32.61 percent (223/685) aflatoxin producing A. flavus from commercial and 16.67 percent (23/140) from home mixed feed were detected by TLC. These aflatoxins (AFs) were identified as AFB1 and AFB2 and AFG1 by HPLC. Amplicon (500 bps) of A. flavus was observed on 2 percent agarose gel. It was concluded that poultry feed may be a source of transmission of disease producing fungi and aflatoxins to poultry birds and human beings.

Key words: Aflatoxin Aspergillus flavus commercial poultry feed High performance liquid chromatography Home mixed poultry feed Polymerase chain reaction

ENZYMATIC AND AFLATOXIN PRODUCTION POTENTIAL OF *ASPERGILLUS FLAVUS*

Fungi especially *Aspergillus* species are potential candidates for production of mycotoxins and industrially important enzymes. *Aspergillus flavus* isolates (129) recovered from soil mixed with animal rations (n=145) had Aflatoxins (17.82%) and Enzymes (10.37%) production potential. Quantity of detected Aflatoxins varied for different isolates i.e., 3.25 to 11622.24ng, 21.34 to 194.47ng and 3.36 to 40.12ng per mL of Sabouraud's dextrose broth in case of AFB_1, AFB_2 and AFG_1 as determined by High performance liquid chromatography. Optimization of non-toxigenic starch hydrolyzing *A. flavus* was carried out at different incubation temperatures (22, 30 and 37°C), pH (4.5, 6 and 7.5) and substrates including maize, wheat bran and rice husk (1, 3 and 5% each) for incubation period of 7 days. In optimization experiments for starch hydrolysis, most of the *A. flavus* (86%) produced highest enzyme (IU) at 37°C and pH 6 quantified by Dinitrosalicylic method. Maximum isolates were able to produce enzymes using rice husk followed by maize. The maximum enzyme production by *A. flavus* was 179.88$\pm$1.71IU using one percent of maize at pH 6 and 37°C. It was concluded that indigenous *A. flavus* can be used in food industry as biological source of starch hydrolyzing enzymes.

Key words: *Aspergillus flavus*, starch hydrolysis, mycotoxins, optimization and substrates

CHAPTER 1
INTRODUCTION

Feed is a major factor in production of meat and milk for human consumption (Sivakumar *et al.* 2014). Cereals, meals and oil seed are integral components of animal and poultry feed (Muhammad *et al.* 2010). Feed and feed ingredients are exposed to different physical, chemical and biological hazards in pre-harvesting, during harvesting and post-harvesting stage. These hazards result in direct and indirect effects on animals and public health respectively. Feed and ingredients used in feed preparations are source of nutrition for animals and a good substrate for microorganism's growth. These organisms may be saprophytes, true pathogens, opportunistic pathogens or toxin producers. The number and type of microorganisms depends upon intrinsic and extrinsic factors of feed materials (Cabarkapa *et al.* 2009). However, feed quality is harmed by presence of microorganisms either bacteria or fungi (Khosravi *et al.* 2008).

The plant-based feedstuff is contaminated by various fungal species via air, dust, water, soil and animals during processing, storage and transportation (Kukier *et al.* 2013). Poor harvesting practices, improper drying, handling, packaging, storage, and transport of raw ingredients and feed contribute to fungal growth and production of toxic metabolites called mycotoxins (Bhat *et al.* 2010). Particularly, mycotoxins build up to injurious level during hot and humid conditions (Aliyu *et al.* 2016; Ibrahim *et al.* 2017). According to Food and Agriculture Organization (FAO), 25 percent of the crops get contaminated by mycotoxins (Trail *et al.* 1995; CAST 1989). This contamination of feed deteriorates nutritional quality of feeding stuff imposes health hazardous to animals and human and causes economic losses.

Mycoflora contaminating the feedstuff determine the hygienic status, nutritional quality and toxicity (Khosravi 2008). *Aspergillus, Penicillium, Fusarium, Rhizopus* and *Mucor* are common contaminants of feed material in pre and post harvesting stage (Raju *et al.* 2016). *Aspergillus Penicillium, Rhizopus* and *Cladosporium* are storage fungi as these fungi contaminate feed material during its storage (Maciorowski *et al.* 2007). Mycotoxin producing fungal contaminants of feed belong to genus *Aspergillus, Penicillium, Fusarium Claviceps*, *Alternaria* and *Stachybotrys* (Zain 2011). However, *Aspergillus* species are the most frequent contaminants of feed and feed ingredients (Ghiasian and Maghsood 2011).

