EVALUATION OF PROTEINS INVOLVED IN GERMINATION OF TOXIGENIC *ASPERGILLUS FLAVUS* CONIDIA AND STUDIES ON PHYTOCHEMICALS AS ANTI-AFLATOXIGENIC AGENTS

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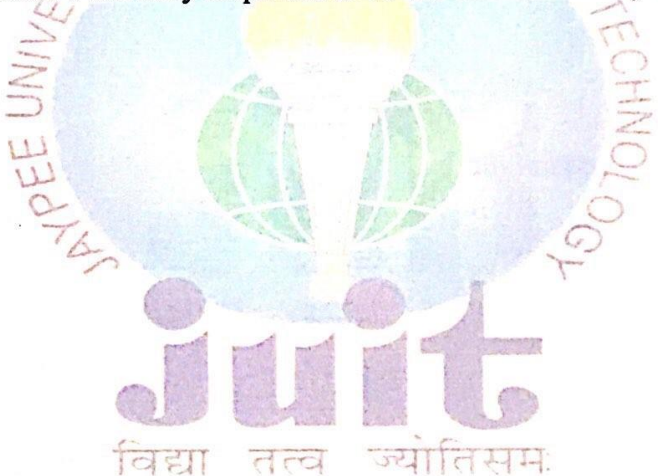
I hereby declare that the work reported in the Ph.D. thesis entitled ***"Evaluation of proteins involved in germination of toxigenic Aspergillus flavus conidia and studies on phytochemicals as anti-aflatoxigenic agents"*** submitted at **Jaypee University of Information Technology, Waknaghat, India,** 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. I am fully responsible for the contents of my Ph.D. Thesis.



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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "***Evaluation of proteins involved in germination of toxigenic Aspergillus flavus conidia and studies on phytochemical as anti-aflatoxigenic agents***" submitted by **Ms. Shraddha Tiwari** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of his original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Dedicated to my Sister

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LIST OF ABBREVIATIONS AND SYMBOLS

List of abbreviations used in alphabetical order

2-DE	2 dimensional electrophoresis
AAA	Aeromatic amino acid
ACP	Acyl carrier protein
AF	Aflatoxin
AT	Adenylyl transferase
BCAA	Branched chain areomatic acid
cAMP	Cyclic adenosine mono phosphate
CF	*Aspergillus flavus* cultured in corn flour
CF_Q	*Aspergillus flavus* cultured in corn flour media with quercetin
CFU	Colony forming unit
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1ropanesulfonate
CYC	Cyclase
DH	Dehydratase
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
DTT	DI thio thretiol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme kinked immune sorbent essay
ER	Enoyl reductase
EU	European union
FAO	Food and agricultural organization
FDA	Food and drug administration
GO	Gene ontology
GOSC	Gene ontology slim categories
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immune virus
HPLC	High performance liquid chromatography
IACR	Indian agricultural institute of cancer research
ICRISAT	International council of agricultural research in semi-arid trophics
IIT	Indian institute of technology
ITS	Internal transcribed spacer
KEGG	Kyoto encyclopedia of gene and genome
KR	Keto acyl reductase
KS	Keto acyl synthase
LCB	Lacto phenol cotton blue
MAPK	Mytogen activated protein kinase
MIC	Minimum inhibitory concentration
MS	Mass spectroscopy
MT	Methyl transferases
MTCC	Microbial type culture collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight

NCBI	National centre of biotechnology information
NOR	Norsolinic acid
NRPS	Non reducing polyketide synthase
OD	Optical density
PBST	Phosphate buffer saline with tween 20
PDA	Potato dextrose agar
PKA	Protein kinase A
PKS	Polyketide synthase
PLGS	Protein lynux global services
PMSF	Phenyl methyl sulphonyl floride
QTL	Quantitative trait loci
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
SDA	Saboraoud dextrose agar
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TCA	Trichloroaceticacid
TLC	Thin layer chromatography
TOF	Time of flight
UPLC	Ultra high performance liquid chromatography
UV	Ultraviolet radiations
VERB	Versicolorin B

List of symbols used in order as they appear first

µ	Micro
α	Alpha
β	Beta
º	Degree
C	Celcius
kg	Kilogram
ml	Millilitre
L	Liter
h	Hour
%	Percentage
$	Dollars
g	Gram
Da	Dalton
Å	Angstrom
min	Minutes
ng	Nanogram
ppm	Parts per million
ppb	Parts per billion

ABSTRACT

Aspergillus flavus is widely present in the environment, having small asexual bodies known as conidia. These conidia swell to form germ tube which develops into hyphae and ultimately forms mycelia. It is main contaminants recognized as a potent aflatoxin producer (carcinogenic secondary metabolite) which is also responsible to cause aflatoxicosis in humans and animals. Germination is the key event in *A. flavus* life-cycle, which needs further exploration to develop effective antiaflatoxigenic compounds and to identify key antiaspergillus targets. Enormus studies have been conducted at *A. flavus* mycelial stage, which includes both proteomic and transcriptomic approaches, suggesting *A. flavus* is potent mycotoxin producer at mycelia phase. However, there is a huge research gap for different morphological conditions at proteomic level to develop novel antiaflatoxigenic and antiaspergillus compound. Thus in our first objective of current study, toxigenic strains of *A. parasiticus* (MTCC8189) and *A. flavus* (MTCC11866) were treated with four different phytochemicals viz. Gallic acid, Ascorbic acid, Caffeine, Quercetin via Poisoned food plate technique and MTT (3-(4,5-Dimethylthiazole-2-yl)-2,5-Diphenyltetrazolium bromide) assay, ranged between 1-400 µg/ml. All the phytochemicals showed considerable downfall in growth in comparison with control, but the inhibitory effect of quercetin was found to be strongest in both *Aspergillus* species with MIC_{50} of 36 µg/ml and 113 µg/ml in *A. parasiticus* and *A. flavus* respectively. Our data suggested that quercetin is the potent inhibitor of *Aspergillus* species, hence could be a promising biological agent as antiaspergillus compound. The second objective was to understand the germination mechanism of *A. flavus* (germ tube stage; 7 h) using proteomic approach. Functional classification of *A. flavus* proteome revealed majority of proteins from cell wall biosynthesis, metabolisms processes (amino acid and carbohydrate metabolism) and translation. Proteins/enzymes from secondary metabolite production (AF biosynthesis) were observed, which suggested the activation and expression of AF biosynthesis during early germination time-points. Dicer-like proteins (1 and 2) mediating post-transcriptional regulation and autophagy proteins (13, 2, 11, 22, 9, 18) were also expressed. MAPK signalling pathway associated protein/enzyme were also observed during the germination. These finding could also be applied to other *Aspergillus* species. The third objective was to understand the mechanisms of phytochemical (quercetin) mediated AF biosynthesis inhibition in *A. flavus* with proteomic as well as HPLC approach. Thus, *A. flavus* cultured in corn flour (CF) and *A. flavus* cultured in corn flour with quercetin (CFQ) revealed expression of 843 and 705 proteins, respectively. Gene Ontology Slim Categories (GOSC) of CF exhibited major transcriptional factors; involved in acetylation and deacetylation of histone proteins, carbohydrate metabolism and hydrolase activity, whereas, GOSC analysis of CFQ showed membrane transport activity, including both efflux and influx proteins. cAMP/PKA pathway was

expressed in CFQ whereas MAPK pathway in CF. Comparative expression of AF biosynthesizing enzyme revealed the downregulation of polyketide synthase A (PksA) and inhibition of major enzymes such as norsolinic acid, noranthrone synthase, NOR reductase etc. in CFQ against CF, which suggested quercetin mediated AF inhibition. Further, AF B1 (AFB1) quantification in CF and CFQ was performed with HPLC analysis at 7 h, 12 h, 24 h and 48 h showed decrease in AFB1 (1%) at 7 h to 24 h in CFQ and (51%) at 48 h time point was observed, which raises possibility to use quercetin as an anti-aflatoxigenic agent. Last objective was to understand quercetin mediated inhibition of AF biosynthesis at molecular level. Thus *in silico* interaction study with *A. flavus* PksA was undertaken. The protein structure of PksA domains (seven) was modeled using SWISS-MODEL server and docking was achieved using Autodock tools-1.5.6. Docking energies of hexanoic acid and quercetin were compared to PksA domains. Binding energy for quercetin was lesser that ranged from -7.1kcal/Mol to -5.25kcal/Mol with respect to hexanoic acid (-4.74 kcal/Mol to -3.54kcal/Mol). Ligplot analysis showed formation of 12 H-bonds in case of quercetin and 8 H-bonds in hexanoic acid. Interaction studies of acyl-transferase domain showed H-bond formation at Arg63 position. Also, in product template (PT) domain quercetin creates four H-bonds and one in hexanoic acid. Overall results showed that quercetin exhibited the maximum level of binding potential with PksA domain against hexanoic acid, thus quercetin possibly inhibits via competitively binding to the domains of PksA concerned to AF biosynthesis. Thus our study paved way to identify molecular mechanism in *A. flavus* germination and suggested the quercetin may be explored as antiaflatoxigenic and antiaspergillus compound against aflatoxicosis in future.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The biological machinery of pathogenic or non pathogenic microbes is complex due to biosynthesis of macromolecules required for several biological functions and for their association with host-pathogen interactions. *Aspergillus* species is well-known producer of mycotoxin such as carcinogenic compound, a leading causing of contamination or disease in plants/animals. Also, fungal diseases such aspergillosis is predominant in various incidences of HIV, cancer patients and immunosuppressive therapies, which increases the mortality rate in infected human. *A. fumigatus*, *A. flavus*, *A. terrus* are the major species responsible for aspergillosis. *Aspergillus* species exists in different morphological stages in their life-cycle viz. conidia, mycelia and hyphae. Recently, trasncriptomic approach as been used to reveal molculae mechanism during these morphological stages. However, transcriptomic approach has certain limitations, as transcriptomic studies reveal the partial picture of exact mechanism of events occurring, in addition, also depends on post transcriptional regulations. Hence proteomic study is essential to contribute the innovation in biological science of microbes. However limited set of proteomic studies are available on *Aspergillus* species during different morphological stages or during the interaction with antifungal or phytochemicals. With the accessibility of annotated proteome of several pathogenic and non-pathogenic *Aspergillus* species (*A. fumigatus*, *A. parasiticus*, *A. flavus*, *A. terrus*, etc.) and experimental limited protein dataset, efforts are now possible to comprehend the biological as well as molecular mechanism of *Aspergillus* biology. However, it is to note worthy that identification of proteins and pathways at different fungal stages of germination will allow discovering novel strategies for drug development to combat *Aspergillus* related disease. *A. flavus* not only contribute to aspergillosis but also a major contributer for aflatoxin (AF) production in food crops. Till now mycelia stage is known to be AF producing stage in *A. flavus* but the protein data on transition from conidia to mycelia of *A. flavus* is limited. So, identifying the unique proteins and active biochemical and metabolic activities at different germination stages could be crucial for antimicrobial agent against

Aspergillus species. Hence, these studies on different morphotypes of *Aspergilli* using proteomic approach may lead to determine common metabolically active processes during the transition from dormant to germinating conidia, and may be a target for antimicrobial agents. In addition it will provide the data on how conidia exit the dormancy. Further, hazardous factors such as chronic and acute poisoning caused by several fungicides and pesticides are major problem in various developing countries. Antifungal agent such as azoles and polyenes targets biosynthesis of ergosterol whereas, echinocandins acts via cell wall biosynthesis inhibition. These literature reviews suggested that fungal cell wall is the crucial target for antifungal drugs. Recent epidemiogical data revealed that the fungal species are emerging with azole-resistance which raises concern for medical community. Plant extracts such as phytochemicals contain antimicrobial properties, though extensively studied, still not practiced commercially. Hence, calls for a sustainable approach to search phytochemicals having anti-*Aspergillus* or anti-aflatoxigenic properties which are inexpensive and eco-friendly against AF production. This approach could help to prevent the spoilage of post-harvest crops due contamination of mycotoxin. Also, it is equally important to understand mechanism of action of phytochemicals on *Aspergillus* as anti-*Aspergillus* and anti-AF compound for developing strategies against AF contamination. Increase incidence of *Aspergillus* induced contamination/disease in recent years have developed the thrust to understand the events of *A. flavus* germination, expression of enzymes in mycotoxin biosynthesis during different germination stage, empirical antifungal therapies, and identification of biomarkers for developing new, safer, specific and effective novel group of antifungal drugs. With this in view, looking in the advances in proteomics and availablility of highthroughput techniques, the current work on "**Evaluation of proteins involved in germination of toxigenic *Aspergillus flavus* conidia and studies on phytochemical as anti-aflatoxigenic agents**" was undertaken with following objectives.

