"DIFFERENTIAL EXPRESSION ANALYSIS OF SELECTED GENES FROM AFLATOXIN BIOSYNTHESIS PATHWAY AT DIFFERENT MORPHOTYPES OF *Aspergillus flavus*"

Dissertation submitted in partial fulfilment of the requirement for the

degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

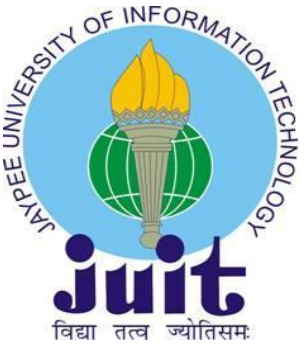
By

Nitin Arora (141811)

Ojus Verma (141804)

UNDER THE GUIDANCE OF

DR. JATA SHANKAR



DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN, HIMACHAL PRADESH, INDIA

MAY - 2018

CONTENTS

DECLARATION BY SCHOLAR

I hereby declare that the work reported in the B-Tech thesis entitled **"Differential expression analysis of selected genes from aflatoxin biosynthesis pathway at different morphotypes of *Aspergillus flavus*"** submitted at **Jaypee University of Information Technology,Waknaghat India,** is an authentic record of my work carried out under the supervision of **Dr. Jata Shankar.** I have not submitted this work elsewhere for any other degree or diploma.

Nitin Arora

Ojus Verma

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat , India

Date:

CERTIFICATE

This is to certify that the work which is being presented in the project title, **"Differential expression analysis of genes selected from aflatoxin biosynthesis pathway at different morphotypes of *Aspergillus flavus*"** for the end semester (8^{th} semester) of Bachelor of technology and submitted in Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of work done by Ojus Verma (141804) and Nitin Arora (141811) during a period from July 2017 to May 2018 under the supervision of **Dr. Jata Shankar**, Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat.

The above statement made is correct to the best of my knowledge.

Date:

_____

Dr. Jata Shankar (Guide)

Associate Professor

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology

ACKNOWLEDGEMENT

Words betray to express our heartfelt sentiments towards the two towering peaks of our lives, our parents who have helped us to surpass all the academic pursuits and our life.

We sincerely express our thanks and heartfelt gratitude to Prof. Dr. Sudhir Kumar, Head of Department of Biotechnology and Bioinformatics and Associate Professor for permitting us to pursue this project.

We owe our sincere thanks to Dr. Jata Shankar, Associate professor, Department of Biotechnology and Bioinformatics, for constantly guiding us in this project.

Our heartiest gratitude to Raman Thakur, Shraddha Tiwari and Sonia Shishodia for their constant guidance, support and motivation.

We extend our cordial thanks to all those who directly or indirectly helped in the successful fulfillment of our work.

Nitin Arora

Ojus Verma

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µL	Microlitre
kb	Kilobytes
Mb	Megabytes
mV	Millivolt
ppb	Parts per billion
mm	Millimetre
^{0}C	Degree celsius
B1,G1	Blue1, Green1
B2,G2	Blue2, Green2
A.flavus	Aspergillus flavus
A.parasiticus	Aspergillus parasiticus
A.niger	Aspergillus niger
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
AFB1	Aflatoxin Blue1
LCB	Lactophenol Cotton Blue
ABPA	Allergic bronchopulmonary aspergillosis
EST	Expressed sequence tags
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-PCR
GAPDH	Glyceraldehdye-3-phosphate dehydrogenase
cAMP-PKA	Cyclic Adenosine Monophosphate-PKA
SSO	Specific spoilage organism
PBST 20	Phosphate Buffer Saline Tween 20
dNTP	Deoxyribonucleoside Triphosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
ITS	Internal Transcribed Spacer
TAE	Tris-Acetate EDTA

ABSTRACT

Fungi are eukaryotic, saprophytic organism having ecological and medicinal importance and acts as major causative agent of food spoilage. *Aspergillus* species such *Aspergillus flavus* is one such opportunistic, toxigenic mould responsible for contaminating cereal grains, dried fruits and vegetables and causing illness in immune-compromised and immune-competent patients worldwide making scientific interventions necessary. Keeping these facts in mind, the aim of the study was to determine the time points of different morphotypes (swollen conidia, germinating tube, hyphae and mycelia) of *Aspergillus flavus* (MTCC11866) which have an effect on the biosynthesis of toxins produced by the fungi and to check the expression of 4 aflatoxin pathway genes (afl D, afl R, afl P, afl S) at 7hours, 12hours and 24hours. It was observed that 7hour, 12hour and 24hour time periods correspond to the germinating conidia (swollen conidia with germ tube), hyphae stage and the mycelia stage of fungus development respectively. Semi-quantitative PCR showed bands for aflD, aflR, aflP genes but no bands for aflS gene. It was further validated by Real time PCR in which aflS gene had a lower transcript value in comparison to the other genes. The current work added knowledge on morphotypes of *A.flavus* and possible role of these genes in morphogenesis.

CHAPTER -1

INTRODUCTION

Food is expected to be nutritious (as is its nature), therefore, food is a rich habitat for microbes, in contrast with the great natural systems, soil, water and plants. Given the correct physic-chemical conditions, only the most fastidious microorganisms are unable to grow in foods, so that factors other than nutrients usually select for particular types of microbial populations. Perhaps the most vital of these relate to the biological state of the food. Living food, particularly, nuts/grains and fresh vegetables/fruits prior to harvest, posses strong defence mechanisms against invasion by microbes. The cardinal factor for spoilage of the living foods is the ability peculiar to some microbes to overcome defence mechanisms. And one of the major causal organisms of food spoilage is fungi.

Fungi, any of approximately 99,000 known types of life forms of the kingdom Fungi, constitute yeasts, rusts, smuts, mildews, moulds, and mushrooms. They are widely spread and found commonly where moisture is present with sufficient nutrients that can support their development.

Molds are a group of fungi characterized by the presence of filamentous hyphae, and producing airborne spores or conidia (asexual propagules).In nature, molds act as decomposers for recycling the organic waste. In medicine, they are producers of antibiotics. Yeasts are fungi which appear oval/elliptical when observed under microscope but do not possess filamentous hyphae.

Most of these fungi including mainly molds that have toxin producing potential and the non-toxin producing species may lend a moldy odour and taste when stored for long durations, both of these fungi contributing to large economic losses in foods around the world, especially in developing countries like India. Yeasts too play an important role in sensory quality of many food- can become major source of microbial food spoilage.

Dimorphic fungi are those that characteristically grow as a mould under certain environmental conditions (usually 25-30°C) and as yeast at a temperature ideal for causing diseases in humans i.e. between 35°C to 37°C. Medically important dimorphic fungi can be very pathogenic and special heed is called for at the time of handling

fungal cultures mainly because of the likelihood of culturing and contracting one of these microbes.

Mycotoxins or toxins produced by microfungi are the secondary metabolites responsible for mortality in people and many animals and does not include the toxins produced by Basidiomycetes, the mushrooms or the macrofungi that are edible. The major toxin producing fungi include *Aspergillus, Fusarium, Penicillium, Claviceps & Alternaria.* The individual fungi are very distinct from each other, which again implies that the potential appearance of a specific mycotoxin is restricted to a certain food number.

Aflatoxins, Patulin, Ochratoxins, Fumonisins produced by these molds were found to have a negative impact on human and animal health like they cause cancer, immune disorders and even prove to be fatal. Mycotoxins, produced by these fungi are a big challenge, especially in maize rendering food unfit for consumption. A large amount (25%–40%) of cereal grains are befouled by the mycotoxins (mostly by aflatoxins) produced by storage fungi throughout the world. Scientists have reported that aflatoxins, once consumed (they have low molecular weight), are quickly absorbed in the gastro-intestinal tract/digestive system via a non-described-passive mechanism, following which these toxic substances circulate as metabolites in blood in less than 30 minutes.

There are a lot of post harvest losses during peak production of fruits like apple because it's harvested over a limited period of time and this temperate fruit crop is of high importance to the states of Jammu and Kashmir, Himachal Pradesh and Uttrakhand.

In the absence of detailed scientific information on the status of the toxin produced by species like and other apple growing states, it is imperative to address the safety concerns of the consumers about this toxin which makes scientific interventions necessary.

Keeping in view the human health hazards of these toxins, the present study was carried out to investigate the presence of spoilage fungi present in food products with special focus on the mycotoxin producing fungi.

Various naturally contaminated food stuffs from different regions of Himachal Pradesh (Solan, Shimla etc)- fruits like apple, pear, orange and cereal grains, wheat flour etc were used; the region showing fungal contamination was carefully excised and plated

on medium favourable for fungi enumeration like Potato Dextrose Agar (PDA) followed by morphological, microscopic and molecular analysis of the isolated fungi. Morphological analysis involved observing the features of the colonies of the microbe grown on the PDA plates kept for incubation. Lactophenol Cotton Blue (LCB) stain was used to observe the microscopic characteristics of the fungi (if any) isolated or observed. The DNA of the isolated fungi was isolated followed by DNA amplification through Polymerase Chain Reaction using appropriate primers for molecular analysis of the fungus.

Aspergillus flavus (MTCC11866) aflatoxigenic strain was then analyzed to obtain information about the different morphotypes of the fungus and whether such changes through the growth phase of fungus has a role in the production of toxins at 7 hour, 12 hour and 24 hour growth periods. Four aflatoxin pathway genes were selected and expression checked using Semi-quantitative PCR/RT-PCR and further quantified by quantitative PCR (Real-time PCR).

CHAPTER 2

REVIEW OF LITERATURE

2.1 Fungi & food spoilage

Fungi are eukaryotes that include microorganisms such as yeasts and molds, as well as mushrooms. Fungi differ in their shape and size, from one-celled, microscopic organisms to multi-cellular forms easily seen with the naked eye.