Mycotoxins are low molecular weight secondary metabolites which are toxic to vertebrates, human beings and animals (Zain 2011). Among several types of mycotoxins, the most important are Aflatoxins, Ochratoxins, Zearalenone, Deoxynivalenol, Fumonisin and T-2 toxin (Fokunang *et al.* 2006). Aflatoxin is a group of 20 biologically similar compounds produced by species of genus *Aspergillus***.** The important aflatoxin producing species are *Aspergillus flavus, A. parasiticus* and *A. nomius* (Njobeh *et al.* 2009; Kumar and Rajendran 2008). Aflatoxins are classified as AFB_1, AFB_2, AFG_1 and AFG_2 based upon their natural ability to fluoresce under Ultra Violet (UV) light and chromatographic mobility (Habib *et al.* 2015). Among these, AFB_1 is the most potent toxin. AFB_1 and AFB_2 are converted to hydroxilated metabolites by liver enzymes termed as aflatoxin M_1 (AFM_1) and AFM_2 respectively. It can be detected in milk, tissues and biological fluids of these animals (Oliveira *et al.* 2013; Murphy *et al.* 2006; Peltonem *et al.* 2001; Oatley *et al.* 2000; Oliveira and Germano 1997).

CHAPTER 2
REVIEW OF LITERATURE

2.1. Mycotoxins

Animals, plants, and microorganisms produce some hazardous substances which are termed as toxins. Phycotoxins are toxic metabolites of plants. Microorganisms produce endotoxins (Gram's negative bacteria) and different exotoxins. Similarly, fungi produced Mycotoxins. The combination of two words makes the term mycotoxin, '*mykes*' a Greek word meaning fungus and *toxicum*' a Latin word meaning toxins (Eman, 2003). Mycotoxins are low molecular weight secondary metabolites excreted by many fungi. Approximately 200 mold species are detected as mycotoxins producers (Turner *et al.* 2009). So far ~ 300 to 400 mycotoxins have been identified with their potential threat to human and animals (Fakih, 2014). The most common groups of mycotoxins which contaminate food are Zearalenone, Ochratoxin, Deoxynivalenol/Nivalenol, Fumonisins and Aflatoxins (Tola and Kebede, 2016). There are certain factors which contribute in production of mycotoxins such as method of cultivation and susceptibility of different crops during processing and storage (Jonathan and Esho 2010). Two types of fungi are responsible for mycotoxin production in feed stuff; these are field fungi and storage fungi. Each type of fungi has its own growth requirements (Adeyeye and Akingbala, 2016).

2.2. Toxigenic Fungal Species

Particularly saprophytic fungi which can grow on a wide range of feed material produce mycotoxins (Turner *et al.* 2009). The toxigenic fungi belong to Aspergillus, Fusarium, Penecillium and Alternaria (Pitt and Hocking 2009) Cladosporium, Helminthosporium and Claviceps (Urughucki and Yamazahi 1978). Some of important mycotoxins are aflatoxins, ochratoxins, zaeralenone, fumonisins, trichothecens and ergot alkaloids. Fusarium, Stachybotrys, Trichoderma, Trichothecium, Memnoniella, Phomopsis and Myrothecium are Trichothecenes producers. Ochratoxins are toxic metabolites of *Aspergillus ochraceus, Aspergillus carbonarius and Penicillium verrucosum.* Fumonisins are toxic secondary metabolites of some Fusarium species. The important species are Fusarium verticillioides, Fusarium graminearum, Fusarium proliferatum. Fusarium culmorum, Fusarium crookwellense, Fusarium equiseti, are known to produce Zearalenone. Ergot alkaloids are mycotoxins produced by *Claviceps purpurea* (Fakih 2014).