Objective 1: Screening of phytochemicals (Ascorbic acid, gallic acid, caffeine and quercetin) against aflatoxin producing *Aspergillus* species and determination of MIC_{50}

Objective 2: To elucidate the proteins/enzymes of biochemical pathways of *Aspergillus flavus* conidia during germination

Objective 3: To understand the mechanism of action of quercetin mediated inhibition of aflatoxin biosynthesis in *Aspergillus flavus* using proeomic approach

Objective 4: Structure-function analysis of interaction of quercetin and hexanoic acid with domains of polyketide synthase A (PksA) of *Aspergillus flavus*

Review of Literature

1.2.1 *Aspergillus flavus*, an opportunistic fungus

Aspergillus genera is a ubiquotus and saprophytic fungi known to have more than 500 species, that grow on a variety of organic materials and facilitates to recycle carbon and nitrogen by decomposing dead organic debris [1]. Link (1809), reported *A. flavus* as second most predominant *Aspergillus* species after *A. fumigatus*. Biological nomenclature of *A. flavus* is described in Table 1.1. *A. flavus* is asexual species as it bears conidia. There are various important food crops which are prone to *A. flavus* responsible to cause eat rot in maize, yellow mold in peanuts and cottonseeds [2-4]. The ability of *A. flavus* to cause infection highly depends on stress factors such as drought stress, damage in plant parts and seeds, osmotic stress, oxidative stress etc. [5]. Another important feature of *A. flavus* is the capability to produce AF which is a carcinogenic secondary metabolite [2]. It also mediates the biosynthesis of cyclopiazonic acid and aflatrem, other class of secondary metabolites [6, 7]. AF causes aflatoxicosis (leads to liver cancer) and invasive growth (aspergillosis) resulting from inhaling or ingesting AF contaminants in larger amount which is frequently lethal in immunocompromised humans [7]. Also, the developing countries of Africa and Asia are prone to aflatoxin related diseases. Recently, outbreak in Kenya (Africa) has been seen after consuming AF contaminated maize [8]. Further, the emergence of drug resistance *Aspergilli* pose great threat to human beings [9, 10]. Hence, *A. flavus* being an agricultural and medical threat become an important fungus to take into consideration. The broad morphological and cultural characteristics, significant stress-tolerance chemistry, capability to penetrate host immune system and colonization or damage to host-tissues, development of resistance against antifungal drugs and other aspect of its eco-physiology collectively makes *A. flavus*, a thriving pathogen [11].

Table 1.1 Biological nomenclatures of *Aspergillus flavus*

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

1.2.2 Ecology and geographical distribution

Apergillus flavus is known to be scattered worldwide both in geography and ecology. This is due to the consequences of *A. flavus* bearing conidia which are airborne and effortlessly disperse with the aid of air moves and insects [12]. Composition of the atmosphere has an exquisite effect on *A. flavus* boom and humidity being maximum crucial variable [13]. Water activity (a_w) within the range of 0.86 to 0.92 and temperature of 37ºC are the optimum conditions for growth of *A. flavus* [14]. However, *A. flavus* increase can be established at different temperature range (12 to 48ºC) which suggests the thermotolerant nature of fungi. In post harvested crops (stored in humid conditions) *A. flavus* produce rots, which suggests that for *A. flavus* pathogenesis, it required humid and hot environment [10]. The capability of *A. flavus* to continue to exist in harsh situations mediates competitive inhibition of other organisms in plants for different substrates [15]. *A. flavus* is highly prevalent in tropical countries [16, 17]. Studies on the distribution of *A. flavus* showed that air of Barcelona was highly contaminated with mostly two types of *Aspergillus* species viz, *A. niger* and *A. flavus* [18]. However, survey in Madrid showed that vital species occurring in the environment was *A. fumigatus*, about 54% [19]. In southern France *A. versicolor* and *A. glaucus* was the predominant *Aspergillus* groups [20]. *A. flavus* was identified as most frequently encountered *Aspergillus* species, isolated from environment of houses and

hospitals in Iran [21]. Additionally**,** various studies showed occurance of *A. flavus* in drinking water [22, 23].

For India, because of the climate variations (50 °C in Rajasthan, hot and humid summer afternoon in Punjab and Uttar Padesh and −40°C in Cargill) causes excessive warming and floods and making friendly enviroment for colonization of *A. flavus*. The *A. flavus* colonization on sutaible substrate results in AF contamination [24]. In India, the phrase 'aflatoxicosis' had has seemed in public information domain in the 1960s when the 2219 chicks were found dead in Mysore poultry farms in Karnataka [25]. Later in 1974, *A. flavus* has been detected as the foremost fungus in contaminating maize crop which later leads to hepatitis in tribal population of Gujarat and Rajasthan. Later, 106 deaths were reported due to hepatitis in 150 villages, because of consumption of AF contamated maize. The analysis confirmed the causative agaents for maize contamination by AF was *A. flavus* [26]. In north-western India various dogs and humans were affected by jaundice, which was the result of toxic hepatitis [27]. AF (produced by *A. flavus*) related outbreaks were also seen in some parts of Andhra Pradesh in 1994, leading death of more than two billion broiler chicken [391]. Ramesh et al. have analyzed the pervelance of *A. flavus* in Chennai [28].

1.2.3. Morphology

Aspergillus bears a compex taxonomy which is ever evolving. *Aspergillus* genus is by far recognised with the aid of its feature conidiophore, however identification and distinction of species is difficult [29]. Macro-morphological characters which might be taken into consideration encompasses color of mycelia and conidia, reverse color of colony of germinated *Aspergillus* species, colony diameter, pigment analysis and occurance of sclerotia. Micro-morphology characters may depend on vesicle size, vesicle shape, seriation, conidia morphology, and morphology of ascospores [30]**.** *A. flavus* is found in colonies which bear yellow to green spores and golden-reddish from lower part known to exist as powdery mass. *A. flavus* exists in three different morphological stages viz., conidia, hyphae and mycelia. Mode of reproduction in *A. flavus* is generally asexual. However, sexual mode of reproduction (development of ascospores inside sclerotia) in *A. flavus* has been recently accounted [31]. *A. flavus* reproduces mostly by asexual mode by reproductive bodies known as conidia. *A. flavus* conidia are small in size generally ranged from 2-5 µm. Conidia are metabolically less active and stay dormant both in air and water which requires triggering

mechanism for germination, which involves utilizing of amino acids, sugars and organic salts [32, 33]. Triggering of conidia leads to isotropic growth, which caused conidial swelling resulting self adhesion, followed by polarization mediated germ tube formation. From germ tube the protburance of hyphae takes place, which is a branched structure and produce mycelia. Mycelia are the fulley developed structure of *A. flavus*, which harvests complex nutrients from different sources. *A. flavus* is known to reproduce asexual spores known as conidiospores (colorless and rough). *A. flavus* bears each type of phialides that is uniseriate and biseriate. Classification of *A. flavus* has conducted depending on sclerotia size that is L strains (sclerotia greater than 400 µm) and S strains (sclerotia less than 400 µm). Both the strains mediate biosynthesis of AFB1 and AFB2 [34]. Morphological representation of *A. flavus* (MTCC9367) is given in Figure 1.1.

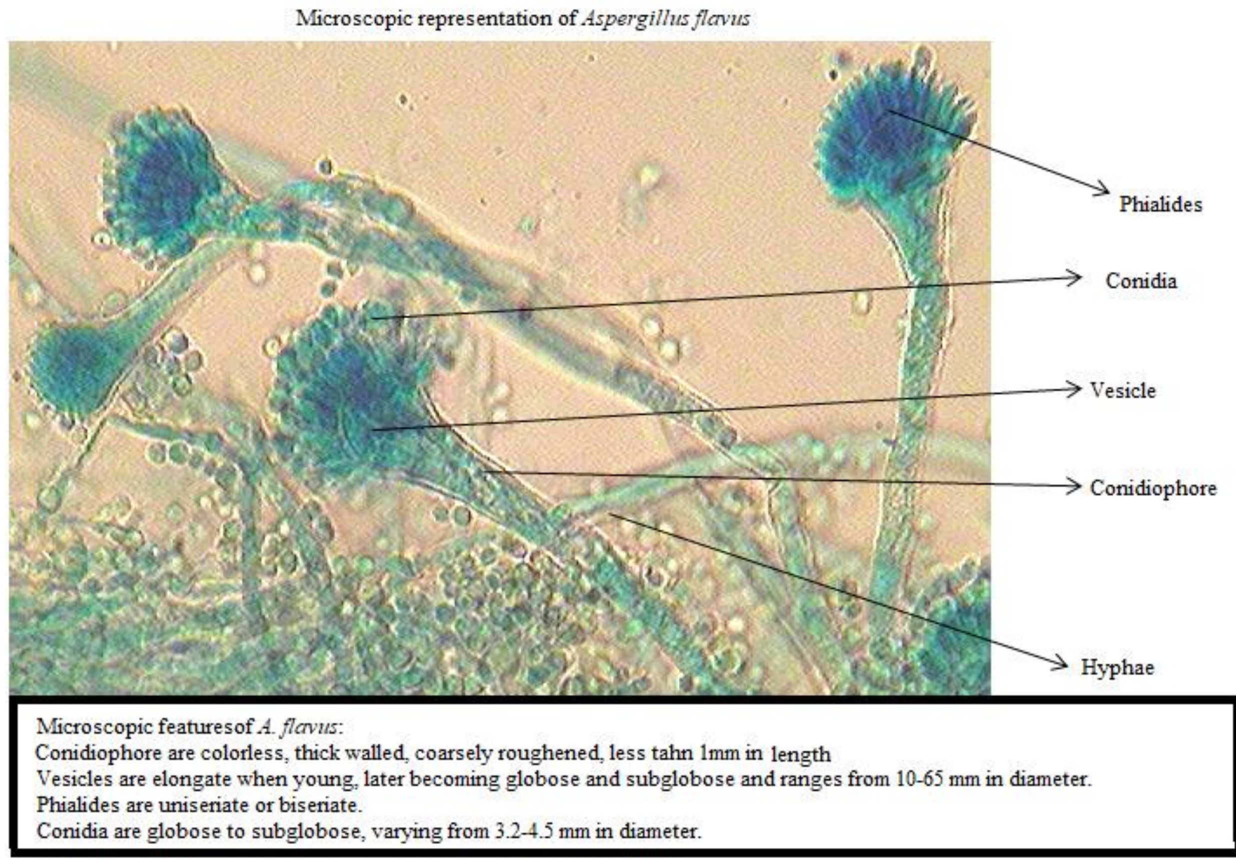


Figure 1.1 Lactophenol cotton blue staining and microscopic representation of *A. flavus* (MTCC9367) mycelia

1.2.4 *Aspergillus flavus* as a model organism

1.2.4.1 Genomic studies on *Aspergillus flavus*

Genomic studies on *A. flavus* have been widely accomplished, and the entire sequence of genome is available in the genomic databases [35-38]. Till now genome sequence of 8 *Aspergillus* species is available, which pave a way for comparative analysis and post genomic researches in *Aspergilli* [11, 39]. Earlier, *A. flavus* was known to bear 7218 unique ESTs from total 19,618 identified ESTs [35]. Later (2005), J. Craig, an eminent researcher from Venter Institute released the entire genome sequence of *A. flavus* [392]. *A. flavus* is about 36.8 Mb in genome size with about twelve thousand anticipated functional genes (54, 234). *A. flavus* genome size barely larger in comparison to other *Aspergillus species*, such as *A. terreus* (30 Mb), *A. fumigatus* (30 Mb), *A. nidulans* (31 Mb) and *A. niger* (34 Mb) [36, 37, 40, 41]. The reason of size difference is due to presence of more copies of specific genes [42]. Inspite of difference in genomic size of *Aspergillus* species, all the species have 8 chromosomes [42, 43]. Karyotyping of *A. parasiticus* and *A. flavus* showed both the fungus shares similar genome [44]. *A. flavus* can be distinctive from different *Aspergillus* species based on its internal transcribed spacer (ITS) region (ITS1 and ITS2) which is located between the 18s and 28s rRNA transcribed region and offers identification sensitivity to *Aspergillus* species but cannot be distinguishes on the basis of 18S rRNA sequence [45]. *A. flavus* telomeric repeat sequence is TTAGGGTCAACA which is similar to *A. oryzae* telomeric sequence, but differs from other filamentous fungi (TTAGGG) [46, 47]. The afltoxin biosynthesis gene cluster (75 kb) of *A. flavus* contains hallmark genes which includes polyketide synthases (25), PKS-like synthases (3), nonribosomal peptide synthases (18), NRPS-like synthases (14), prenyltransferases (8) and PKS-NRPS enzymes (2) [35, 48].