The molds form large aggregates which are not unicellular of long branching filaments, called hyphae which are of 2 types- vegetative hyphae and reproductive hyphae. It is these tube-like hyphae that give the macroscopic mold colony a fluffy appearance. Symmetrical breakage in hyphae forms germ tubes or branch initial (Lin X. et al.). Hyphae and other structures combine to form an extensive network called as mycelium; for reproduction they form sporangia which produce spores. Mycelia eventually form aerial conidiophores having multiple cell types (stalks, vesicles, metulae, phialides, and conidia).

For successful adaptation of fungus to a particular environment, changes in both physiology and morphology needs to be present (Butler et al. 2009; O'Connor et al. 2010). The environmental/extracellular factors and genetic factors induce and execute morphogenesis in fungi respectively.
Both fungi and bacteria tend to grow under the same conditions of warmth and moisture. It's for this reason that fungal infections pose a serious problem to troops in the tropics. Besides, they are also common spoilage organisms of food.

Some microbes are commonly found in many types of spoiled foods while others are more selective in the items they procure; multiple species are often identified in a single spoiled food item but there may be one species (a specific spoilage organism, SSO) primarily responsible for secreting substances imparting bad odours and flavours.

Chemical reactions that cause bad sensory changes in foods are mediated by a variety of microbes that require food as a carbon and energy source. These creatures incorporate prokaryotes (microorganisms), single-celled life forms lacking characterized

cores/nuclei and different organelles, and eukaryotes, unicellular (yeasts) and multicellular (molds) living beings with cores and different organelles

Fungi including yeasts and molds (majorly) are able to colonize all kinds of food products and may result in various kinds of food's spoilage like off-flavours, toxins, changes in color, rotting or formation of allergic/pathogenic propagules. The exoenzymes like lipases, proteases, carbohydrases produced by fungi during growth (Bieglis, 1992) often results in the deterioration of these organo-leptic properties of the food on which it grows. After gaining entry into the food, these chemicals may proceed with their exercises free of devastation or evacuation of the mycelium and may offer ascent to flavors like rioy espresso beans, smelly scents in stopper and wine or dried organic products after the action of enzymes on them (Filtenborg O.et.al.1996)

These saprophytic organisms, which are of a great environmental and medical importance, causes a major contamination of food grains during post-harvest storage accounting up to 30% (Chhokar R, 2001). The majority of the black *Aspergillus* species, for example *A.niger,* are identified in grapes, onions, maize, and peanuts, where they are cited as pathogens causing such diseases as peanut and maize seedling blight, & maize kernel rot (Palencia et. al.2010). More than 300 fungal secondary metabolites have been reported as mycotoxins and spoil more than 25% of the world's food crop which is the most vital aspect of mould food spoilage (Galvano et al.2001).

The mycotoxins produced by mycotoxigenic molds are produced in fruits and vegetables, grain crops, pulses and cereals, nuts,dried fruits and other commodities.

Out of the many moulds known for their ability to secrete toxins, a limited number of genera, *Penicillium, Aspergillus, Fusarium, Claviceps & Alternaria* are of considerable importance in food. The most important mycotoxins, esp. in maize include Aflatoxins, Ochratoxins, Fumonisins and Deoxynivalenol (Kumar D and Kalita P., 2017).

As is the nature of secondary metabolites in general, these are produced only by a limited number of species. According to Frisvad et al., aflatoxins are produced only by the closely related *Aspergillus flavus*, A.*parasiticus* and *A. nomius*. In some cases like pomaceous fruits (apple, pear), the mycotoxin Patulin is produced by molds like *Aspergillus*, *Byssochlamys* and *Penicillium*, most notably *P.expansum* (causing blue mold rot in apples, cherries etc). Patulin, earlier being used as an antibiotic, was later found to be harmful to both plants and animals and reclassified as a mycotoxin (Puel O.

et al 2010). Ochratoxins and cyclopiazonic acid produced by species of *Aspergillus* and *Penicillium,* Gliotoxin released by *A.terreus, A.fumigatus* and species of *Gliocladium.*
With the discovery of mycotoxin production in food the importance of moulds in food stuffs is highlighted and there has been an increase in knowledge and understanding of the role played by these food spoilage fungi especially of the genus *Aspergillus.*

2.2 *Aspergillus*

Aspergillus is a big genus constituted of less than 200 accepted anamorphic species with teleomorphs distributed in 9 different genera. The genus is sub-divided in 7 subgenera and further put into sections (Klich M.A.2002).

Aspergillus taxonomy seems to be complex and ever evolving which is true for all fungi. The species of this genus can be easily identified by their characteristic conidiophore, identifying and differentiating them is quite a task, for it is traditionally based on a range of morphological features.
Many of the species belonging to this class of imperfect fungi are producers of secondary metabolites (beneficial) like *A.terreus* which produces lovastatin (a potent cholesterol lowering drug). Some secrete antibiotics (cephalosporin), antifungals (grieofulvin) and anti-tumor drugs (terrequinone A) (Hoffmeister, D.; Keller, N.P 2007). However, harmful secondary metabolites too are produced by them making the genus one of the most common producers of mycotoxins in food crops and somehow adversely affecting human and animal life (Shankar *et al.,* 2005, Bheteriya *et al.,* 2009).
As of now, around 20 species have been reported to be opportunistic pathogens in humans worldwide (Dagenais and Keller 2009).They influence health by causing diseases ranging from localized infections to fatal diseases either producing allergic responses on inhaling conidia (aspergillosis) or by intoxication (mycotoxicosis). The toxins mentioned earlier produced by these Aspergillus species are hepatocarcinogenic, nephrogenic and immunological in nature (Gautum et. al.2011).

Within the genus *Aspergillus, A.flavus* is the most notorious and economically important as it produces aflatoxins and is the second leading cause of the deadly invasive aspergillosis after *A.fumigatus.*

2.3 ***Aspergillus flavus***

Aspergillus flavus is the most commonly encountered saprotroph in the soil and feeds on dead and decaying organic matter, storage areas of crops, cotton, animal food and also on animals and humans having a weak immune system (Klich, M.A.1998). It is found to infect both plants and animals. Ubiquitious in nature, it is found in a wide range of climates, surviving in temperature range of 12^0C to 48^0C and temperature optima from 28^0C to 37^0C. It is capable of surviving at high temperature and hence a pathogen of humans and other warm-blooded animals.

Table 1: Scientific classification of *Aspergillus flavus*

Kingdom	Fungi
Division	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*Flavus*

A.flavus's, which is genetically similar to *A.oryzae*, assembly sequence shows that the genome size is 36.3 Mb, has eight chromosomes and around 13,071 predicted genes.

2.3.1 Cultural and microscopic characteristics

Appear as yellowish-green colonies having yellow or no color on its reverse.Its conidiophores (<1mm in length) are coarsely roughened, colorless and heavily walled. The vesicles appear quite elongated when young and later develop into globose to sub-globose with varying diameter. Uniseriate or biseriate phialides project from vesicles in the shape of a flask. Branched septate hyphae are there and conidia have a diameter of 3.5mm to 4.5mm which may be globose or sub-globose (Gautam A. K. and Bhadauria R,2012).

For most of its lifecycle, the fungus overwinters in the form of mycelium or asexual spores (conidia). When conditions are unsuitable such as dearth of adequate nutrients or

water, the fungal mycelium changes to resistant structures called sclerotia, which can live in the extremes of conditions. The fungus manages to survive either as spores, sclerotia, or as mycelium in debris. With the onset of favourable conditions, the sclerotia are found to mature directly to form new colonies or conidiophores containing conidia (Cotty P.1988).

2.4 Aspergillosis

Aspergillus flavus, A.parsiticus,A.fumigatus and Aspergillus niger are the causal agents of allergic bronchopulmonary aspergillosis (ABPA) in immune-competent hosts worldwide (Shankar *et al.*, 2004).

A.fumigatus is the dominant cause of hypersensitivity in humans followed by *A.flavus* for invasive and non invasive aspergillosis. In patients with immune-compromised immune system such as in those who have recently undergone a transplant, have leukemia or are HIV positive, the immune system fails to recognize and remove the *Aspergillus* conidia making way for invasive aspergillosis. Given the opportunistic nature of these molds, they quickly invade tissues and cause their destruction (Shankar J.2013).

Table 2: Total burden of aspergillosis in world and India (Agarwal et al 2014, WHS)

	INDIA	**WORLD**
Total population	1.2 billion	7.1 billion
Asthmatic patients	27.6 million	200 million
ABPA burden	1.3 million	5 million
Invasive aspergillosis	0.17 million	0.37 million

2.5 Aflatoxicosis

A. flavus, mostly observed in temperate and tropical climates and *Aspergillus parasiticus*, are considered the most dangerous of the mycotoxin (aflatoxins-B1, B2, G1, G2) producing species. Aflatoxin B1 and B2 are specific to *A.flavus* and B1, B2, G1 and G2 are produced by *A.parasiticus*. These four noteworthy aflatoxins are named

according to their blue (B) or green (G) fluorescence under UV light, and their relative mobility by thin-layer chromatography on silica gel.

Aflatoxins were discovered in *Aspergillus flavus* (thus the name "a-fla-toxin") about fourty years ago post an outbreak of a disease named Turkey X in Britain (Klich, M. A. et. al, 2000). Other significant members of the aflatoxin family, M_1 and M_2, are oxidized forms of aflatoxin B_1 transformed in the digestive tracts of some animals and isolated from milk, urine, and stool.

The order of acute and chronic toxicity is AFB1 > AFG1 > AFB2 > AFG2. The International Agency for Research on Cancer (IARC) includes B1, G2, B1, G2 in Class I potent human carcinogen (IARC 1987). Among these compounds, AFB1 is normally predominant in concentrations in cultures as well as in food products. Long term exposure suppresses the immune system, causes malnutrition, bile duct proliferation, hepatic necrosis, fatty infiltration of the liver, lesions on the liver and hepatomas. In our body, the liver is main site of biotransformation of aflatoxins (Bennett JW, Klich MA 1992). AFB1 detoxification by a cytochrome P450 monooxygenase changes the toxin into a more carcinogenic by-product i.e. the AFB1-8, 9-epoxide form of aflatoxin which binds to guanine residues of DNA, forms adducts of guanyl-N7, and induces mutations like commonly in p53 tumor suppressor gene thus linked with the initiation of hepatic cancer (Jiujiang Yu,2012).