2.3. Statement of Problem

Aflatoxins are toxic metabolites produced by Aspergilli. The aflatoxin producing fungi include mainly *A. flavus, A. parasiticus and less commonly by A. nomius, A. pseudotamarii, A. tamarii, A. bombycis, A. ochraceoroseus*. The species which are responsible for production of Difurocoumarocyclopentenone are *A. flavus, A.nomius A. arachidicola, A. bombycis, A. minisclerotigenes, A. parasiticus, A. pseudotamarii, A. ochraceoroseus, A. rambellii, Emericella venezuelensis.* While second series of aflatoxin named as Difurocoumarolactone are produced *by A. flavus, A. nomius, A. arachidicola, A. minisclerotigenes and A. parasiticus. A.flavus* is the most commonly isolated from agricultural products and others are less frequently occurred (Udomkun *et al.* 2017; Varga *et al.* 2011).The study has been designed with following objectives:

- Purification of Ochratoxins
- Characterization of Ochratoxins

CHAPTER 3
MATERIALS AND METHODS

Poultry Feed, animal rations and feed ingredients were processed for mycological quality evaluation. Different contaminating fungi were purified and characterized based upon their macroscopic and microscopic features. Mycotoxin production potential of fungal isolates was determined using standard protocols. Aflatoxin producing fungi were confirmed by polymerase chain reaction (PCR). Growth and Aflatoxins production was optimized under different physical and nutritional conditions to obtain maximum yield. Different Aflatoxins were extracted and purified to check their stability in relation to different storage conditions to be used as standards.

3.1 Sampling

Fungal isolation was carried out from feed and feed ingredients. These materials were evaluated for fungal load as well. Five different feed material were selected including commercial poultry feed, home mixed poultry feed, commercial animal rations, grains (stored rice, maize, wheat) and cotton seed cakes following the method described by (Saleemi *et al.* 2010) from District Lahore. A total of 140 samples (n=20, each) were collected in properly labeled sterile polythene bags from feed production units and commercial market. A total of three kg feed was collected from three different sites of a feed lot and pooled. One kg from pooled selected feed samples was transported to Mycology Laboratory, Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

3.2 Fungal Load

Fungal load in each of the selected samples was calculated by Dilution plate method and Mycoflora determination following protocol described by Pitt and Hocking (2009).

3.2.1 Media preparation

Sabouraud's dextrose agar (SDA) was selected for determination of fungal load (ANNEXURE I). Medium was prepared following the instructions of manufacturer. Briefly, medium was prepared by mixing 65 grams of dried powder in distilled water (1L final volume) and dissolved by gentle heat. pH was adjusted to 5.6 using diluted HCL and poured (30 mL each) in labeled (1 to 10) universal bottles. It was sterilized by autoclaving at 121 ºC for fifteen minutes in autoclave under pressure of 15 pounds per inch square (psi). Medium was cooled (45 to 50ºC) and antibiotics (Streptomycin 30µg/mL and gentamicin 50 µg/mL) were added to inhibit the growth of bacterial contaminants in samples. It was poured in labeled glass petri plates (90 mm) already sterilized by dry heat for 30 minutes at 180 ºC using hot air oven (Nagano *et al.* 2008; Merz *et al.*1976).

3.2.2 Preparation of sample dilutions

Fungal load was calculated in one gram of each selected sample. Briefly, tenfold serial dilution of each sample was prepared using sterilized peptone water (ANNEXURE II) in labeled (10-1 to 10-10) screw capped glass test tubes (09 mL each). Samples were crushed to fine powder and ten grams from

each sample was added to 90mL sterile peptone water (0.1%) in a 250mL blue capped sterile bottle. Sample was mixed properly and one mL from this suspension transferred to a peptone water test tube labeled as 10-1 using disposable sterilized syringe followed by gentle mixing. One mL from tube 10-1 was transferred to tube labeled as 10-2. Similar procedure was repeated up to 10-10 test tube and one mL discarded from this tube (Saddozai 2012). All the experimental work was carried out under sterilized conditions in safety cabinet (Biotroll/BSC Class 2 B2).

CHAPTER 4
RESULTS

Mycological quality of poultry and animal feed/feed ingredients procured from local market was observed. Fungal load in each sample was calculated and compared statistically. Different contaminating fungi were isolated, purified and identified preliminary based on macroscopic and microscopic characters. Isolated fungi, particularly Aspergillus flavus, were screened for mycotoxin production potential using thin layer chromatography (TLC) followed by high performance liquid chromatography (HPLC). Aflatoxins producing A. flavus isolates were confirmed by polymerase chain reaction (PCR).