1.2.4.2 Proteomic studies on *Aspergillus flavus*

To understand the convolutions of biological functions, expression of genes and interaction mechanism of gene products, proteomic research could be an assest. In latest years various proteomic studies were conducted on different *Aspergillus* species for the identification and development of diagnostic and therapeutic targets [49-51]. Transcriptional studies assist us to identify various biological mechanisms in the terms of expression of genes. They

additionally facilitate us to know the interactions between host and pathogens throughout in-vitro and in-vivo studies. The drawback of mRNA studies is that it cannot offer the entire image at protein level (function unit). Thus it is far crucial to review the expressed protein, produced after post translational modification at specific condition. There are few proteomic studies which have been conducted on *A. flavus*. In 2013, Pechanova et al. showed the expression of *A. flavus* mycelial proteins using 2DE and MALDI-TOF-MS/MS analysis and found 581 uniquely expressed proteins which showed AF biosynthesis enzymes [52]. Proteomic studies on the *A. flavus* secretory proteins were conducted by Medina et al. in three different growth conditions using 2-DE and also MS/MS, which resulted into 51 uniquely, expressed proteins [53]. GO studies showed that secretory proteins were majorily concerned to proteolysis, electron transport, carbohydrate metabolism and peptidolysis. Another research on secretomes of *A. flavus* showed the expression of 73 secreted proteins which majorily involved proteins of rutine degradation pathway [54]. To understand the effect of post transcriptional regulation in *A. flavus* proteome, Bai et al showed expression of 2823 proteins from 3886 total identified proteins, which suggested that post-transcriptional regulation is a crucial event in protein related product formation [55]. Temperature is an important factor in the *A. flavus* development. There are proteomic and transcriptomic studies which have been conducted on different temperature conditions and the results revealed that at 28°C various proteins and transcripts were found to be upregulated [55]. Along with temperature, activity of water (a_w) is also the key factor in the development of *A. flavus* and also mediates secondary metabolite biosynthesis. Proteomic studies have been conducted on *A. flavus* in different a_w conditions using iTRAQ method. Results showed differential expression of 837 proteins from a total of 3566 *A. flavus* proteins. Increase in a_w showed increased hydrolase activity, which may be possibly linked to biosynthesis of AF in *A. flavus* [56]. In the recent MS proteomic studies on *A. flavus* (isolated from infected cornea) showed total of 637 exoproteome which showed hydrolase proteins were predominant exoproteome [57].

1.2.5 Secondary metabolism; Aflatoxin production

1.2.5.1 Chemistry of aflatoxins

AF are the chemical compounds produced by mostly two types of *Aspergillus* species specially, *A. flavus* and *A. parasiticus* [58]. AF are the difuranocoumarin derivatives that are produced with the aid of polyketide pathway. Majorily AF can be distinguished into four types (B1, B2, G1 and G2), which can be distinguished on the basis of fluorescence [59]. The UV radiations require to fluorescence AF is 365 nm. B indicates blue fluorescence and G indicates green fluorescence in UV light and 1 and 2 indicating the relative distance of migration on a TLC plate [60]. There are two other class of AF (M1 and M2), which are generally found to be present in the milk produced by lactating animals [61]. The precursor molecule in AF biosynthesis is versiconal hemiacetal acetate, which is common for all types of AF [62]. The molecular characterization of different AF and there three-dimensional structures is listed in Table 1.2. *A. flavus* is a predominant producer of AF and is known to produce AFB1 and AFB2. In several studies on *A. flavus* four other AF such as M1, M2, B2A and G2A were also isolated successfully, which suggests *A. flavus* can produce different categories of AF. Among all the categories of AF AFB1 is the most carcinogenic one [63]. Chemically AFB1 is yellow to pale-white crystalline and odorless compound. AF are soluble in different solvents such as acetone, acetonitrile, methanol and chloroform. The potency of carcinogenicity, mutagenicity and toxicity of AF are **AFB1 > AFG1 > AFB2 > AFG2** which is determined by LD50 values [64].

Table 1.2: Molecular characterization of different aflatoxins produced by *Aspergillus* species

Aflatoxin	Molecular formula	Molecular weight (Da)	Melting point	Fluorescence under 365 nm	Structure
AFB1	$C_{17}H_{12}O_6$	312	268-269	Blue	
AFB2	$C_{17}H_{14}O_6$	314	286-289	Blue	
AFG1	$C_{17}H_{12}O_7$	328	244-246	Yellow green	
AFG2	$C_{17}H_{14}O_7$	330	237-240	Yellow green	
AFM1	$C_{17}H_{12}O_7$	328	250-299	Blue	
AFM2	$C_{17}H_{14}O_7$	330	181-184	Blue	

1.2.5.2 Aflatoxin biosynthesis pathway in *Aspergillus flavus*

Genomic collection of *A. flavus* incorporates characteristic enzymatic genes which are related which biosynthesis of secondary metabolites.These enzymes, at the site of different cluster enzymes are known to produce dimethylallyl transferase which is generally termed as polyketides [65]. AF biosynthesis cluster is known to be conserved in different fungal species which includes *A. zhaoqingensis, A. parasiticus, A. rambellii, A. nidulans, A. toxicarius, A. bombycis, A. ochraceoroseus*, *A. pseudotamarii* [66-69]. In *A. flavus* AF are the polyketide which are synthesized using genes clusterd at telomere of chromosome 3 [48]. *aflR* is the regulator of *Zn(II)2Cys6* (transcriptional factor) which is involved in the expression regulation of biosynthetic genes of AF/sterigmatocystin pathway [70]. *aflR* ones overexpressed, mediates the production of AF by activating clusteral genes of AF biosynthesis [71, 72]. Usually, fungal AflR is known to bind at consensus motif (5-TCGN5CGR-3) which is present in the AF/sterigmatocystin genes promoter regions [73]. However, in *A. flavus* found another binding site (5-TTAGGCCTAA), which mediates autoregulation of transcripts of *aflR* [74, 75]. The genes involved in AF biosynthesis pathway are named on the basis of substrate such as norsolinic acid (*norA* and *norB*), averantin (*avnA*), averufin (*avfA*), versicolorin A (*verA* and *verB*). Some genes are classified on the basis of enzymatic functions such as fatty acyl synthases (*fas1* and *fas2*), polyketide synthase (*pksA*), alcohol dehydrogenase (*adhA*), esterase (*estA*), VERB synthase (*vbs*), O-methyltransferase (*dmtA*), O-methyltransferase (*omtA* and *omtB*), oxidoreductase-A (*ordA*), cytochrome P450 (*cypA*), cytochrome P450 monooxygenase (*cypX*), monooxygenase (*moxY*). *A. flavus* genes concerned to AF biosynthesis from early precursors is given in Figure 1.3.

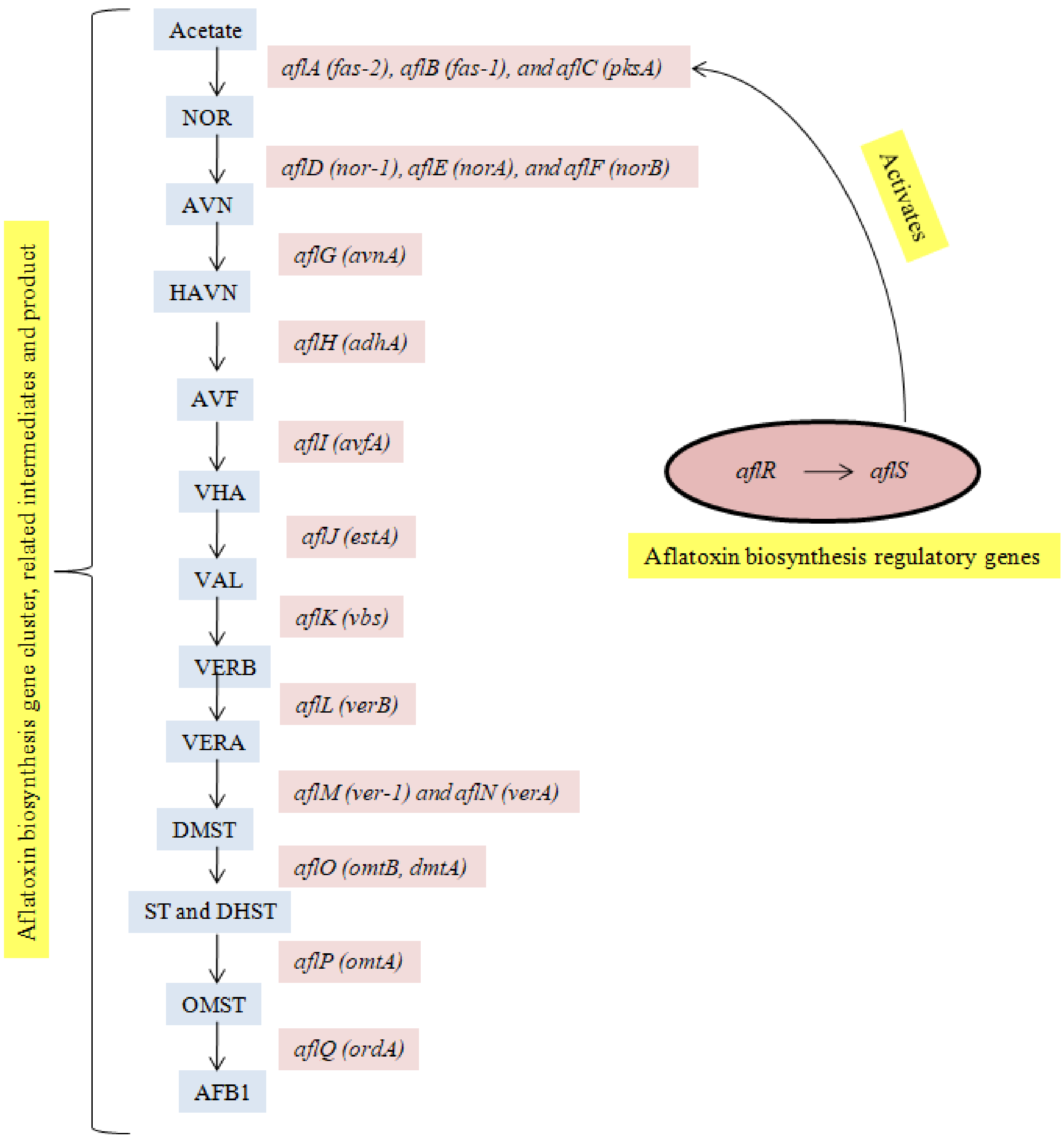


Figure 1.2 *Aspergillus flavus* genes involved in the major conversion steps from early precursors to AFB1

1.2.5.3 Factors affecting aflatoxin production

A latest observation has shown that on a global scale, diseases and pests are migrating towards the poles at the speed of 3 to 5 km per year [76]. *A. flavus* is known to contaminate an extensive form of food commodities which includes oilseeds, maize, groundnuts, spices,

milk, dried fruit and tree nuts [77, 78]. Production of AF depends on various environmental conditions such as rainfall, drought stress, variation in temperature etc. and also on other factors such as agricultural practices and insect damage [79]. The factors involved in contamination of post harvested food crops by *A. flavus* are processing of food, food storage, and transportation. *A. flavus* is known to colonize two major crops peanuts and maize, which are the extreme source of aflataoxins for humans [79]. It has been shown that climate has a great impact on AF contamination as well as *A. flavus* growth, for example, change in concentration of CO_2 also affects the AF productivity rate [80, 81]. Other factors such as availability of water and modifications in temperature have shown various effects in expression of regulatory genes of AF pathway (*aflR* and *aflS*) in *A. flavus* [81, 82]. Studies on effect of temperature on expression of AF biosynthesis genome cluster showed that, temperature above 37°C negatively regulates biosynthesis of AF (AFB1), but does not have any effect on the growth and development of *A. flavus* [83].