Out of the 400 mycotoxins identified, aflatoxins are the best known and the number is by all accounts expanding at a quick rate. Roughly 4.5 billion individuals dwelling in developing nations are presented to unregulated measure of aflatoxin which prompts intense aflatoxicosis (Obrian G. et al. 2007, Tiwari S. 2018) and if stringent measures are not put into place, it will get much worse.

To control the exposure of aflatoxin, the European Commission has fixed a mark of 20 ppb by most countries, and 8 ppb for AFB1(Van Egmond HP, Jonker MA, 2005). Periodic monitoring of toxin producing mycoflora of the agro-based feeds and foods is an important pre-requisite for developing strategies to contain or mitigate mycotoxins exposure to the animals and man.

2.5.1 Genetics and Molecular Biology of Biosynthesis of Aflatoxin

The aflatoxin biosynthesis pathway involves around 27 steps catalyzed by enzymes with as many as 30 genes having a potential role in the biosynthesis. In *A. flavus* and *A. parasiticus* the genes that participate in aflatoxin production are clustered within a 75-kb region of fungal genome on chromosome III roughly eighty kb away from telomere as shown in the figure 1.

Analysis of 7218 unique Expressed Sequence Tags (ESTs) identified by Yu *et al. (*2004b) provided information about the genes of the aflatoxin pathway. The functional genes of the pathway are designated from *aflA* to *aflQ* mediating the initial conversion of fatty acids to end product aflatoxins and *aflR* and *alfS (aflJ*) for the 2 transcriptional regulators.

The homologous genes of Sterigmatocystin (ST) synthesis in *A. nidulans* and their involvement with the biochemical pathway are normal to aflatoxins and ST (Figure). *Aspergillus nidulans* doesn't contain the aflP orthologue and consequently, produces ST instead of aflatoxin.

Aflatoxins or the polyketide-derived secondary metabolites are produced via the following conversion path: acetate → polyketide → anthraquinones → xanthones → aflatoxins. (Jiujiang Yu et al.2004).

A hexanoyl unit (sugar, lipid) acts as a primer for the initiation of the biosynthetic pathway. This induction causes the transcriptional activation of the pathway genes *pksA (*polyketide synthaseA*), nor-1(*norsolorinic acid-1*), ver-1(*versicolorin-1), *omtA(*O-methyltransferaseA),and the regulator gene aflR.

PksA along with fatty acid synthases (*fas-1, fas-2*) encode enzymes responsible for the conversion of the first acetate units to polyketides. A reductase coded by *nor-1* catalyzes the conversion of substrate *nor* to *avn* (averantin). NOR is the first stable precursor of the aflatoxin. The dehydrogenases (*ver-1* gene) and 0-methyltransferases (*omtA* gene) facilitate the end steps of the pathway. The change of substrate Versiconal to Versicolorin B by the enzyme cyclase/ Versicolorin B synthase is one of the key steps of the route as it causes the closure of aflatoxin's bisfuran ring ; bisurfan ring is the reason for aflatoxin's toxic and carcinogenic nature (Jiujiang Yu. 2012). This

formation of VerB is a critical branch point that decides which aflatoxin will be formed (AFB1/AFG1 or AFB2/AFG2) during the conversion from VerB to VerA.

The aflR gene has sequences for AflR, a sequence-specific zinc-finger DNA-binding protein (47 kDa) having a role of activating the conversion of DNA to RNA (transcription) of almost all the genes specific to the path of conversion. In *A.flavus*, the over-expression of aflR gene and the up-regulation of transcription of genes and the subsequent accumulation of the toxin are inter-linked (Flaherty J.E. and Payne G.A1997). aflR gene has been found to be negatively regulated by cAMP-PKA signalling pathway during transcription and post-transcription (OBrian G.R.et al,2003).

The AflS is a co-activator of the process that forms RNA of the aflS gene affecting the synthesis mechanism. The Quantitative PCR showed that in the *aflS* knockout mutants, the absence of *aflS* transcript corresponds to a 5 to 20-fold reduction in expression of the aflatoxin pathway genes namely *aflC* (*pksA*), *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*). Also, the aflR and aflS don't influence the transcription of each other (Jiujiang Yu,2012).

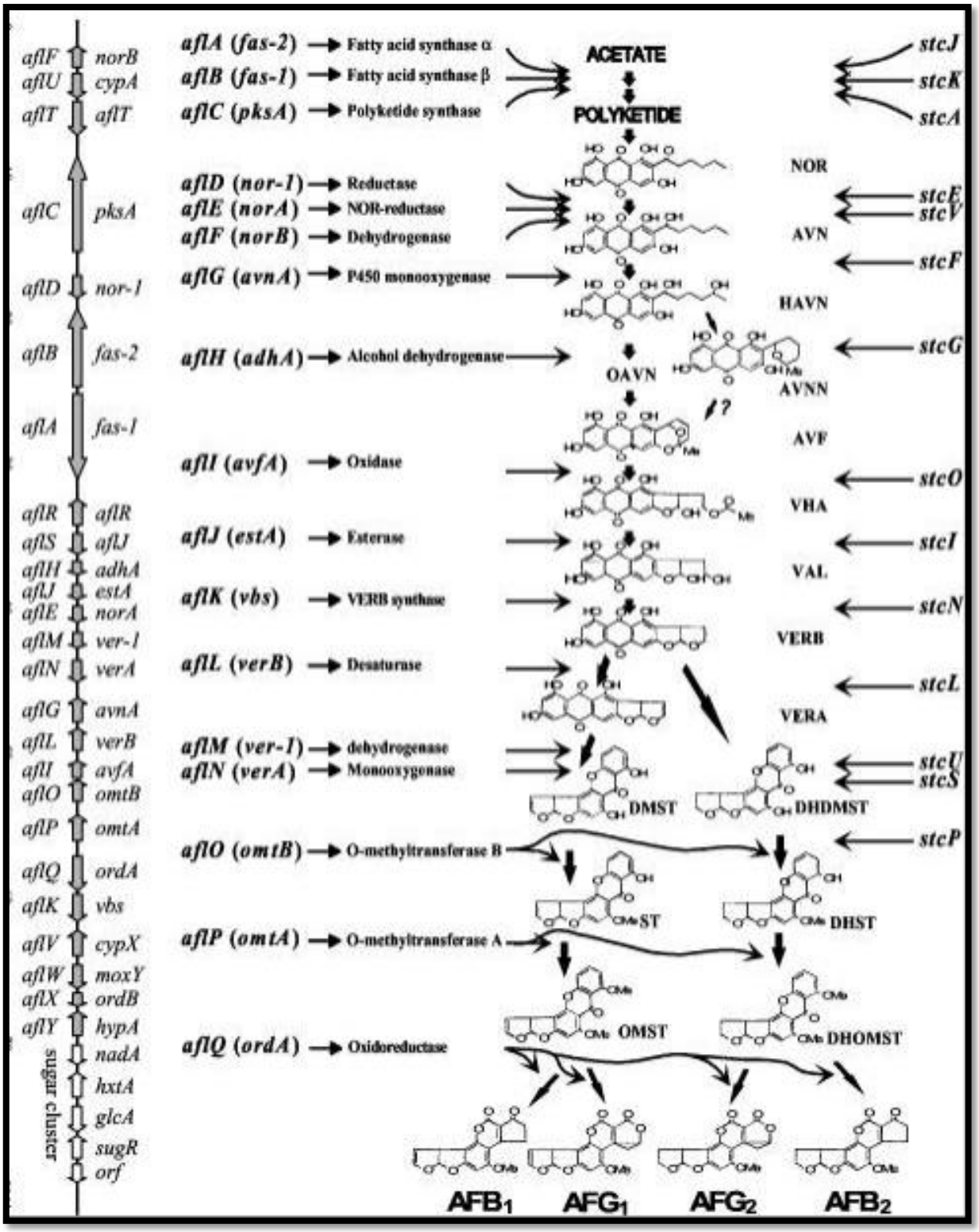


Figure 1: Gene cluster and Aflatoxin biosynthesis pathway (Yu et al.2004)

2.5.2 Factors responsible for Aflatoxin biosynthesis

Various environmental and physiological factors like temperature, pH of the medium, water activity (a_w) and nutritional sources such as Carbon, Nitrogen play a role in synthesis of the toxin in the host system (Bennett, J.W.; Papa, K.E, 1988).

Carbon and Nitrogen: Sugars such as glucose or maltose or lactose and media containing asparagine, alanine, ammonium sulphate and proline support alfatoxin production. Substrate containing lipids are also ideal for aflatoxin production.

Temperature: Optimum temperature for production of these toxins is 30 °C (from 28 °C to 35 °C).At temperatures above 37^0C, no more aflatoxin is produced. The genome wide gene profiling by making use of microarray and RT-PCR clarification (O Brian, G.R.2007) showed that high temperature leads to a lower expression of the genes involved. It was observed that higher temperature affects the activity of the pathway genes especially the regulatory genes aflR and aflS. The transcript level of aflS is changed more than aflR.

Oxidative stress: Oxidative stress has been found to be a reason for the production of AFB1 in *Aseprgillus flavus* and *A.parasiticus*. Treatment of *A.flavus* with antioxidants like caffeic acid, gallic acid, ascorbic acid had an inhibitory effect on the synthesis of these toxic substances (Jiujiang Yu, 2012). The protein profile of *A.flavus* strain treated with another phytochemical Quercetin indicated the production of proteins having antioxidant activity and enzymes such as Heat shock protein 70, catalase A etc (Tiwari S. 2018).