4.1 Fungal load

Fungal load in commercial poultry feed, home mixed poultry feed and major feed ingredients including stored rice, wheat and maize grains (n=20 each) was calculated and compared statistically by one-way ANOVA (Analysis of Variance) followed by Duncan's multiple range posthoc test. Fungal load ranges recorded were 2.0x101 to 6.0x103, 2.0x102 to 1.6x104, 2.0x103 to 2.7x104, 5.0x102 to 1.2x104 and 9.0x103 to 5.9x104 in commercial poultry feed, home mixed poultry feed, stored rice, wheat and maize, respectively. Highest Statistical mean fungal load (4.45±.24) was observed in maize and lowest (2.96±.54) in commercial poultry feed samples. Fugal loads of wheat differed non-significantly with home mixed poultry feed and rice, while significantly with mean load of commercial poultry feed and maize. Fungal loads of each sample of all categories are presented at table (4.1), log fungal loads at table (4.2).

Table 4.1: Fungal load (CFU/g) in commercial, home mixed poultry feed, stored rice, wheat and maize grains (n=20 each).

Sr. No.	Commercial feed	Home mixed feed	Stored Rice	Wheat grains	Maize grains
1	$2.2x10^3$	$7.6x10^3$	$6.0x10^3$	$2.0x10^3$	$9.0x10^3$
2	$1.0x10^3$	$3.4x10^3$	$6.0x10^3$	$3.0x10^3$	$2.5x10^4$
3	$1.1x10^3$	$7.4x10^3$	$3.0x10^3$	$1.2x10^4$	$1.2x10^4$
4	$1.8x10^3$	$4.6x10^3$	$1.2x10^4$	$7.0x10^3$	$1.4x10^4$
5	$2.6x10^3$	$4.5x10^3$	$6.0x10^3$	$1.2x10^4$	$3.2x10^4$
6	$2.9x10^3$	$4.4x10^3$	$3.0x10^3$	$9.0x10^3$	$5.0x10^4$
7	$9.0x10^2$	$3.0x10^3$	$2.7x10^4$	$5.0x10^2$	$2.5x10^4$
8	$7.0x10^2$	$1.0x10^4$	$1.9x10^4$	$4.5x10^3$	$4.2x10^4$
9	$5.0x10^2$	$6.5x10^3$	$3.0x10^3$	$8.0x10^3$	$2.5x10^4$
10	$6.0x10^3$	$9.2x10^3$	$5.0x10^3$	$1.0x10^3$	$2.2x10^4$
11	$7.0x10^2$	$5.7x10^3$	$1.2x10^4$	$2.5x10^3$	$4.8x10^4$
12	$1.0x10^3$	$1.6x10^4$	$4.0x10^3$	$5.0x10^3$	$4.8x10^4$
13	$3.8x10^3$	$6.7x10^3$	$5.0x10^3$	$6.0x10^3$	$4.4x10^4$
14	$1.3x10^3$	$6.0x10^2$	$2.0x10^3$	$2.5x10^3$	$5.5x10^4$
15	$1.2x10^3$	$2.0x10^2$	$2.0x10^3$	$2.0x10^3$	$3.4x10^4$
16	$1.0x10^3$	$8.0x10^2$	$6.0x10^3$	$1.0x10^3$	$5.9x10^4$
17	$7.0x10^2$	$3.0x10^2$	$1.7x10^4$	$6.0x10^3$	$4.3x10^4$
18	$8.0x10^2$	$1.0x10^3$	$2.5x10^3$	$1.0x10^4$	$1.1x10^4$
19	$2.0x10^1$	$6.0x10^2$	$6.0x10^3$	$1.9x10^3$	$2.5x10^4$
20	$1.0x10^2$	$6.0x10^2$	$5.0x10^3$	$6.0x10^3$	$3.6x10^4$