1.2.6 *Aspergillus flavus* mediated aflatoxin contamination and related hazards

1.2.6.1 Epidemiology in crops

A. flavus is known to colonize most of the food crops and spices such as peanuts, cottonseeds and maize. For *A. flavus* to cause disease cycle in plants colonization is the major event which requires appropriate environmental conditions (dry and hot) and also the carriers such as birds and insects [84, 85]. According to a survey it has been shown that *A. flavus* contamination on aerial crops (cotton, maize) is predominant than *A. parasiticus* contamination in US [86]. Also, in stored maize *A. flavus* contamination has been reported causing rot in the post harvested crop [87]. Some studies have shown that *A. flavus* also causes peaches rot which was reported in Greece [3].

1.2.6.2 Economic losses

Contamination caused by *A. flavus* does no longer necessarily decreases the product yield, however reasons financial losses via AF contaminated seeds. So it is not wrong if we say that AF is main causative agent of this trouble. It is difficult to estimate the losses occurring from AF contamination because of unawareness about AF contamination in farmers or normal peoples within the developing and underdeveloped nations. Whereas, in USA the

average loss due to AF contamination was approximately $1 billion, representing AF as a major contaminant [88]. US have faced fourty lakh dollars loss annually, only with the AF contaminated cottonseeds in Arizona and seventy lakh dollars in Texas. In Georgia, the AF related loss in groundnut was estimated to be 2 crore dollars annually. In Mississippi losses from AF contaminated corn was estimated to be three crore dollars, which implies the AF related hazards in food crops [89]. However in underdeveloped and developing countries (Africa and Asia), no such data is available but these are more prone to AF contamination in comparison to US, which calls for the awareness in the peoples to understand the AF hazards [90]. A survey on the African exporters of food and related products showed that, due to regulations of EU on food safety measures and not meeting the standards, Africans faced the loss of six hundred seventy million dollars in 2001 [91]. In another survey in Africa, showed no big impact on the export of groundnut, as the groundnut in Africa is used domestically, which lowers the food quality for the local peoples [91].

1.2.6.3 Aspergillosis

Dixon and Walsh defined the term aspergillosis as diseases which are caused due to *Aspergillus* to humans/animals [92]. Next to *A. fumigatus*, *A. flavus* is the second-most opportunistic pathogen responsible to cause aspergillosis (invasive and noninvasive) [4]. Aspergillosis is clinically classified as invasive, allergic, colonizing and saprophytic. Aspergillosis leads to asthma, allergic alveolitis, bronchopulmonary aspergillosis, pulmonary infections [93]. Spores of *A. flavus* are transmitted through primary (respiration) and secondary means (wounds and infection) [94]. In the recent studies in North America it has been shown that *A. flavus* was the predominant fungus known to cause aspergillosis in 65% childrens [95]. Recently, mycotic keratitis (cornea infected with fungi) has shown to be caused by *A. flavus* [96]. Animals together with rabbits, ducks, chickens, turkey and geese are preety vulnerable to aspergillosis as a result of *A. flavus* contamination [97]. *A. flavus* is also known to cause disease in honeybees known as stonebrood disease [98].

1.2.6.4 Carcinogen

From past decades, it has been understood that AFB1 is a potent carcinogen for both animals and humans. Studies showed that AFB1 induces cancer of liver known as hepatocarcinogen and also causes the formation of tumors kidney, colon and lung in both animals and humans. Cancerous effects of AF were shown by Liu & Wu, who revealed that

approximately 4.6% to 28.2% of world's hepatocellular carcinoma (HCC) is due to exposure of AF in emerging countries [99]. The mechanism of AF mediated HCC is studied extensively and is known to implicate p53 protein involved in tumor suppression. AFB1 on interaction with enzymes of mitochondria converts to AFB1-epoxide and AFB1-exo-epoxides which intercalate between DNA bases and integrate itself in the codon 249 of p53 gene, causing mutation of AGG to AGT which is often seen in patients suffering from HCC [100]. HCC cases are predominant in developing countries (East Africa or China) and the rate of occurance of HCC in developed countries is >3% [101]. HCC has lead more than 83% of deaths in sub-Saharan Africa and East Asia [77, 102].

1.2.6.5 Aflatoxicosis

AF produced by *A. flavus* reasons fitness issues to humans/animals referred to as aflatoxicoses. Aflatoxicosis is often a hepatic disorder, which majorily goals liver of humans/animals. For the proper functioning of liver, neurotransmission is important which is mediated by branches chain-amino acids (BCAA; leucine, valine and isoleucine) and aromatic-amino acids (AAA; tyrosine, phenylalanine, and tryptophan). Consumption of AF beyond tolerance limit causes disturbance of BCAA and AAA balance which leads to oedema, hepatic diseases, mental changes, bleeding, jaundice, degradation of heme into bilirubin, etc. [103, 104]. The mechanism of action of AF on liver is represented in Figure 1.4. *In-vivo* studies on laying hens showed that exposure of AF resulted in swelling of liver and also found to lower production of eggs [105]. There are majorily two types of aflatoxicosis viz. acute and chronic aflatoxicosis. Acute aflatoxicosis is caused due to intake of high concentrations of AF in one or some exposures. On the other hand, daily intake of low doses of AF for prolonged time leads to chronic aflatoxicosis [106].

1.2.6.5.1 Acute exposure to aflatoxins

High exposure of AF leads to acute aflatoxicosis in animals/humans which is known to cause oedema hemorrhage, acute liver damage and sometimes death in humans [107]. Factors which are responsible for acute aflatoxicosis in animlas/humans involve environmental factors, limited food and most importantly unavailability of proper systems to monitor and manage AF contamination [108]. Acute aflatoxicosis is majorily caused by the mutation in liver DNA. Also, in some cases acute aflatoxicosis causes liver falure mediated by binding and inactivation of liver proteins [109]. In a case study in Kenya, 12 peoples

were found dead due to acute aflatoxicosis caused by the consumption of contaminated maize [110]. In 1981, more than 100 calves were found dead due to outbreak of acute aflatoxicosis caused in Australia [111]. Again in 2004, 317 peoples were found to be ill, among which 125 of them died due to acute aflatoxicosis [110, 112]. In 2007, a number of animal were found dead in Argentina due to consumption of highly AF contaminated food [113]. Acute aflatoxicosis is known to cause hundreds of death yearly.

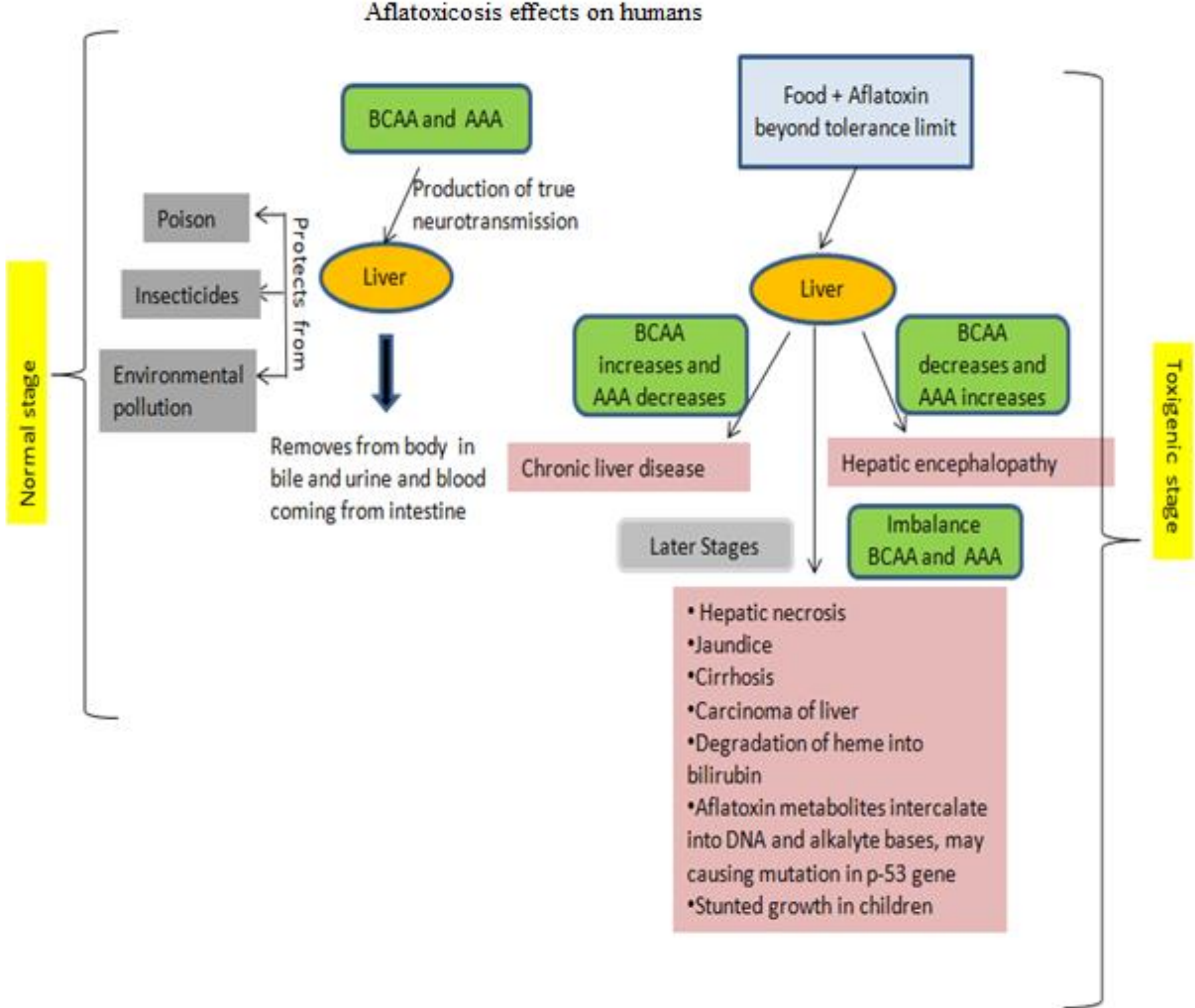


Figure 1.3 Mechnaism involved in aflatoxin related health problems in human lungs

1.2.6.5.2 Chronic exposure to aflatoxins

Chronic aflatoxicosis is the exposure of low dose of AF for prolonged time resulting in the HCC. Chronic aflatoxicosis is found in the in persons suffering from the infection of chronic hepatitis B virus (HBV) [114, 115]. Person suffering from HBV infection may develop 30 folds more chances of liver cancer on consumption of AF [115]. These combinations (HBV-

AF) however observed more frequently in underdeveloped nations [99]. Studies on animal models have confirmed that if exposure of AF is for prolonged time could severe effects immunity (immune suppression), impaired reproductivity, increase in mortality rate, jaundice, anemia and growth inhibition [116]. More than 85% of African childrens showed the detectable levels of AF-albumin adducts in serum or AF in urine [117-119]. Reseach on the breast milk showed the traces of AF (AFM1 and AFB1), confirming the AF contamination in females [120, 121]. Yearly, on a worldwide basis, more than five to six lakhs cases of HCC have been reported from which approximately one lakh instances are on account of AF consumption [99]. Chronic aflatoxicosis is known to cause 90,000 deaths yearly.