Stages of development: Secondary metabolism takes place when sporulation and sclerotia formation starts. The formation of spores and production of secondary metabolites go hand in hand. A change in the morphotype of the fungus implies a change in the aflatoxin producing potential of the fungus. The morphotypes such as swollen conidia, germinating tube, hyphae and later mycelium mediate the survival of the fungus in adverse and ambient conditions by secreting various substances thereby making the fungus virulent and pathogenic (Shankar et.al.2018).

CHAPTER -3

OBJECTIVE

1. To determine the time points of different morphotypes (swollen conidia, germinating tube, hyphae and mycelia) of *Aspergillus flavus*.
2. To check the expression of 4 aflatoxin pathway genes (afl D, afl R, afl P, afl S) at 7 hours, 12 hours and 24 hours

CHAPTER -4

MATERIALS AND METHODS

PART A

4.1 Isolation of fungus from food products

1. Three food samples : Red apple(sample1), golden delicious apple(sample2) and wheat flour (sample3) were exposed to air for 7 days
2. In sterile conditions, small portions of the tissues were cut and plated on Potato Dextrose Agar (PDA) petri plates.
3. The plates were incubated at 37^0C and 25^0C for a week.
4. For each of the food sample, microscopic slides were prepared which were stained with Lactophenol Cotton Blue.
5. The slides were observed under the light microscope
6. The fungi obtained on plates inoculated with contaminated apple were carefully passaged on PDA plates and slants to obtain a single colony and grown for several days.

Figure 2: Contaminated food products (Red apple, golden apple and wheat flour), in order

4.2 Extraction of DNA from the fungus isolated from golden delicious apple

The following solutions and reagents were used:

1. Liquid nitrogen
2. Buffer solution for extraction (200mM Tris-Hcl pH 7.5; 25mM EDTA pH 8; 250mM NaCl and 0.5% SDS)
3. Cold phenol:chloroform (1:1)
4. Cold isopropanol and 70% ethanol

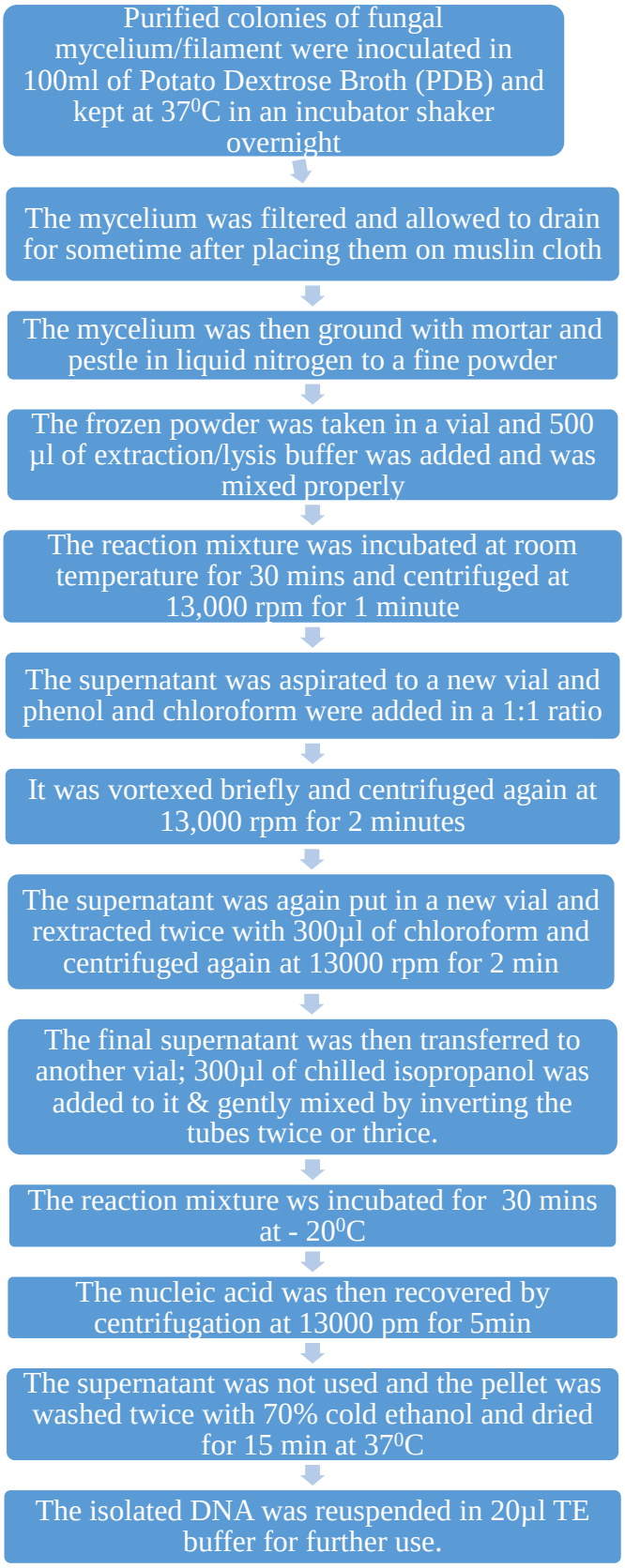


Figure 3: Schematic representation of DNA isolation from fungus

Agarose Gel electrophoresis of the DNA (Sample 2) was performed:

1. 100 ml of 1% agarose gel was prepared by dissolving 1g of agarose in 100ml of 1X TAE buffer in a flask and heating to dissolve.
2. 5µL of ethidium bromide (10mg/ml stock) was added to the flask.
3. The gel and the tray (for casting) containing wells forming comb were put. Post the solidification of the gel, casting tray was placed in an electrophoresis tank with 1X TAE buffer and the comb was taken out.
4. The isolated DNA for Sample 2 along with 1000bp ladder was pipetted into the wells.
5. The gel was run at 100mV and 1 hour and observed under gel documentation system

4.3 Molecular identification using Polymerase Chain Reaction (PCR)

1. A PCR was performed in a total volume of 15µl (2X).
2. The reaction for 1X was set up as follows and was done for isolate from Sample 2 to check with house-keeping genes Tubulin, GAPDH and terelysin.

Table 3: PCR reaction components for golden apple with housekeeping genes

Reagent	Volume (in µL)
Master mix	7.5
Forward primer	0.25
Reverse primer	0.25
Genomic DNA	1
Distilled water	6

3. The vials were placed in a thermal cycler and the PCR was maintained at the following time-temperature profile:

Initial denaturation: 95^0C for 5minutes

Denaturation: 95^0C for 45 seconds

Annealing: 61.6^0C for 30 seconds

Extension: 72^0C for 45 seconds

Final extension: 72^0C for 7minutes

Hold at 4^0C

4. It was then further primed (15µL) by using ITS region specific primers (ITS1 and ITS4) having sequence:

   ITS1 (F): 5′TCCGTAGGTGAACCTGCGG3′

   ITS4 (R): 5′TCCTCCGCTTATTGATATGC3′

Table 4: PCR reaction components for golden apple with ITS primers

Reagent	Volume (in µL)
Master mix	7.5
Forward primer	0.25
Reverse primer	0.25
Template DNA	1
Distilled water	6

5. Vials were placed in the thermal cycler and the PCR was run at the following conditions of reaction:

   Denaturation (Initial): 95^0C for 5minutes

   Denaturation: 95^0C for 45 seconds

   Annealing: 55^0C for 30 seconds

   Extension: 72^0C for 45 seconds

   Extension (Final): 72^0C for 7minutes

   Hold at 4^0C

6. PCR products (expected size: 700-800bp) so obtained were resolved by gel electrophoresis in a 1% agarose gel and visualized along with 4µl of 100 base pair ladder.

PART B

4.4 Growth of *A.flavus* (MTCC11866)

1. *A.flavus* (MTCC11866) conidia were inoculated on Potato Dextrose Agar (Himedia, India) and kept for incubation for a week at 37°C.
2. The harvesting of spores was done by gently rinsing the colonies with Phosphate buffered saline (PBS) and 0.05 % Tween 20 (PBST) having pH 7.4, centrifugation (10,000 rpm, 10 min, 4°C) followed by washing twice with PBS.
3. The extract was then collected in centrifuge tubes.

4.5 Large scale culture for Total RNA extraction

1. The cells of *A.flavus* were counted using haemocytometer:
    - The spore suspension was collected in small vials
    - The surface of the haemocytometer was cleaned
    - 10µL of the cell suspension was pipetted in one of the 2 counting chambers and the cells count done under the microscope.
    - Counting done using the formula:

      Cells/mL: (n) × 10^4× dilution factor

      Where n= the avg. cell count per square of the four corner squares taken into account
2. *A. flavus* spores (1×10^6 cells/ml) were inoculated in Potato Dextrose Broth (100ml) in 3 flasks.
3. The flasks were kept in shaker incubator at 37^0C for 7hrs, 12hrs and 24hrs
4. Each of the flask was filtered using a muslin cloth and kept at -80^0C for further use.
5. LCB staining was carried out and observed under the microscope to observe the morphotypes in the culture grown in each of the flask.
6. RNA was extracted from each of the flask using TRIzol method (Figure 4).
7. 2µL of the RNA each isolated for 7hrs, 12hrs and 24hrs were mixed with 2µL loading dye and 4µL of 1kb ladder (1kb plus gene ruler, Fermentas) were loaded in 1.5% agarose gel and run at 100mV for 1hr.Observed under the gel-doc system.