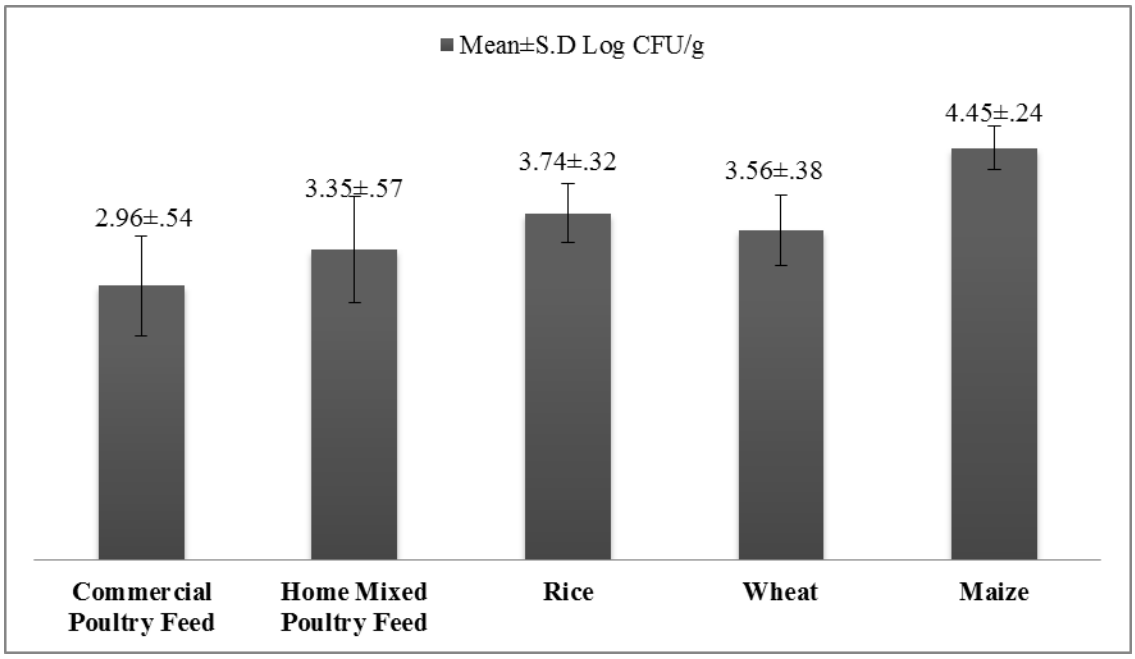

Figure 4.1: Mean fungal load of different poultry feed and ingredients

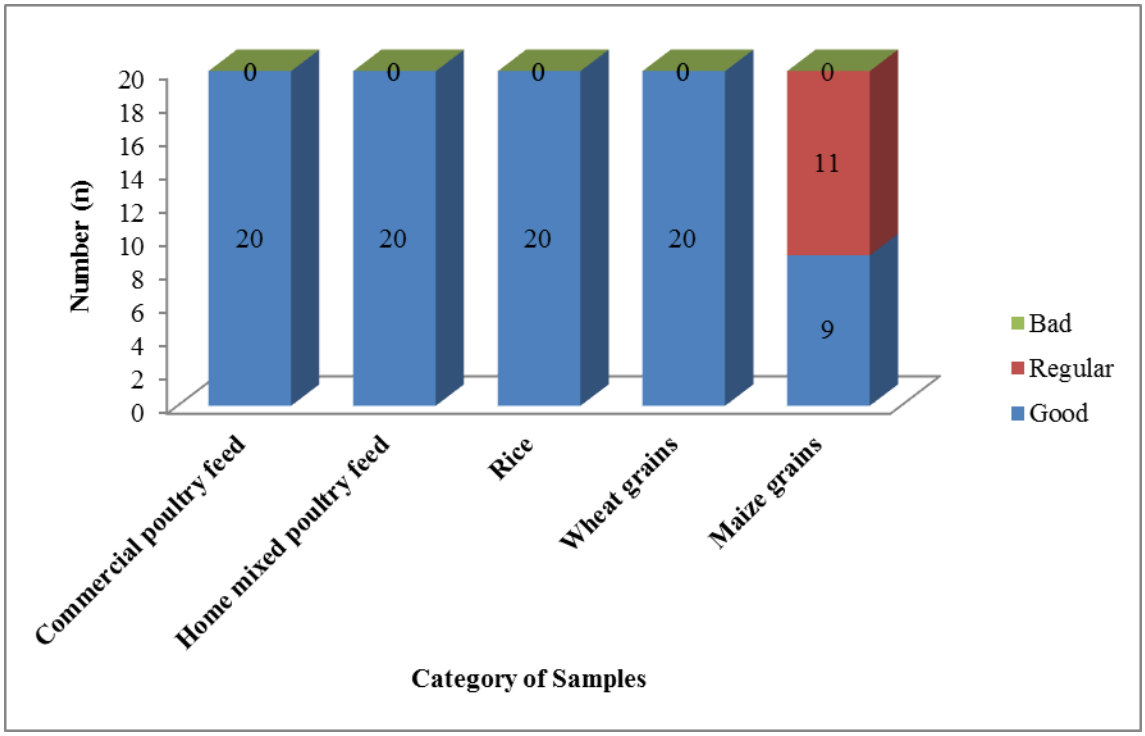

Figure 4.2: Mycological quality of poultry feed and feed ingredients

CHAPTER 5
DISCUSSION

The livestock feed and ingredients are carrier of fungi. Due to ubiquitous nature of fungi, it is not possible to keep feed and ingredients free of these organisms. The presence of toxigenic fungi may lead to toxin build up and causes serious health issues not only in animals but also in human (Morrison *et al.* 2017). Presence of mould species in poultry feed is a public health concern. Mould species not only affect the organoleptic properties of feed and but also produce mycotoxins (Greco *et al.* 2014).

Fugal loads of poultry feed, animal rations and feed ingredients were calculated. Isolated fungal species were identified to calculate the risk associated with true pathogens, opportunistic pathogens and toxigenic fungal contaminants. Toxin producing Aspergillus flavus isolates were selected among toxigenic contaminants for aflatoxins production optimization under different physico-chemical conditions. Thin layer chromatography and High-performance liquid chromatography were used to characterize and quantify aflatoxins respectively. Stability of aflatoxins was evaluated using different solvents and stabilizers ad exposure to light by placing in transparent, opaque and brown storage vials.

In present study, fungal load in home mixed and commercial feed recorded were 1.64 x 104 and 6.0 x 103 CFU/g respectively which were slightly lower than 6.5x106 CFU/g of poultry feed evaluated in Iraq by Shareef (2010). Fungal load of 8.1 x 105 CFU/g in Nigarian bird's feed (Matthew *et al.* 2017) observed were little higher. Similarly, slightly higher fungal loads were recorded in range from 3.0 × 104 CFU/g to 9.6 × 105 CFU/g in Nigerian poultry feed by (Ukaegbu-Obi *et al.