1.2.6.6 Stunting and wasting

Stunted growth and wasting are the two disorders which have been accounted for AF related disease in recent years. The sign and symptoms related to stunting in youngesters are cognitive troubles and susceptibility towards infection [122, 123]. According to a survey in childrens, one hundred and seventy million childrens were found to be stunted, which showed underweight related issues. The analysis disclosed the accumulation of 30 to 40 % higher adducts of AF (30-40%) in blood stream of susceptible childrens when compared with the blood of normal children [124, 125]. In Kenya and Gambia, another type of disorder was reported due to exposure of AF known as wasting [126]. These studies showed that, there is a negative correlation between AF and the birth weight, and also found predominantly in females in comparison to males [126, 127]. Hence, it has become important to know the mechanism behind growth impairment caused by *A. flavus* related AF contamination [117].

1.2.7 Regulations

According to a survey of FAO (Food and Agricultural Organization) which states that world’s 25% of food crops are prone to mycotoxin contamination. This leads to the economic loss along with the reduction in the food supply all over the world [128]. Taking into consideration of these hazards, several countrywide and international agencies have set regulatory levels for consumption of AF in more than ninety nine countrie [89]. Levels of regulatory limits show discremenancy from one nation to other. According to the regulations of FDA in United States the limits of AF consumption in human food is 20 ppb

(µg/kg), for milk is 0.5 ppb and for animal feed (cottonseeds and corn) is not more than 300 ppb [129]. However, according to EU, AF consumption by any means are regulated at the level of 4 ppb while in India AF regulatory levels for the entire food products is 30 ppb and in milk for AFM1 and AFM2 is 0.5 µg/kg [130] . Among all set of laws, EU regulations for AF contamination is most stringent in comparison to other regulations globally. AFB1 is the potent carcinogen among all afltoxins hence EU has set limits for AFB1 as 2-12 µg/kg and from 4-15 µg/kg for total flatoxins. According to EU regulations, the conent of AFB1in infant's food should be not above than 0.10 µg/kg and for AFM1 should not be above than 0.025 µg/kg [131]. The comparison of above data from the data of Indian food commodities showed that AF contamination in India is 2 to 7.5 times high in India according to EU regulations [132]. Seeing the hazards related to AFB1 contamination various nations has set certain limits for AFB1 consumption from food which is tabulated in Table 1.3.

Table 1.3 Regulations of AFB1 consumption limits in various countries in various food products (2002)

Country	Aflatoxin B1Permissible limits (µg/kg)
India	30
Zimbabwe	5
Japan	10
Argentina	0
Nigeria	20
Brazil	15
Poland	0
China	10
South Africa	5
Czech Republic	5
Hungary	5

1.2.8 Therapies to control aflatoxin contamination

To reduce or vanish AF contaminations various food safety measure are important to take into consideration. There are various technologies which are available for decreasing contamination tiers at different stages [79]. Some of the strategies that have been implemented in latest years have been discussed below.

1.2.8.1 Physical treatment

Contamination of AF is a global problem which can be primarily controlled upto some extent by physical ways viz roasting, extrusion, boiling, irradiation, cooking, microwave heating, etc. However, inactivation of mycotoxins by thermal stress is not upto mark as they are heat-stable [133]. For degrading AF by means of thermal stress requires certain factors such as humid moisture content, time factors and temperature range. Degradation of AF was found to be achieved at heating (200°C) for prolonged time [134]. Degradation was found more efficient at elevated moisture content [135]. Photosenstivitiy of AF makes it prone towards gamma radiations, UV light and sunlight. In the previous studies AFB1 was removed from different oils (groundnut and olive) using sunlight [136, 137]. Susceptibility of AFB1 has shown to be enhanced in broth in comparison to solid media. Liu et al. showed the reduction of AFB1 mutagenecity and cytotoxicity in broth using UV radiations [138]. However, use of UV radiations for fungal inoculate has found to increase the AF content in broth [139]. Further, ozone treatment has been found to be most effective method to inhibit AFB1 from animal feed upto 90% and also effectively overcomes the mutagenesis and toxic factors, making food contamination free [140, 141].

1.2.8.2 Chemical treatment

Degradation of AFB1 by chemical means is an efficient method but the major drawback is formation of end product also known as degradation product, which may exert some harmful effects. The effect of citric acid has been studied extensively for the inhibition of AFB1, which showed hydrolysis reaction and converts to AFB2a [142]. Acids, such as lactic acid, has been widely studied in AFB1 inhibition which leads to the formation of AFD1, AFB2a and AFB2, showing the incomplete degradation [143]. In other combinational studies to inhibit AFB1, lactic acid provided in combination with the heat stress, showed only one end product that is AFB2a [144]. At higher temperature treatment

with hydrochloric acid had shown the most effective results, as complete destruction of AFB1 [145]. Previous studies showed the effect of other acids (anthranilic, sulphamic, propionic, sulposalicylic, boric, salicylic, oxalic, benzoic) on AFB1 inhibition was found to be more than 90% [146]. Other than acids, alkalis were also used for the inhibition of AFB1, which mediates hydrolysis of lactone ring present in AFB1 [147]. In some studies, AFB1 level was found to be efficiently reduced by using hydrogen peroxide and sodium hydroxide [148]. Sodiun hydroxide was found to reduce the level of AFB1 from contaminated maize upto 93%, which further reversed the effect aflter acid treatment [147]. To reduce the mutagenicity and toxicity of AFB1 it was efficiently converted in non-toxic AFD1 with the help of ammonia [149].

1.2.8.3 Enzymes

Enzymes are another means to control AF contamination. Researchers have used various fungal enzymes for inhibiting AFB1 such as a multienzyme produced by *Armellaria tabescens*, which efficiently destroys AFB1 by indulging in the difuran ring and inhibiting it [150]. Also, *A. flavus* and *A. parasiticus* are known to produce a class of enzyme, peroxidase which contains property to degrade AFB1 and AFG1 [151]. *Raphinus sativa* (plant) specific enzyme, horseradish-peroxidase has also shown AFB1 degradation [152].

1.2.8.4 Host resistance

There are several proteomic and transcriptomic studies which are available on host-pathogen interaction and the expression of certain proteins/enzymes involved in host resistance against AF contamination or as anti-*Aspergillus* [153]. However, this is a promising approach against AF contamination and accumulation. To develop the resistant varieties against AF contamination may involves selection (direct or indirect) of resistant factors against both fungi as well as AF accumulation, which may include morphological trait selection (kernals, ears etc.) and biotic as well as abiotic stresses [154, 155]. Selection of genetic factors which includes resistant germplasm may be useful for the resistance towards *A. flavus* infection and afltoxin contamination. From the advantegous point of view this technique is inexpensive, can able to maintain uniform disease burden, can be phenotypically screened against AF. Other technique is the screening of kernals, which gives the information about resistant germplasm of maize against *A. flavus* colonization and

accumulation of AF [156]. Also there are some studies related to development of human resistance against fungal infections [157].

1.2.8.5 Detection of aflatoxin content by genomic approaches

Today, there are several strategies to detect, quantify and inhibit AF contamination, but it is also important to develop robust and inexpensive throughput techniques for analysis of AF or related genes at different stress conditions on large scale [158, 159]. Also, AF is known to be low level toxins due to their high sensitivity, methods of detection needs to be accurate. For quick analysis of AF in maize the fluorescence assays have been performed which showed blue to green fluorescence, but can not be conducted for quantitative estimation [160]. Recently, International Crop Research Institute of the Semi-Arid Tropics (ICRISAT) an international organization which conducts agricultural research for rural development, headquartered in Patancheru (Hyderabad, Telangana, India) have developed an ELISA technique which is cost-efficient and can be performed inhouse for usual detection and AF quantification [161]. The expenditure of this assay is two to three dollars per sample and can mediated large scale screening in different traits. Further, various genomic approaches has also been implemented to inhibit AF contamination. Another approach is selection of marker that are linked to QTL or genes involved in AF resistance which can be forwarded to breeding programs followed by selection [162]. Also, DH technology (double haploid) is an approach in the development of inbread lines which includes genes of AF resistance and traits of agronomic importance [163]. This approach requires two to three seasons, which is far less time required in breeding techniques. This technique has been used against *A. flavus* and AF contamination and found to be resistant in preharvested stage, in-vitro stage of seed colonization and also in AF production [164]. ICRISAT, India has also undertaken breeding research against AF contamination for the development of AF-resistant pre-harvested seeds [165, 166]. The maize research focus of ICRISAT is development of early mature breeding lines which should be stress tolerant (heat) and resistant to AF accumulation [167]. Other genomic approaches include *in-vivo* mutational studies conducted to identy mutations related to AFB1 exposure. Researcher have conducted studies on human cell lines, liver tumore, hepatitis infected mice and wildtype mice to analyze the mutagenic effect on AFB1 exposure. The results showed more than fourty thousand mutagens, which can be used as markers in disease incidences [168].

1.2.8.6 Biocontrol with *Aspergillus flavus* atoxigenic strains (NRRL21882, AF36, K49, CT3 and AF051)

Non-toxigenic strains of *A. flavus* are developing as foremost biological control weapons in plant pathology sector. This approach involves use of nontoxigenic strains as a competitive inhibitor for toxigenic strain in terms of substrate utilization from host. Thus, for effective inhibition in agricultural environment, nontoxigenic strains need to be predominant in comparison to toxigenic strains of host-crop. Atoxigenic strains of *A. flavus* have been demonstrated by Cotty [169] against AF contaminated cotton in Arizona. It has been developed to study the AF inhibition on large scale [170]. Till now, Environmental Protection Agency of US has registered two non-toxic strains *of A. flavus.* NRRL21882 strain of *A. flavus* has been isolated in Georgia from peanut has been found to inhibit AF biosynthesis. Mechanism behind AF inhibition by this strain is it lacks cluster genes starting from *hexA* upto the telomers involved in AF biosynthesis.This strain have successfully accomplished field tests for prolonged time and also mediated the development of Afla-Guard (biopesticide/ biocontrol agent which contains non toxicogenic strain of *A. flavus* NRRL21882). Afla-Guard has been successfully used on corn and peanuts for anti fungal properties [171]. Another avirulent strain of *A. flavus* is AF36, which have shown promising effect on AF inhibition from cotton-seeds. This strain defects in *pksA* gene of *A. flavus* which causes AF biosynthesis inhibition [172]. Further, K49 and CT3 are some other atoxigenic strain of *A. flavus*, which showed remarkable results in reducing contamination of AF from corn, in US [173]. BN30 is another atoxigenic *A. flavus* strain of Africa which when provided with toxigenic strain, mediates reduction of AF from maize [174]. In recent studies, AF formation in peanuts was found to be inhibited upto 95% when provided with atoxigenic strains in Australia [175]. Studies conducted in China showed, 99% inhibition of *Aspergillus* species when treated with AF051 (atoxigenic *A. flavus* strain) [176]. Looking at the remarkable results of atoxigenic *A. flavus* strains, current efforts have been intended to identify additional non toxigenic strains for global application [177].