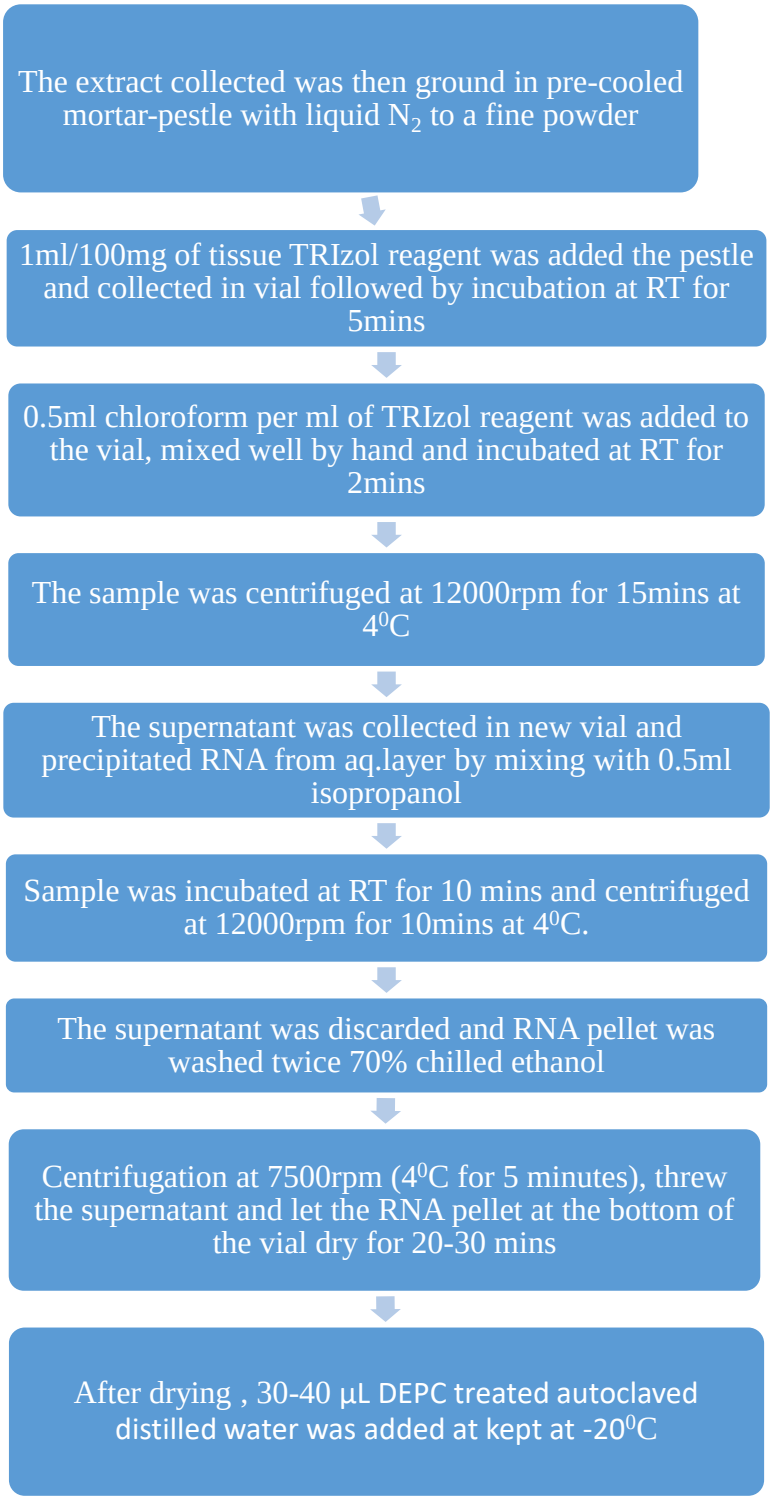


Figure 4: Schematic representation of RNA isolation from fungus by TRIzol method

4.6 cDNA synthesis

1. Before cDNA preparation, the RNA obtained was quantified using NanoDrop Spectrophotometer and the readings were considered to get a volume, which would have the same RNA concentration in all the test samples. This is done so that equal similar concentration of RNA is used.
2. TAKARA BIO INC. cDNA synthesis kit was used
   20µL reaction mixture was prepared in PCR vials for each RNA sample

Table 5: PCR reaction components for cDNA synthesis

Reagent	**Volume (in µL)**
5X prime script buffer	4
dNTP mixture	2
OligodT primer	1
RNase free water	10
Reverse Transcriptase Enzyme	1
RT Enhancer	1
RNA Template	1

The following conditions were employed in thermo cycler for cDNA synthesis:

Stage 1: 42^0C for 30mins

Stage 2: 95^0C for 2mins

Stage 3: 4^0C

3. To check the cDNA synthesized, a semi-quantitative PCR was done for each sample using a housekeeping gene Tubulin and has a product size of 122 base pair.
   It was performed for a total reaction of 12.5µL (Table 6).

Table 6: PCR reaction components with Tubulin (cDNA check)

Reagent	Volume (in µL)
10X buffer	2
dNTP	0.5
Forward primer (tubulin F)	0.5
Reverse primer (tubulin R)	0.5
Taq polymerase enzyme	0.25
Nuclease free water	7.75
cDNA template	1

The following reaction conditions were set up in the thermocycler :

Initial denaturation: 95^0C for 5minutes

Denaturation: 95^0C for 45 seconds

Annealing: 56.3^0C for 30 seconds

Extension: 72^0C for 45 seconds

Final extension: 72^0C for 7minutes

Hold at 4^0C

4. The amplified products were loaded in a 1.5% agarose gel. A 100bp ladder was loaded and the results observed under the gel-doc.
5. Another RT-PCR was carried out w.r.t the 4 aflatoxin pathway genes that were selected for analysis i.e. aflD, aflR, aflP and aflS having product size of 124bp, 113bp, 100bp and 109bp respectively. The reaction mixture totalled to 20 µL (Table 7).
6. The products of the PCR along with 100bp ladder (Thermo Scientific) were loaded onto a 1.5% agarose gel prepared and run at 100mV for 1 hour followed by observation under gel-doc system.
7. To check the expression of the aflatoxin genes, a Quantitative PCR/ qRT- PCR (CFX96 BIO-RAD Real-Time System) was performed by amplification of the cDNA synthesized with primers specific to the aflD, aflR, aflP and aflS genes.

8. Results were obtained in the form of amplification curves & cycle threshold, C_t values, as the fluorophore/dye (SYBY Green, BioRad) emits the light which reveals differential expression of genes (Table 8).

   The following conditions were employed:

   Separation of ds-DNA: 95^0C

   Annealing: 50-60^0C

   Polymerization: 68-72^0C

Table 7: PCR reaction components with aflatoxin pathway genes

Reagent	Volume (in µL)
10X buffer	4
dNTP	1
Taq polymerase enzyme	0.25
Forward primer	1
Reverse primer	1
Nuclease free water	11.75
cDNA	1

Table 8: Real-time PCR reaction components with aflatoxin pathway genes

Reagent	Volume (in µL)
SYBR-green	6
Reverse Primer	0.5
Forward Primer	0.5
cDNA	0.5-1 (200ng)
Water	4.5

Table 9: Sequence of primers used

Primer	Annealing Temperature	Sequence
Tubulin	53.6^{0}C	F 5'-GGAATGGATCTGACGGCAAG-3' R 5'-GGTCAGGAGTTGCAAAGCG-3'
aflD	54^{0}C	F 5´-ATATGGGCGACCAAGGAG-3´ R 5´-AAGTGCCCCGATGTAGTC-3´
aflR	54^{0}C	F 5´-CCTCCCTAGTATGATGGGC-3´ R 5´-CCAATAGGTTCACCAGCG-3´
aflP	55^{0}C	F 5´-TGCGCAAGTAGGGGAATG-3´ R 5´-CAAACCCCACGAATTAGGGC-3´
aflS	55^{0}C	F 5´-GAGCGATCTCTCCTTACTGC-3´ R 5´-AACTTCCCGCATCACCAC-3´

CHAPTER -5

RESULTS

PART A

The morphological /microscopic and molecular characteristics were examined for the identification of the fungi isolated, along with the available literature.

5.1 Morphological and microscopic characteristics

Table 10: Morphological characteristics of the colony

Morphological characteristics of the colony			
Food sample	**Surface color**	**Reverse-side color**	**Possible species**
Red apple (Sample1)	Greenish to yellowish	Colorless	*Aspergillus flavus*
Golden apple (Sample2)	Black	Colorless	*Aspergillus niger*
Wheat flour (Sample3)	Dense white growth	White	*Rhizopus*

Table 11: Microscopic characteristics

Food sample	**Microscopic characteristics**	**Possible species**
Red apple (Sample1)	The erect branch of hyphae arising from the foot cell is the conidiophore, which enlarges at its top to form an elliptical/club-shaped vesicle and colorless spores (conidia)	*Aspergillus flavus*
Golden apple (Sample2)	The conidiophores can be seen, giving rise to circular (globose) vesicle surrounded by black spores (conidia)	*Aspergillus flavus*
Wheat flour (Sample3)	The sporangiophore giving rise to a columella having sporangium over it can be seen. The sporangium contains sporangiospores.	*Rhizopus*

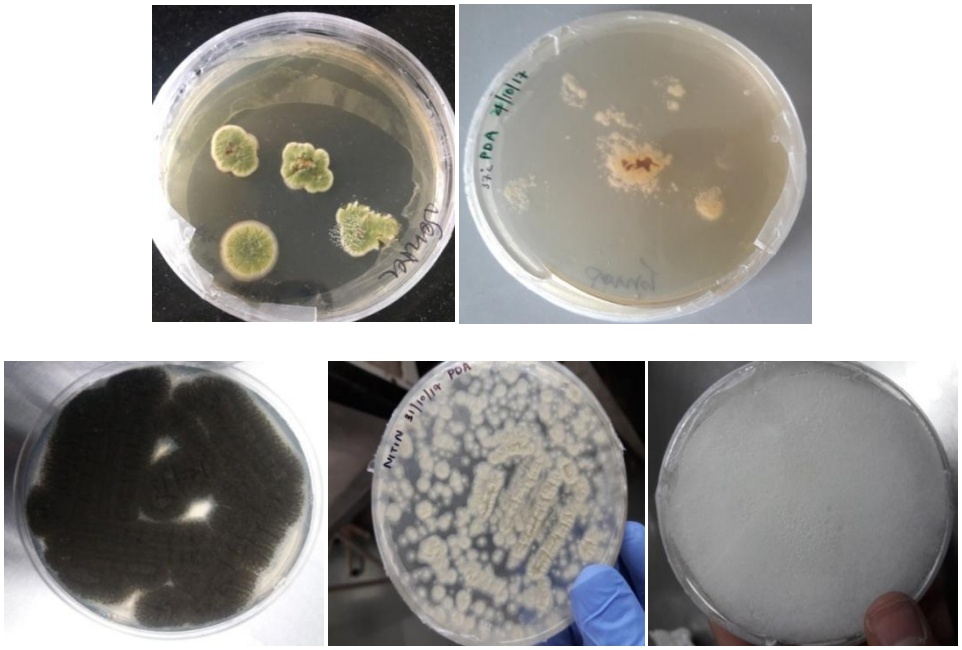


Figure 5: Surface and reverse side characteristics of different fungal species on PDA for red apple, golden apple and wheat flour, in order