* 2017). (Krnjaja *et al.* 2008) determined 1-9 x 104 of fungi per grams of commercial poultry feed samples in Serbia. The results showed slightly higher load of fungi in Serbia's commercial poultry feed. (Rashid *et al.* 2017) investigated commercial feed of broilers and found it contaminated with fungi (4.72 × 103±0.708) in agreement with present study commercial feed. Results are in accordance with 42 × 103 CFU/g of poultry feed in Nigarian (Kehinde *et al.* 2014), 8.7x103 CFU/g of poultry feed in India (Banerjee and Shetty 1992) and poultry feed in Iran (1.2×103 to 1×101 CFU /g). (Ghaemmaghami *et al.* 2016) documented in accord fungal load in commercial poultry feed and slightly different from fungal of home mixed poultry feed. The differences in fungal load are attributed to variations in source of crop, storage conditions, and geographical differences.

Fungal load rages in rice, wheat and maize recorded were, 2.0x103 to 2.7x104, 5.0x102 to 1.2x104 and 9.0x103 to 5.9x104 respectively. (Cabarkapa *et al.* 2009) recorded 6.5 X 105 CFU/g of fungi in maize in contrast to present study findings. (Pereyra *et al.* 2010) reported that maize grains had 2. 9 × 105 CFU/g of fungi in comparison to status of maize grains contamination in present study.

5.1. Conclusion:

AFB1 was the most stable toxin and the highest stability was recorded in chloroform. In brown color bottles having chloroform AFB1, 55.27 percent reduction was detected by HPLC. AFB2 showed least reduction (65.63%) in methanol stored in opaque vials for six months. AFG1 was remained stable up to four months in sucrose stored in brown vials. After six months complete (100%) was detected under all storage condition. AFG2 was stable (35.53% reduction) in acetonitrile stored in opaque vial after four months. After six months no AFG2 was detected under all storage conditions.