1.2.8.7 Bacteria and Yeast

Another approach against AF accumulation is use of different bacterial and yeast strains. *Bacillus thurengenesis* (Bt) is a bacterial strain which has efficiently used to develope Bt-crops (cotton), which have shown resistance against *Aspergillus* colonization and AF

contamination [178]. This bacterium is known to produce a toxin which bears insectisidal activity. Toxin reduction by Bt in various crops such as corn highly depends on environmental factors [179]. However some studies did not found any effect in AF inhibition on Bt-corn, which showed contrasts in the activity of Bt-corn [180, 181]. Survey by Wu and his coworkers on Bt-corn showed profit of 14.1 million dollars against AF contamination [182]. Other than *B. thorengenesis,* other bacterial species viz, *Lactobacillus* species, *Bacillus subtilis*, *Ralstonia* species, *P. solanacearun*, *Pseudomonas* species and *Burkholderia* species have also found effective against *Aspergillus* mediation AF production [183, 184]. The drawback is, it is very complicated to carry cells of bacterial at *Aspergillus* mediated infection sites in field experiments [177]. Yeast (*Pichia anomala* and *Candida krusei*) have also been tested against *A. flavus* contamination in lab- conditions [185, 186]. Also, they have found potential as anti-AF agents [177].

1.2.8.8 Proteomic approach

Availability of genome of different *Aspergillus* species have opened the gateway to understand post-genomics and increased the knowledge against the important fungal genus [187]. The evaluation of proteome provides a organised understanding of events or functions or organism at cellular and molecular level, which helps in identifying different targets [188]. Proteomic approaches in AF research have been considered to diminish the contamination in post- and pre-harvest crops. Biosynthesis of AF does not only depend upon *Aspergillus* species but also on various environmental factors and host [189]. Reviews associated with proteomic approaches in fungal biology have been studied extensively [190]. Additionally, Bhatnagar and his coworkers have reviewed the capability of OMICS technologies (genomics, metabolomics and proteomics) to overcome problems realted to AF contamination [189]. Recent approaches using proteomics against AF requires identification of resistant proteins against AF accumulation in host plant (example; corn), to devlope resistant variety of AF prone crops [153]. Researchers has analyzed corn proteins of diverse genotypes, which showed resistance or susceptibility towars *A. flavus* and found a 14-kDa protein which may be used as as a selectable marker for host-resistance against *A. flavus* [191]. In a further study authors conducted proteomics studies to recognize possible markers in maize which was found to be resistant against colonization of *A. flavus* [192]. Also, Chen and coworkers have identified maize kernel endosperm proteins which have shown

resistance against AF contamination [193]. Another review described the proteins related to AF resistance [194] and also studied the negative relationship between PR10 (pathogenesis protein) expression and kernel resistance against infection due to *A. flavus* [153]. Further, they observed a considerable PR10 protein reduction in RNAi-silenced lines. Different proteomic approaches showed that, maize which is resistant against fungal conatmination have elevated levels of storage proteins and stress related proteins when compared with susceptible lines [192, 195]. In the future the resistance associated global proteome analysis has a potential to identify novel breeding markers. On the basis of several researches on AF resistance, proteomic approaches can be useful for scientists to identify mechanisms behind host resistance and stress. Results could be helpful to understand factors effecting AF biosynthesis and can direct the novel ways in increasing plant resistance against contamination of *Aspergilli.*

1.2.8.9 Drug targets and development of antifungal compound

High rist hazards due to *A. flavus* needs the treatment from preventive and therapeutic antifungal compounds. There are several class of antifunfal compounds viz, alkaloids, flavanoides, azoles, isoflavones, etc. *A. flavus* mediated death rate and morbidity are associated with limited antifungal drugs and their toxic effects. Drugs used against *Aspergillus* infection are voriconazole and terbinamine, which are nephrotoxic in nature [196]. Previously, some proteomic studies were conducted on *Aspergillus* species to percieve the interaction studies with antifungal drugs such as AmB, caspfungin [49, 197]. Also, study showed the inhibition of transition fron mycelia to yeast is the target of some antifungal drugs, such as 17β-estradiol [198]. In the recent years *A. flavus* has acquired resistant mechanism against present antifungal drugs by developing biofilms and overexpression of efflux pump proteins [199]. Thus, new strategies is required to improve treatment against fungal responses which can be accomplished by developing new formulation of existing antifungal drugs, discovering a new drugs and utilization of better carrier molecules like nano particles. Studies have been conducted, to identify effective antifungal targets against *A. flavus*, one of the targets is calcium signaling pathway. It includes calcineurin protein, which is associated with various biological processes such as cell morphogenesis, stress response and resistance against antifungals in fungi [200]. It also

regulates β-glucan, chitin and ergosterol. In recent times a new compound, triphenylethylene was found to inhibit calcineurin pathway by activating calcineurin along with calmodulin [201, 202]. Further, Hsp (Hsp-90) is also identified as a new target against fungi [203]. Furthermore, there have been continuous efforts in the recent years to develope new antifungal formulations and structural modifications to achieve antifungal drugs of lower toxicity. For example, AmB derivative, N-methyl-N-D-fructosyl AmB methyltransferase, is known for its limited toxicity to humans [204]. Latest classes of antifungal drugs are echinocandins, which targets fungal cell wall glucan [205]. Various drugs such as PC945, PC1244, CD101 and F901318 are under clinical development and have shown promising results against fungi [206-208]. Despite of immense efforts to develop antifungal compound for controlling antifungal resistance, modification in existing drugs have not proved to be a crucial approach in terms of drug toxicity and resistance acquired by fungal pathogenesis. This scenario therefore calls for alternative approach to develop ideal alternatives which should be economic, non-hazardious, non-toxic and have efficient antifungal properties [209]. One of such approach is use of botanicals (phytochemicals) which are now emerging with anti-afltoxigenic along anti-fungal properties, which are safe for human kind in every way. Phytochemicals are derivatives of plant natural products ranges from flavanoides to alkaloids. Till now various phytochemicals have been tested against *A. flavus*, which have also shown the promising results. AFB1 mutagenesity was efficietly suppressed by various plant extracts such as piperine, xantophylls, lutein and carotenoids [210, 211]. Further, essential oils extracted from *Cymbopogon martini*, *Illicium verum*, *Zataria multiflora*, *Cinnamon zylenium*, *Cinnamomum jensenianum*, *Eucalyptus globules* and *Ocimum sanctum* has shown significant anti fungal and anti-AFB1 activities [212-215]. Also, *Cymbopogon martini* extracted essential oil showed anti-AFB1 activity along with quality control of stored melon seeds [216]. Furthermore, in last decades growth of *A. flavus* and AFB1 production has been efficiently inhibited by clove oil which contains eugenol [217-219]. Neem, betel, oregano and garlic extracts has also found to be effective compound against growth of *A. flavus* and AF production (B and G) [220-223]. All the phytochemicals have their different mode of actions [224]. Some plant species such as *Cymbopogon citrates*, *Occimum gratissimum*, *Sizygium aromaticum*, *Xylopia aethiopica*, *Cinnamomum verum*, etc. has shown to inhibit *A. flavus* mediated biosynthesis of AF by inhibition precursor molecule

formation (non-sorbic acid) [225, 226]. Also, onion containing thio-propanol-S-oxide has shown the promising effects against *A. flavus* [227]. In the recent studies different phtochemicals such as gallic acid, piperine, ascorbic acid, quercetin etc. also showed the antiaflatoxigenic properties against *A. flavus* among which quercetin (flavanoid) was found to be most efficient [228, 229].

1.2.8.10 Diagnostic markers using bioinformatic approaches

Another approach which is in account now a days is use of *in-silico* methods, still are in emerging stage, however use of protein identification, interactions, identifying targets and drug designing make it more proficient [230, 231]. With time, the analysis using several docking software has become more reliable and accurate [232].Various softwares and databases are now in field such as "Maestro" designed by Schrodinger, "Sanjeevni" which is designed by IIT Delhi, India etc. which showed a huge contributions in the area of biotechnology and for the development of anti-fungal drug targets [233, 234]. Bioinformatical approaches have been used against AF contamination, through analysis of resistance host (maize) or *A. flavus* isolates (may be atoxigenic isolates) [235-237]. Also, bioinformatical approaches have been used to associate a population map for *A. flavus* mediated AFB1 accumulation and the maize rsesistance, which can be used to identify the resistant markers present in maize or susceptible markers present in *A. flavus* [238].

CHAPTER 2

Screening of Phytochemicals against Aflatoxin Producing *Aspergillus* Species and Determination of MIC_{50}

2.1 INTRODUTION

Aspergillus flavus colonization allows contamination of AF in pre- and post-harvested food crops and related losses up to 30% [239]. Twenty five thousand million US greenbacks annual global crop loss became predicted due to fungal pathogens [240, 241]. It is correct to say kingdom fungi is one of the destroyers of foodstuffs and grains all through garage, making it inappropriate for human consumption via destroying their nutritive cost often by means of generating mycotoxins [242, 243]. These usually involve 3 fungal species, *Aspergillus*, *Fusarium* and *Penicillium*. As per Galvano et al., over 300 fungal metabolites are the potent mycotoxin producer, accountable to contaminate over twenty fifth of world's food crop [244].

Aspergillus parasiticus is closely associated with *A. flavus*, as both are chargeable for the AF biosynthesis (B1, B2, G1 and G2) [245]. These two fungal pathogens are vital pathogens infecting peanuts, corn, cotton, and alternative oil-seed crops therefore manufacturing toxins both within field and into the course of storage.These fungi are classified as group-1 cancer agents through International Agency for Research on Cancer (IARC). Contamination caused by AF in food crops has awakened many nations to have strict limits on AF intake in animal feed [246].

Hazardous effects which include chronic and acute poisoning as a consequence of several fungicides and pesticides are the foremost drawback in numerous developing countries [247, 248]. Bio pesticides (botanical pesticides) are the rising area due to their benefits in having precise target of action. Plant assets as antimicrobial compounds, referred to as phytochemicals, though studied appreciably, still now not have been able to practice at the industrial level. Natural phytochemicals (phenolic compounds, thiols, carotenoids, flavonoids, anthocyanin and tocopherol) extracted from different plant parts (fruits, veggies and herbs) have showed a good vary of biological results, such as anti-inflammatory,

antimicrobial and antioxidant actions. This state of affairs therefore requires alternate procedures which can be eco-friendly and economically feasible to control the disease and growth the yield [249, 250].

Dangerous affects, for example, excessive and perpetual harming caused due to fungicides and insectisides are the real trouble in low income countries [25, 26]. Bio-pesticides are the rising range due to their focal points in having uniqueness in action. Plant sources as antimicrobial blends, called phytochemicals, considered extensively, still not have possessed the capacity to rehearse at the business level. Trademark phytochemicals (phenolic, thiols, carotenoids, flavonoids, anthocyanin and tocopherol) extracted from various plant parts, for example, herbal products, herbs and vegetables have tested in depth sort of organic affects, together with cell reinforcement, antimicrobial and inhibitory activities. This situation consequently, requires interchangable approache which might be financially viable and eco-accommodating to control the disorder and amplify the yield [27, 28].