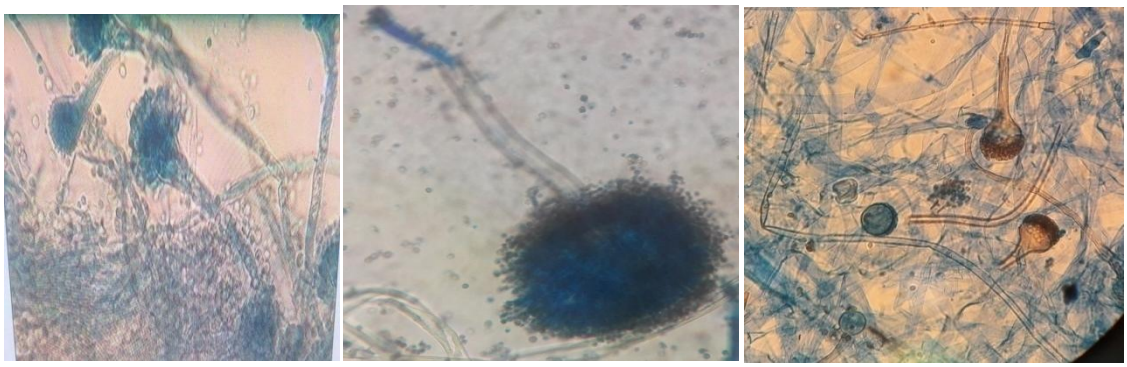


Figure 6: Microscopic characteristics of different fungal species for red apple, golden apple and wheat flour in order, as observed under microscope at 40X

5.2 Molecular analysis

The DNA was successfully extracted from the fungus isolated from golden delicious apple (Sample 2) with RNA contamination.

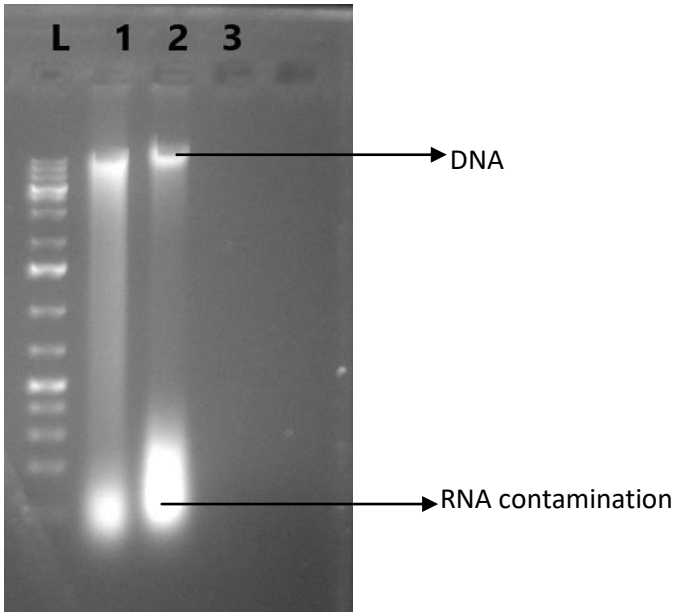


Figure 7: Results of gel electrophoresis of DNA sample. Lane L= Ladder, Lane 1, 2= DNA isolated.

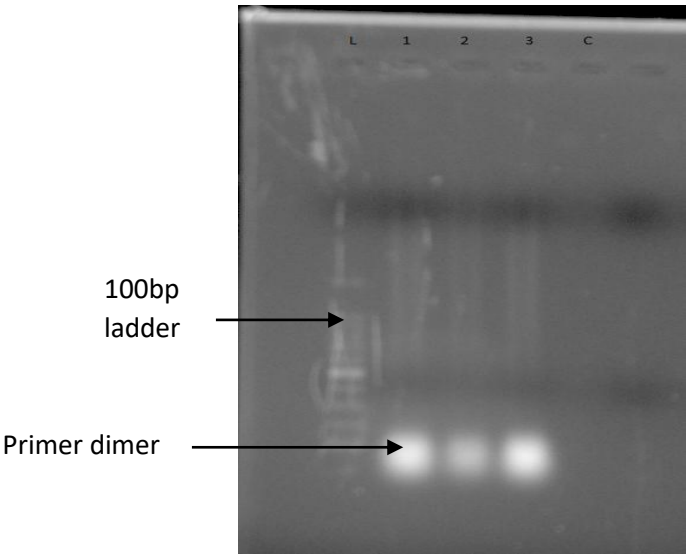


Figure 8: Bands for sample 2 resulted from PCR reaction primed by Tubulin, GAPDH and Terelysin. Lane L= 100bp ladder; Lane C= Control

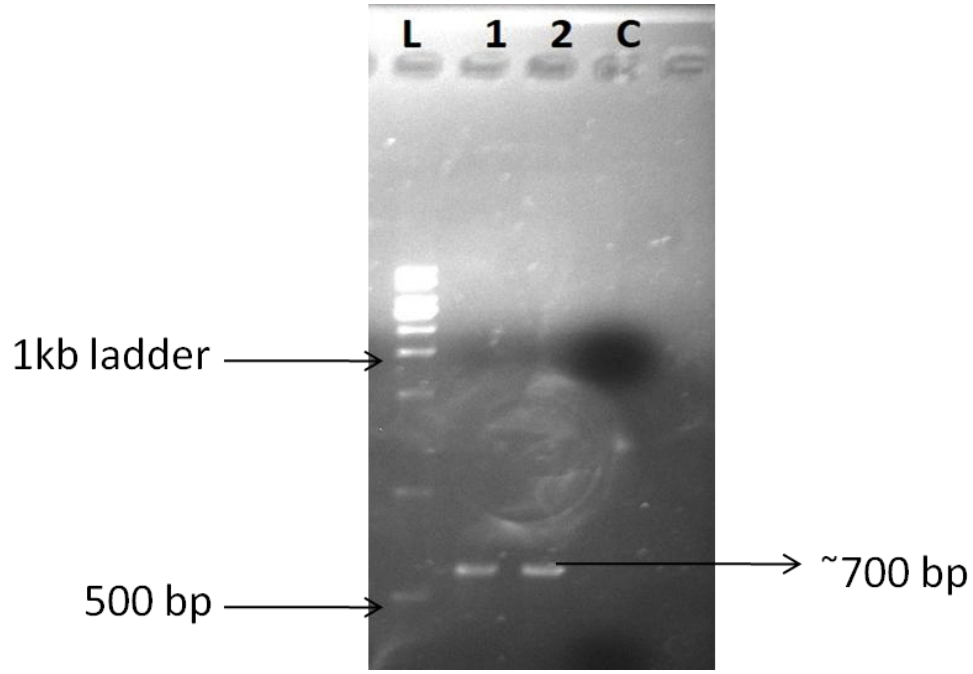


Figure 9: Bands for sample 2 resulted from PCR reaction using ITS1 and ITS4. Lane L= 1kb ladder, Lane 1, 2= PCR products, Lane C= Control

PART B

5.3 Growth of *A.flavus* (MTCC11866)

Figure 10: *Aspergillus flavus* (MTCC11866) on Potato dextrose agar plate.