5.2. Suggestions:

The stability studies of aflatoxins under different conditions will help to long term storage and use of aflatoxin standards.

CHAPTER 6
SUMMARY

Agricultural commodities are contaminated with aflatoxin producing fungi. Under favorable conditions, these fungi grow and produce aflatoxins. The poultry and animal feed based on agricultural products contains aflatoxins higher than permissible limit. These aflatoxins are transferred to human via consumption of poultry and dairy products such as meat, milk and eggs. The aflatoxins cause serious health hazards to animal, poultry and human. Animal feed, poultry feed and feed ingredients harbor aflatoxin producing fungi. These fungi produce aflatoxins and destroy nutritional quality of feed. These aflatoxins are extracted, purified and standardized for accurate detection of aflatoxin. A total of n=140 samples including commercial poultry feed, home mixed poultry feed, commercial animal rations, cotton seed cake, maize grains, wheat grains and stored rice were evaluated for fungal load. Samples were serially diluted and inoculated on Sabouraud's dextrose agar. The inoculated samples were incubated for three days at 25 ºC, colony forming units (CFU) were calculated and isolates were purified by spot culture technique. Purified isolates were identified by their macroscopic and microscopic feature on purified growth and by slide culture techniques/cellophane tap respectively. Isolation frequency and relative density of fungal genera and species were determined. Preliminary screening of isolates for aflatoxin (AFB1, AFB2, AFG1 and AFG2) production carried out by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Aflatoxin producing A.flavus isolates were confirmed by polymerase chain reaction using specie specific primers and used for physicochemical optimization for biomass and aflatoxins (AFB1, AFB2, AFG1 and AFG2) production under different temperature, pH and supplementation of inoculating media with three substrates in three different concentrations. Finally, aflatoxins were extracted, purified, quantified and mixed (100ng/mL) with solvents (chloroform, methanol and acetonitrile) and solids (egg yolk, skim milk and sucrose). These were placed at four degree Celsius in different storage vials (brown, opaque and transparent) for six months

CHAPTER 7
LITERATURE CITED

Abbas HK, Shier WT, Horn BW, Weaver MA. 2004. Cultural methods for aflatoxin detection. J Toxicol Toxin Rev. 23(2-3): 295-315.

Banerjee, Shetty HS. 1992. Microbial load in poultry feed and detection of aflatoxin B1 using monoclonal antibody based enzyme linked immunosorbent assay. Lett Appl Microbial. 15: 89-91.

Cardile V, Russo A, Formisano C, Rigano D, Senatore F, Arnold NA, Piozzi F. 2009. Essential oils of Salvia bracteata and Salvia rubifolia from Lebanon: chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells. J Ethnopharmacol. 126: 265-272.

Deng Y, Yu Y, Luo H, Zhang M, Qin X, Li L. 2011. Antimicrobial activity of extract and two alkaloids from traditional chinese medicinal plant stephania dielsiana. Food Chem. 124: 1556-1560.

El-Hamaky AM, Hassan AA, El Yazeed HA, Refai MK. 2016. Prevalence and Detection of Toxigenic *A. flavus, A. niger and A. ochraceus* by traditional and molecular biology methods in feeds. Int J Curr Res. 8: 25621-25633.

Garcia ME, Blanco JL, Suarez G. 1994. Aflatoxins B 1 and G 1 solubility in standard solutions and stability during cold storage. Mycotox Res. 10(2): 97-100.

Mabbett T. 2004. Keep feeds free from fungi. African Farming: 15-16.

Soliman KM, Badeaa RI. 2002. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food Chem Toxicol. 40(11):1669-1675.

Umar S, Munir MT, Shah MAA, Shahzad M, Sohoo MUR, Khan RA, Khan AU, Ameen K, Rafia-Munir A, Saleem F. 2015. Outbreak of aflatoxicosis in local cattle farm in Pakistan. Veterinaria. 3(1): 13-17.

Urughucki K, Yamazahi, M. 1978. Toxicology, biochemistry and pathology of mycotoxins. Hoboken: John Wiley & Sons.

Yu J. 2012. Current understanding on aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins. 4(11): 1024-1057.

Zain ME. 2011. Impact of mycotoxins on humans and animals. J Saudi Chem Soc. 15: 129-144.