In this study, 4 different phytochemicals were examined for their anti-fungal interest. Caffeine is a water-solvent alkaloid, which is shown to inhibit development and polyketide mycotoxin production in some of *Aspergillus* and *Penicillium* species. It is stated to repress AF G1 and G2 in a study performed via Maraqa et al. [251]. Quercetin belongs to plant pigments known as flavonoids. Flavonoids are omnipresent in photosynthesizing cells and are typically acccumulated in fruits, honey, vegetables, tea, wine, flower, nuts, propolis and seeds. Antifungal activity of quercetin has not been studied notably. Quercetin can stop the conversion of AFB1 to the carcinogenic product AFB1-8, 9-epoxide [252]. Gallic acid is the hydrolysable tannins, free acids and esters, found in plant sources. Gallic acid has been established as an efficient antimicrobial compound against different fungai and yeasts such as *Corynobacterium accolans*, *Staphylococcus aureus*, *Erwinia carotovora* and *Candida albicans* [253]. Gallic acid is understood to exhibit suitable antifungal interests against *Magnaporthe grisea* and Erysiphe *graminis*. In *M. grisea* mediates inhibition of germinating conidia (64%) and also inhibited appressorium formation upto 5% [254]. Ascorbic acid is a 6-C compound associated with aldohexose, predominant in citrus fruits and plenty of vegetables. Also, it is a widely-known anti-microbial compound. It is proverbial to show 14.3% inhibition against *A. flavus* at 1% concentration of ascorbic acid according to

Obaleye et al., during a study of fungitoxic effects of ascorbic acid on four well known strains of fungal species *Trichophyton* species, *Penicillium* species, *A. flavus* and *A. niger* [255].

The objective of the study was to evaluate different phytochemicals under *in-vitro* conditions to discover most effective antimycotic agent, against *A. flavus* and *A. parasiticus*. The developing and underdeveloped countries of Asia and Africa are suffering from health issues related to intense mycotoxin consumption present in contaminated pre- and post-harvest crops. Eventhough, effective and economical management on seed borne pathogenic fungi can be done by the utilization of artificial chemical fungicides, the same can't be applied to grains for motives of chemical toxicity [256, 257]. Therefore there is a necessity to look for another techniques to develop atoxigenic grains/cereals which might be eco-friendly and non-expensive. By better knowledge about the restrictive outcomes of numerous phytochemicals on fungal growth, we can establish new compounds which are beneficial for dominant fungal toxicity throughout food processes and storage.

2.2 MATERIAL AND METHOD

2.2.1 *Aspergillus flavus* isolates

Aspergillus flavus (MTCC 11866) and *A. parasiticus* (MTCC 8189) were used in our study [258]. The toxigenic strains were treated in BSL2 biosafety cabinet (Telstar, Life Science solutions, Spain).

2.2.2 Chemicals

Analytical grade reagents and solvents were used in the study obtained from Sigma Chemical Co. Potato dextrose agar (PDA) media (Himedia, Mumbai, India) and RPMI 1640 medium powder with L-glutamine and sodiumbicarbonate (Himedia, Mumbai, India), were used as a culture media. Other chemicals such as MTT and DMSO wae obtained from Himedia, Mumbai, India and Fischer Scientific, Mumbai, India, respectively. All the phytochemicals viz. ascorbic acid, gallic acid, caffeine and quercetin were obtained from Loba chemie Pvt. Ltd., Mumbai, India.

2.2.3 Spore harvesting

Potato dextrose agar slants were used to culture *A. flavus* and *A. parasiticus* culture. And spores were harvested after 72 h using phosphatebuffered saline (PBS) with 0.05% Tween 20 (PBST). Centrifugation was performed at 10,000 rpm for 10 min at 4°C and washed with PBS twice, followed by CFU count using haemocytometer. 1×10^6 cells/ml was used as a working conidial culture in our studies.

2.2.4 Preparation of Stock solution of Phytochemicals

Four phytochemicals were used: ascorbic acid, gallic acid, caffeine and quercetin. Stock solutions of all the phytochemicals were prepared at 500 µg/ml, from which different dilutions of working solution was prepared (1 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml, 300 µg/ml, 350 µg/ml, 400 µg/ml), respectively, with 90% methanol, and stored at 4°C for further use.

2.2.5 Antifungal assays

2.2.5.1 Poisoned-food technique

Grover and Moore (1962) method was used to evaluate the antifungal activity of phytochemicals [259] with slight modification [260]. All the four phytochemicals at the concentration of 300µg/ml was used against both the *Aspergillus* species used in our study. 1×10^6 conidial cells inoculated on phytochemicals treated PDA using sterilized paper disk (5mm). Plates were incubated up-side down at 37ºC. Control plates does not contain phytochemical within it. Plates containing 2 ml methanol served as methanol control. Asceptic conditions were made using Laminar Air Flow (S.M. International limited, India). Diameter of mycelia was calculated aflter 2 days and 3 days, followed by significantly calculating % mycelial inhibition using following formula in comparison to control.

$$\text{Mycelial Inhibition (\%)} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

2.2.5.2 MTT Assay

RPMI 1640 medium containing L-glutamine and sodium bicarbonate at pH 7.4 was used to adhere and grow *A. flavus* and *A. parasiticus* conidia upto final concentration of 1×10^6 spores/100µl. 10^6 spores with RPMI media (100 µl) were added in the different wells of radiation-sterilized 96-well flat-bottom microtiter plate (Tarsons, Kolkata, India). Different concentrations of all the 4 phytochemicals (1 to 400µg/ml) were added to make the final volume up to 200 µl in each well. Wells without phytochemical were served as control. Microtiter plate was incubated for 24h at temperature range of 37°C. Supernatant was replaced with fresh RPMI media (200 µl) followed by addition of 10 µl MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). The plates were again incubated for 3 to 4 h at 37°C. Plates were centrifuged at 3000 rpm, followed by removal of supernatant. The lysis of leftover cells was performed by Dimethyl Sulfoxide (DMSO) followed by centrifugation (3000 rpm) and transferring the supernatant to fresh microtitre plate [49]. Further, OD was taken at 570 nm and compared with control for the analysis of viable cells. Standard curve was plotted against control by Graphpad Prism (version 5) to calculate the percent inhibition using following formula [261]

$$\text{Growth Inhibition (\%)} = \frac{OD_{(\text{Test sample})} - OD_{(\text{Blank})}}{OD_{(\text{Control})} - OD_{(\text{Blank})}} \times 100$$

The experiment was performed in triplicates followed by taking mean of MIC_{50}, which were further considered for analysis. Statistical analysis was performed by using graph pad software (verson 5.0) and the dose response curve was generated between OD at 570 nm of survived cells (Y axis) and phytochemical concentration in µg/ml (X axis).

2.2.6 Statistical analysis

All dataset were obtained as mean ±SD and analysed using column search by paired t-test which is a nonparametric test. The analysis was carried out to determine differences in significant values between groups mean dring all experiments. P-values < 0.05 showed significant differences. Software used for statistical analysis was GraphPad prism (5.0).

2.3 RESULTS

2.3.1 Mycelial inhibition by poisoned-food method

In comparison with control, both the *Aspergillus* species were found to be inhibited (less than 50%) at 300µg/ml concentration of different phytochemicals at 48 and 72 h. Results revealed that quercetin was the most significant inhibitor among all phytochemicals against both *Aspergillus* species viz. 42.8% for *A. flavus* and 46-50% for *A.parasiticus*. Gallic acid (37-40%) and caffeine (40-45%) also showed significant results in *A.parasiticus*. However, caffeine (24-31%) showed significant inhibitory effects in *A. flavus*. Among all the four tested phytochemicals, ascorbic acid was found to be least effective against both the tested *Aspergillus* species. Comparative analysis of PDA plates including mycelia diameter is represented in Figure 2.1 and Table 2.1 includes mycelia inhibition (%) of both *A. flavus* and *A. parasiticus*. All the phytochemicals showed inhibition against both the fungi therefore subjected to MTT assay, which is a more appropriate method for calculation of minimum inhibitory concentration.

Table 2.1: Mycelial inhibition (%) to determine the inhibitory effect of phytochemicals at 300 µg/ml

Phytochemicals (300 µg/ml)		**Percent Mycelial Inhibition**	
		A. flavus	***A. parasiticus***
Ascorbic acid	48hrs	21	20
	72 hrs	14.2	20.8
Gallic acid	48hrs	10.5	40
	72 hrs	14.3	37.4
Caffeine	48hrs	31.5	40
	72 hrs	24	45
Quercetin	48hrs	**42.1**	**50**
	72 hrs	**48**	**46**

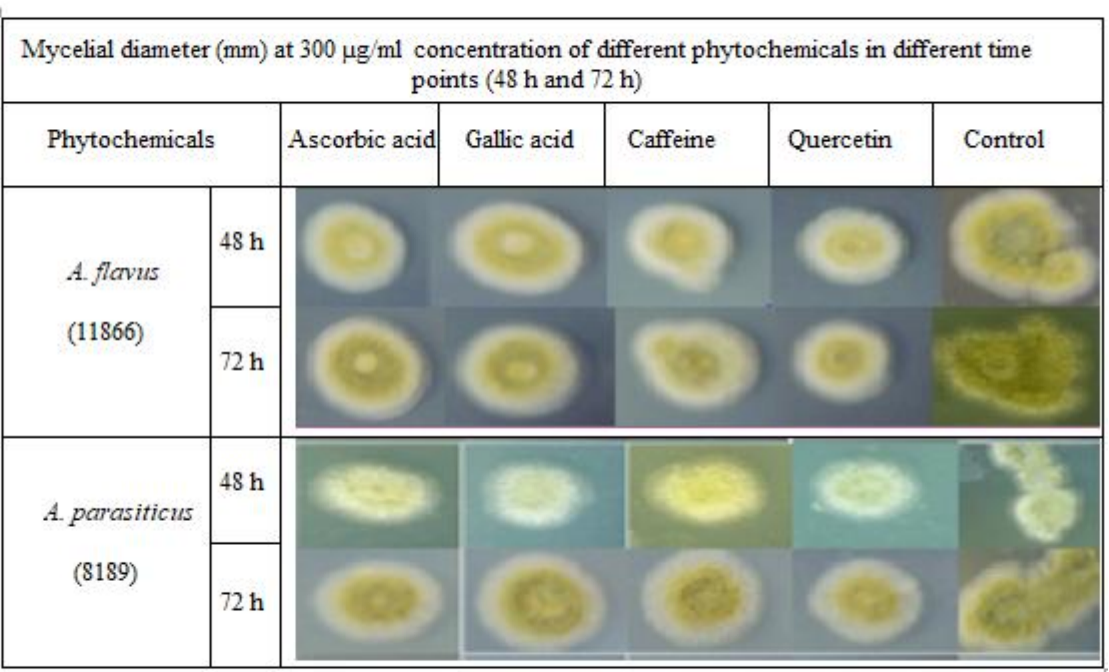


Figure 2.1: Determination of mycelial diameter calculated in mm at phytochemical concentration of 300 µg/ml at 48h and 72 h of *Aspergillus flavus* and *A. parasiticus*

2.3.2 MTT assay

Graph plotted against phytochemicals (concentration) v/s OD (at 570 nm) was used to calculate MIC_{50} value of different phytochemicals against both *A. parasiticus* and *A. flavus*. The results were found to be significant as p value was found to be < 0.05 (Figure2.2). Comparative analysis among four different phytochemicals reveled that quercetin is a promising antifungal compound against both the tested *Aspergillus* species. For quercetin, MIC_{50} value was 36 µg/ml for *A. parasiticus* and 113 µg/ml for *A. flavus*. Gallic acid (MIC_{50}value~153 µg/ml) showed significant results for *A. flavus* inhibition whereas, caffeine (MIC_{50} value~160 µg/ml) showed significant results for *A. parasiticus* inhibition. Ascorbic acid was least effective for both the *Aspergillus* species. Overall assay revealed that quercetin is a potent phytochemical having anti-Aspergillus properties. Results are mentioned in Table 2.2.