5.4 Morphotypes at 7 hours, 12 hours and 24 hours

5.4.1 7 hours

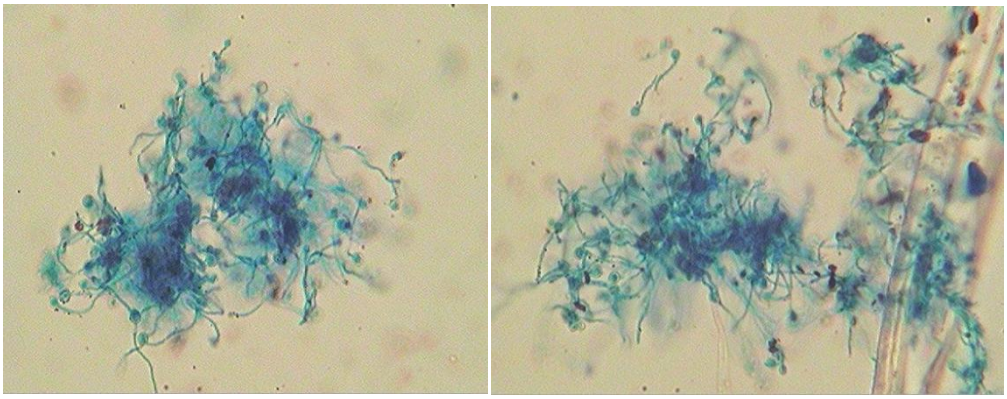


Figure 11: Germinating conidia after 7 hours

5.4.2 12 hours

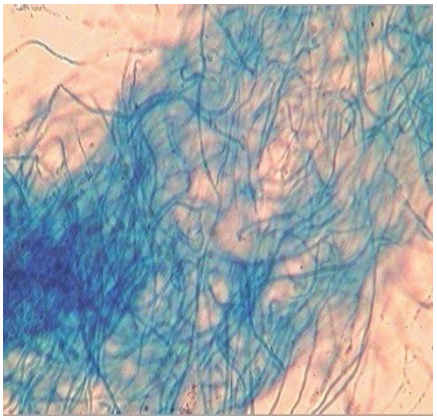


Figure 12: Conidia with mycelia formation after 12 hours

5.4.3 24 hours

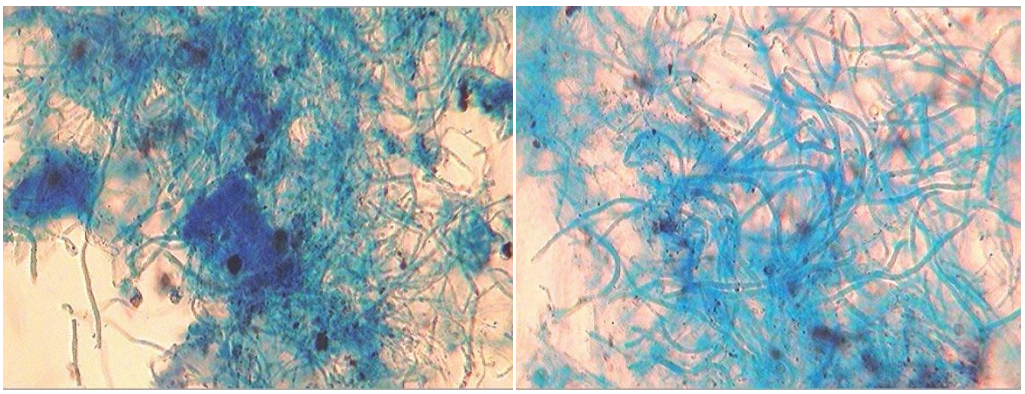


Figure 13: Heavy growth with germinating conidia, hyphae and mycelia formation after 24 hours

5.5 Extracted RNA at 7 hours, 12 hours and 24 hours

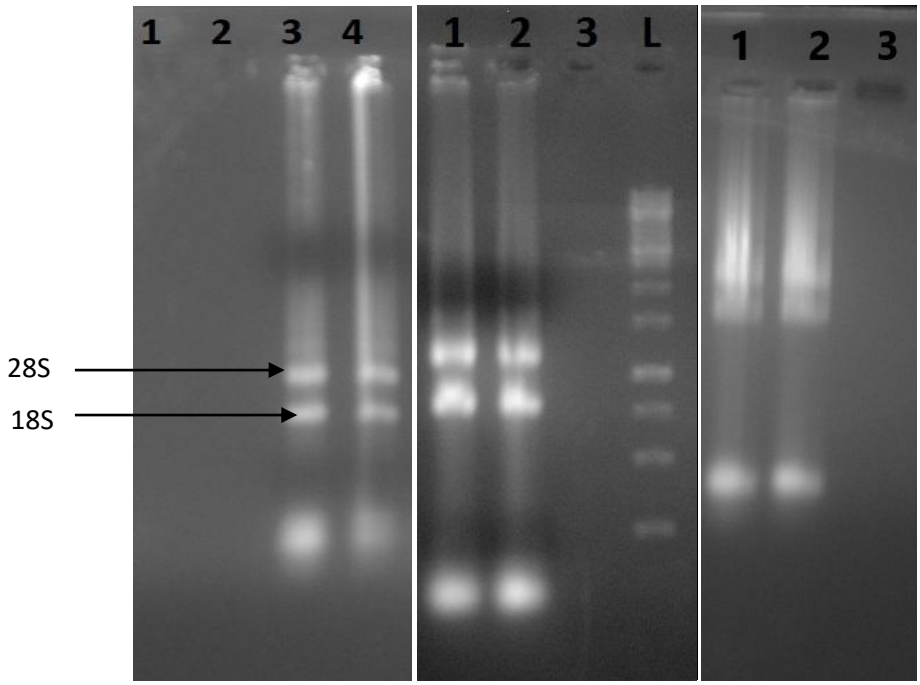


Figure 14: Bands of rRNA (28S and 18S) isolated at 7hrs, 12hrs and 24hrs, in order

Table 12: RNA quantification using NanoDrop Spectrophotometer

Time period (hours)	Concentration of RNA(ng/µL)
7	1040
12	2440
24	2830

5.6 Molecular analysis

5.6.1 Semi-quantitative PCR w.r.t Tubulin (cDNA check)

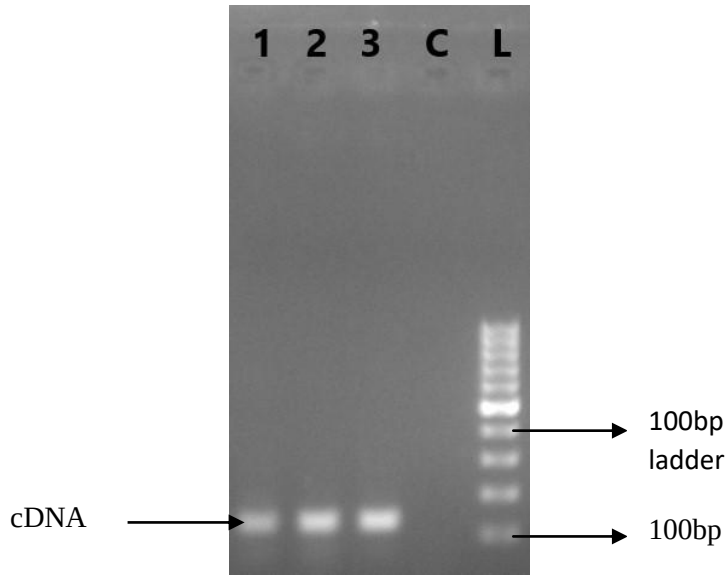


Figure 15: cDNA bands with Tubulin. Lane 1, 2, 3 = cDNA bands at 7hrs, 12hrs and 24hrs resp. Lane C=Control, Lane L= 100bp ladder

5.6.2 Semi-quantitative PCR w.r.t aflD and aflR

Different band intensity for aflD and aflR genes at 7hrs, 12hrs and 24hrs was observed, implying the presence of their transcripts.

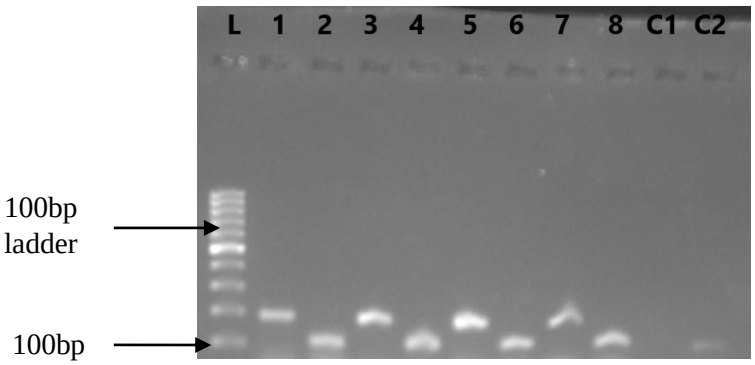


Figure 16: Gel picture of amplified cDNA product with aflD and aflR genes. L=100bp ladder, Lane 1,3, 5,7: amplified aflD gene at 7h,12h and 24h resp., Lane 2,4,6,8: amplified aflR gene at 7h, 12h and 24h resp., C1 and C2: Control

5.6.3 Semi-quantitative PCR w.r.t aflS and aflP

No bands were observed for aflS gene while aflP showed same intensity bands at 7hrs, 12hrs and 24hrs.

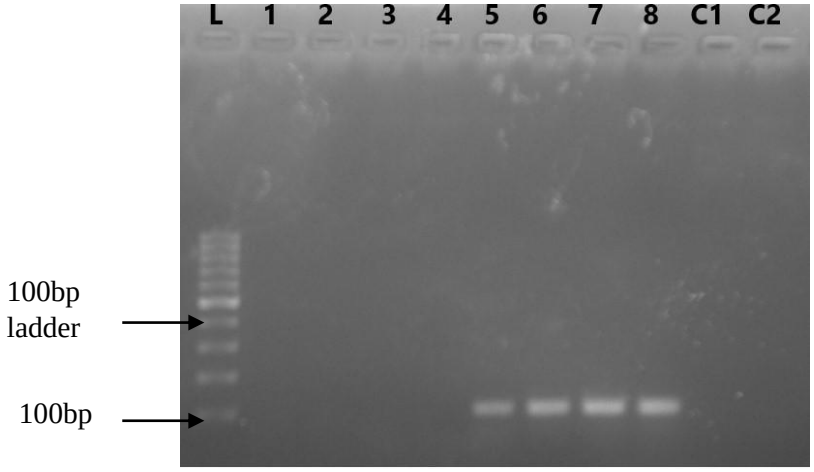


Figure 17: Gel picture of amplified cDNA product with aflP and aflS genes L=100bp ladder, Lane 1,2,3, 4: aflS gene at 7h,12h and 24h resp., Lane 5,6,7,8: amplified aflP gene at 7h, 12h and 24h resp., C1 and C2: Control

5.6.4 Real-time PCR for selected aflatoxin pathway genes

Table 13: Ct (cycle threshold) value of selected genes wrt tubulin

Time period	Genes				
	Tubulin	**aflD**	**aflR**	**aflP**	**aflS**
7h	21.39	26.3	26.9	22.83	28.7
12h	20.75	24	26.1	24.57	29.8
24h	20.46	22.9	23.5	22.8	23.8

Table 14: Normalized C_t values of selected genes wrt tubulin

	aflD	**aflR**	**aflP**	**aflS**
7h	4.91	5.51	1.44	7.31
12h	3.25	5.35	3.82	9.05
24h	2.44	3.04	2.34	3.34

From the normalized C_t value data, following are the interpretations:

1. At 24 hour time period (mycelia stage), aflD gene was found to be up-regulated than at germinating tube and hyphae stage.
2. The aflP gene was up-regulated at the germinating tube stage (7 hours), stating its role in melanin biosynthesis pathway.
3. The regulatory genes, aflS and aflR controlling the formation of secondary metabolite formation, were up-regulated at mycelia stage.

CHAPTER 6

DISCUSSION AND CONCLUSION

Contamination of food products by microbes like fungi occur where adequate storage facilities are not available having hot and humid conditions, especially in developing countries. Different types of fungi like *Penicillium*, *Rhizopus*, *Aspergillus* etc. are found to feed on a variety of foods like apples, wheat, grains and nuts with *A.flavus* being the most notorious responsible for secreting deadly toxins rendering food crops waste and on ingesting, causing mycoses in people with weak immune system and in-tact immune system alike. The genes for production of secondary metabolites in general are linked to each other.

Aflatoxin produced by *A.flavus* and *A.parasiticus* was found to be produced after the conidial germination occurs. Around 25 genes have been identified to be involved with the synthesis of aflatoxin like regulatory aflR and aflS gene, noranthrone synthase, polyketide synthase, from α and β(sub units) of"sterigmatocystin fatty acid synthase to nonribosomal peptide synthetase 10 as well as noranthrone monooxygenase. In *A.flavus*, MAPK"signalling pathway has been observed which may be involved to make proteins responsible for oxidative stress and ultimately, to synthesis of aflatoxin. On treatment of the fungus with plant-based substances like quercetin, there was as switch to cAMP-signalling pathway and inhibition of the enzyme pksA. The first product which is stable among the other products of the production pathway is norsolorinic acid and when this is stopped from being formed, no further synthesis of proteins takes place to carry on the synthesis of the aflatoxin, which are of great concern.

The entire idea of the project was to identify, isolate and characterize the different types of spoilage fungi present in the environment that contaminate food products like grains, fruits and vegetables etc. Also, to check the expression of four selected aflatoxin pathway genes (aflD, aflR, aflP, aflS) during the morphogenesis of *Aspergillus flavus* (MTCC11866) at three time points - 7 hours, 12 hours and 24 hours obtained from Objective 1.

It was observed that germinating conidia were obtained at 7 hours, followed by more differentiation (hyphae growth) at 12 hours and a dense growth of fungi showing mycelia formation at 24 hours, staining by Lactophenol Cotton Blue. RT-PCR results showed that amplification of aflD, aflR, aflP genes but not aflS at any of the time period which necessitated the need for validation by quantifying the expression by Quantitative Real-time PCR. The aflP gene had a higher expression at spore geminating stage (7 hours) which may be involved in a separate pathway for formation of melanin. Also, the regulatory genes were up-regulated at the mycelia stage (48 hours), showing regulation at later stages of the development and secondary metabolite production in the fungus. Hence, the morphogenesis and metabolism of secondary metabolites (toxins) may be related and further research needs to be carried out to get a greater detailed picture for deciphering the role played by each growth phase.

REFERENCES

1. **Agarwal R, Denning DW, Chakrabarti A** (2014) Estimation of the burden of chronic and allergic pulmonary aspergillosis in India. PloS one 9 (12):e114745
2. **Bennett, J.W.; Papa, K.E.** The aflatoxigenic *Aspergillus* spp. *Adv. Plant Pathol.* **1988**, *6*,265–279
3. **Bennett JW, Klich MA** (1992). Aspergillus: Biology and industrial applications. Boston, Butterworth-Heinemann
4. **Bhetariya P J, Shankar J, Singh Y, Madan T, Varma A, Basir S F and Sarma P U** (2009), "Multiplex PCR for Detection of *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*", *J. Allergy Clin. Immun.*, Vol. 123, No. 2, pp. S160-S160.
5. **Bigelis. R.** (1992) Food enzymes. In: D.B. Finkelstein and C. Ball (editors), Biotechnology of Filamentous Fungi. Technology and Products. Butterworth-Heinemann, Boston, MS, pp. 361 -415
6. **Butler G, RasmussenMD, LinMF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, et al**. (2009). Evolution of pathogenicity and sexual reproduction in eight Candida genomes. Nature 459: 657–662.
7. **Chhokar R** (2001) Development and use of herbicides. Pestic Inf 27:25-27
8. **Cotty, P.** Aflatoxin and sclerotial production by *Aspergillus flavus*: Influence of pH. *Phytopathol* 1988, *78*, 1250–1253
9. **Dagenais T R and Keller N P** (2009), "Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis", *Clin. Microbiol. Rev.*, Vol. 22, pp. 447-465
10. **Filtenborg O., Frisvad J.C, Thrane U**. Moulds in food spoilage. International Journal of Food Microbiology 33 (1996) 855102
11. **Flaherty J.E. and Payne G.A.** Overexpression of aflR Leads to upregulation of pathway gene transcription and increased aflatoxin production in *Aspergillus flavus*. Applied and Environmental Microbiology, Oct. 1997, p. 3995-4000
12. **Frisvad, J.C. and Thrane, U.** (1995) Mycotoxin production by food-borne fungi. In: R.A. Samson, E,.S.Hockstra, J.C. Frisvad and 0. Filtenborg (editors), Introduction to Food-Borne Fungi, 4th edn. Centraalbureau voor Schimmelcultures, Barn. pp. 251 -260

13. **Galvano F, Piva A, Ritieni A, Galvano G** (2001) Dietary strategies to counteract the effect of mycotoxins: a review. J Food Prot 64(1):120-131
14. **Gautam K. Ajay, Sharma S, Avasthi S, Bhadauria R**. Diversity, Pathogenicity and Toxicology of *A. niger*: An Important Spoilage Fungi. Research Journal Of Microbiology 6(3): 270-280,2011
15. **Gautam A. K. and Bhadauria R.** Characterization of *Aspergillus* species associated with commercially stored triphala powder. African Journal of Biotechnology Vol. 11(104), pp. 16814-16823, 27 December, 2012
16. **Hoffmeister, D.; Keller, N.P**. Natural products of filamentous fungi: Enzymes, genes, and their regulation. *Nat. Prod. Rep.* **2007**, *24*, 393–416
17. **Jiujiang Yu**. Current understanding of aflatoxin biosynthesis and future perspective in reducing aflatoxin contamination. Toxins, 2012
18. **Jiujiang Yu, Perng-Kuang Chang, Kenneth C. Ehrlich, Jeffrey W. Cary, Deepak Bhatnagar,Thomas E. Cleveland, Gary A. Payne, John E. Linz, Charles P. Woloshuk,and Joan W. Bennett.** Clustered pathway genes of aflatoxin biosynthesis. Appl Environ Microbiol. 2004 Mar; 70(3): 1253–1262
19. **Klich,M.A**. Identification of common Aspergillus species, CBS, Netherlands (2002)
20. **Klich, M.A.** Soil fungi of some low-altitude desert cotton fields and ability of their extracts to inhibit *Aspergillus flavus*. *Mycopathologia* 1998, *142*, 97–100
21. **Klich, M. A., E. J. Mullaney, C. B. Daly, and J. W. Cary.** 2000. Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ochraceoroseus*. Appl. Microbiol. Biotechnol. 53**:**605-609
22. **Kumar D and Kalita P**. Reducing Postharvest Losses during Storage of Grain Crops to Strengthen Food Security in Developing Countries.
23. **Lin X., Alspaugh J.A., Liu H., Harris S**. Fungal Morphogenesis.Cold Spring Harb Perspect Med doi: 10.1101/cshperspect.a019679z
24. **OBrian Gregory R., Fakhoury A.M., Payne G.A**. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus prasiticus.* Fungal Genetics and Biology 39 (2003) 118–127
25. **Obrian G, Georgianna DR, Wilkinson JR, Yu J, Abbas HK, Bhatnagar D, Cleveland TE, Nierman W, Payne GA** (2007) The effect of elevated

temperature on gene transcription and aflatoxin biosynthesis. Mycologia 99:232–239

26. **OBrian, G.R.; Georgianna, D.R.; Wilkinson, J.R.; Yu, J.; Abbas, H.K.; Bhatnagar, D.;Cleveland, T.E.; Nierman, W.C.; Payne**, G.A. The effect of elevated temperature on gene transcription and aflatoxin biosynthesis. *Mycologia* **2007**, *99*, 232–239
27. **Palencia R.E, Hinton M.D., Bacon W.C.** The Black *Aspergillus* Species of Maize and Peanuts and Their Potential for Mycotoxin Production.Toxins (Basel) 2010 Apr; 2(4): 399–416
28. **Puel O.,Galtier P.,Oswald I.P**.Biosynthesis and toxicological effects of patulin. Toxins(Basel). 2010 Apr; 2(4): 613–631.
29. **Shankar J, Nigam S, Saxena S, Madan T,and Sarma P U** (2004), "Identification and assignment of function to the genes of *Aspergillus fumigatus* expressed at 37 degrees C", *J. Eukaryot. Microbiol*., Vol.51, pp. 428-432.
30. **Shankar J**. An overview of toxins in Aspergillus associated with pathogenesis. *Int. J. LifeSc. Bt & Pharm. Res. 2013*
31. **Shankar J., Tiwari S., Shishodia S., Gangwar M,, Hoda S, Thakur R. and Vijayaraghavan P.** (2018) Molecular insights into development and virulence determinants of Aspergilli: A proteomic perspective. Front. Cell. Infect. Microbiol. 8:180 | doi: 10.3389/fcimb.2018.00180
32. **Tiwari Shraddha, Shankar J.** Integrated proteome and HPLC analysis revealed quercetin-mediated inhibition of aflatoxin B1 biosyntheis in Aspergillus flavus. 3 Biotech (2018) 8:47
33. **Van Egmond HP, Jonker MA** (2005) Worldwide regulations on aflatoxins. Aflatoxin and food safety. CRC Press, London, pp 77–94
34. **Yu J, Whitelaw C A, Nierman W C, Bhatnagar D and Cleveland T.E** (2004b),"*Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops", *FEMS Microbiol. Lett*., Vol. 237, pp. 333-340