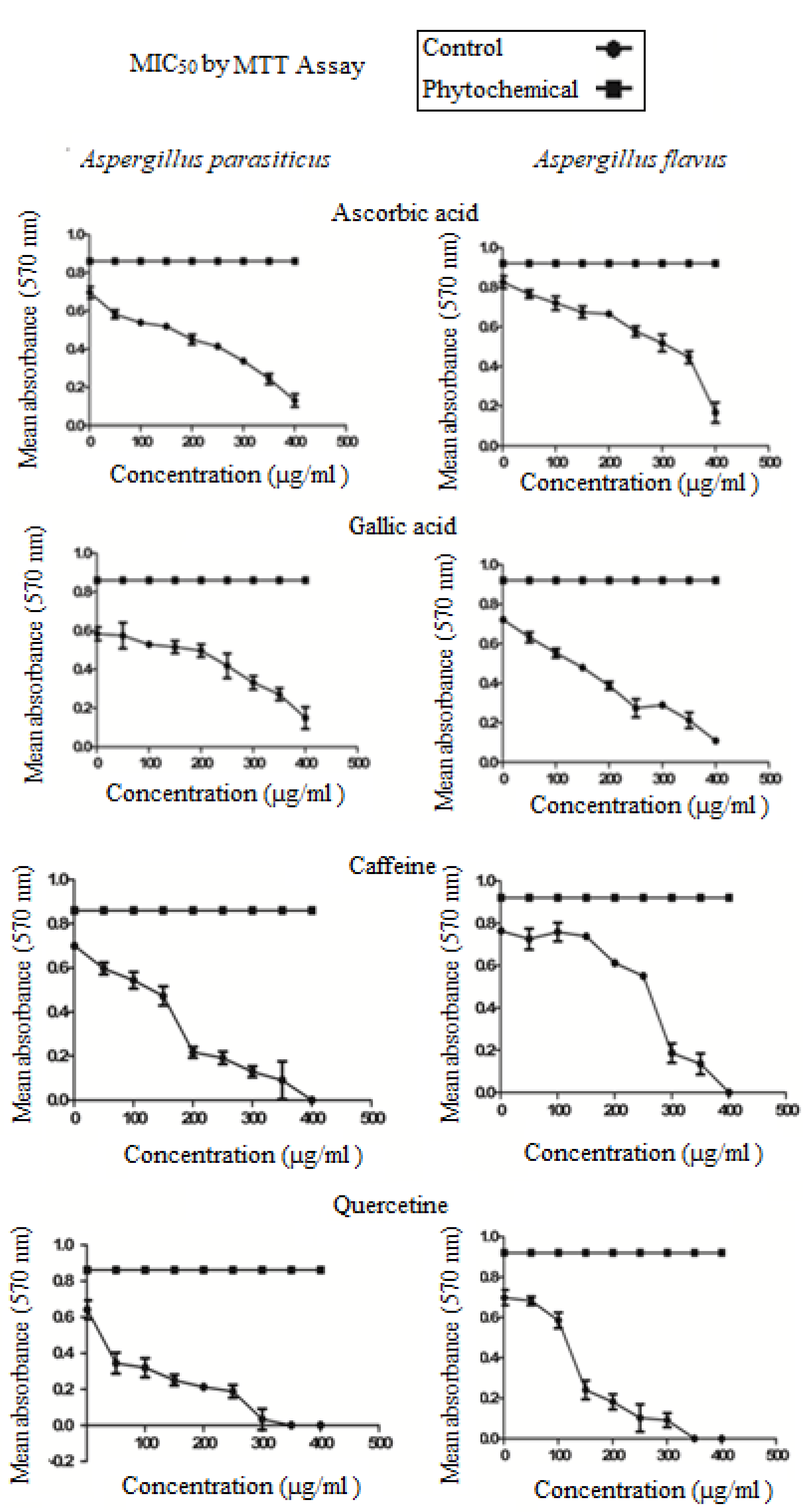


Figure 2.2: Graphical representation of inhibitory activity of phytochemicals used in our study in both the *Aspergillus* species ranged from 1-400 µg/ml using mean ± SD (n=3, per condition) and p value is < 0.05

Table 2.2: MIC_{50} value of different phytochemical against *Aspergillus flavus* and *Aspergillus parasiticus*

Phytochemicals	MIC_{50} (µg/ml)	
	Aspergillus flavus	*Aspergillus parasiticus*
Ascorbic acid	322	321
Gallic acid	153	238
Caffeine	257	160
Quercetin	**113**	**36**

2.4 DISCUSSION

"Food safety is essential as to reap healthy human nutrients" changed into the major topic discussed inside the Second International Conference on Nutrition (ICN2), Rome in November 2014. Government needs to take fundamental steps in establishing and imposing powerful food safety system which guarantees the responsibility of food producers and providers along with entire food chain operators, to deliver safe food to purchasers [262]. Phytochemicals have won the eye of the researchers within the recent years, as plant extracts are proved to be a promising antimicrobial agent with none poisonous effects on animals. Phytochemicals can be extracted from diverse plant components like leaves, stems, flowers etc. [263]. Phytochemicals could also be phenolic which might act as number one antioxidants [264] or flavanols or tannins which usually belong to Leguminosae-Mimiosoideae family [265]. Leal et al. showed that impact of phytochemicals relies upon the fungal species, which can range between groups of fungi. *In-vitro* cytotoxicity assays are the first step to evaluate whether a compound with potential antifungal activity is promising to become a future antifungal agent [266]. Poisoned food technique is the first step to quantify the fungal cells viability in vitro, followed by more suitable technique viz. MTT assay as it is very critical to decide the ratio between the safety and potency of a compound for development of therapeutic products [267, 268]. Results in our study showed significant effects of all phytochemicals against both *A. flavus* and *A. parasiticus*.

Hamza et al., showed that plant extracts having antimicrobial residence are classified on the premise of their MIC values [269]. Extracts displaying MIC of 0.5 mg/ml or less are the sturdy inhibitors of fungal growth, on the basis of MIC classification by Aligiannis et al. [270] who categorized plant extracts as strong inhibitors (MIC of 0.5 mg/ml), moderate inhibitors (MIC between 0.6 and 1.5 mg/ml) and weak inhibitors (MIC above 1.6 mg/ml). The examined molecules differed greatly in their activity against fungi among which quercetin showed best inhibitory activity and found to be significant in both the *Aspergillus* species. Our present data correlated with the data of Walid et al. which showed quercetin exhibiting remarkable anti-*A. niger* and anti-*C. albicans* properties [271]. Studies conducted by Zhou et al. confirmed quercetin as a potent inhibitor of *A. flavus* mediated AFB1 production at 800µg/ml concentration [228]. Our results showed that quercetin inhibited 50% of *A. flavus* and *A. parasiticus* population at 113µg/ml and 36µg/ml respectively confirming quercetin as a significant anti-*Aspergillus* compound. Caffeine also exhibits antifungal properties at >5000 µg/ml [272]. In other studies on *C. albican*, caffeine is a potent inhibitor at the concentration of 12.5mM (MIC) [273]. In our present finding caffeine exhibited remarkable effects by means of inhibiting *Aspergillus* species at 257 µg/ml (*A. flavus*) and 160µg/ml (*A. parasiticus*), which confirms caffeine as promising antifungal compound. The antifungal activity of gallic acid was studied by Seo et al., who showed ≥800 µg/ml concentration is required to inhibit AFB1 production by *A. flavus* [228]. Also, gallic acid was found to inhibit *Fusarium solani* at 500 ppm [274]. In our findings, gallic acid showed significant inhibitory effect on *A. flavus* (MIC_{50} value~153µg/ml) in the assessment to *A. parasiticus* (MIC_{50}value~238µg/ml). Pesarico et al., showed inability of antifungal property of ascorbic acid as it does not showed any inhibition on *C. albicans*. However, when mixed with different antioxidants, such as curcumin, anticandidial effect of ascorbic acid significant increased. These findings suggested that ascorbic acid, alone have poor antifungal activity [275]. In the current studies, among the individual compounds, ascorbic acid showed the least MIC_{50} value in both *A. flavus* (322µg/ml) and *A. parasiticus* (321µg/ml).The overall discussion concludes that quercetin can be taken into consideration as a promising antifungal compound against *Aspergillus* species. However, mechanism of antifungal responses of quercetin needs to be investigated.

SUMMARY

Studies on phytochemicals as antiaflatoxigenic compounds, quercetin was found to be most significant among four different compounds. Quercetin was found to most effectively inhibit germination of both the *Aspergillus* species conidia.

CHAPTER 3

To Elucidate the Proteins/Enzymes Involved in Germination of *Aspergillus flavus* Conidia

3.1. INTRODUCTION

Aspergillus flavus is a distinctive fungus in *Aspergillus* genera. It mediates infection/disease in immunocompromised individuals [276]. *A. flavus* mediated infection is visible across many phyla of animals called aspergillosis [277]. According to a survey in Asia and central-east showed *A. flavus* was the predominant fungus followed by *A. fumigatus* isolated from 2117 patient samples suffering from aspergillosis [278]. *A. flavus*, being cosmopolitan in distribution, is a major AF contaminant in several pre and post harvested food crops such as maize, peanuts, cotton etc. which exerts carcerous effects and massive economic burden [279]. Several epidemiological studies associated with AF, responsible for hepatocellular carcinoma, which is the third largest cancer worldwide [97, 280].

Aspergillus morphotypes mainly comprised of conidia, hyphae and mycelia. Genomic studies on *A. flavus* is widely conducted that showed 13,071 genes in total [281]. AF biosynthetic pathway is a 70-kb gene cluster which includes 24 structural genes controlled by two regulatory genes (*aflR* and *aflS*) [282]. Isotropic growth has been observed as the fungus converts from conidia to swollen conidia stage which includes water activity and decrease in the cytoplasm viscosity [283, 284]. Isotropic growth is followed by polarised growth which undertakes conversion of swelled conidia into germ tube stage with the activity of cytoskeleton proteins, activation of signalling pathways and vesicle trafficking [285]. Pechanova et al. performed *A. flavus* proteomics at mycelia stage which resulted into 538 unique proteins involved in cellular metabolite and biosynthesis processes [52]. Fifty one unique secreted proteins were identified from proteomic studies of *A. flavus* mycelia stage [286]. Temperature mediated (37°C and 28°C) proteomic studies of *A. flavus* was conducted by D. Ryan Georgianna et al., which showed transcription inhibition along with inhibition of AF biosynthesis at 37°C which was shown to be upregulation at 28°C [287]. Proteomic studies have been conducted at the conidial and

mycelia stages of different *Aspergilli* [288-290]. *A. niger* germinating conidial transcriptomic studies have been conducted by Van Leeuwan et al., that showed protein synthesis and cell wall modulation etc. are the major events involved in the germination of *Aspergillus* species [291]. *A. fumigatus* transcriptomic data at germinating conidial (30, 60 and 90 minutes) is also available, which showed dormant conidia involves metabolism of cellular proteins, transportation, RNA metabolism which fluctuates to synthesis of proteins, carbohydrate metabolism, protein complex assembly, and RNA binding protein, after 30 min of germination [292]. However, there is no proteomic data available at the germ tube stage in *Aspergillus* species.

Azoles, echinocandins and polyenes are the class of drugs which are used as antifungals for clinical use, which goals fungal biosynthesis of cell wall [293]. Ergosterol biosynthesis is mainly targeted by polyenes and azoles whereas β-1,3-glucan synthase is majorly targeted by echinocandins [49, 294]. Itraconazole is a class of azole which recently showed the alteration of proteome profile of *A. fumigatus*, which can be used as effective anti-*A. fumigatus* target [295]. Since, fungal cell wall is the major drug target for *Aspergillus* species, is widely necessecitated to understand the mechanism of cell wall biosynthesis at early germination stages.

Transcriptomic and proteomic studies on *A. flavus* at two different temperatures viz. 28°C and 37°C resulted in 664 differentially expressed proteins which were found to be involved in protein synthesis, substrate metabolism and biosynthesis of secondary metabolism. This finding showed less similarity with the transcriptomic data, which suggested that genes may go through post-transcriptional regulation [55]. Thus, to understand the sequential events of germination of *A. flavus* global proteomic approaches profiling may be useful. As observed in previous chapter, selected phytochemicals showed inhibition of germination *Aspergillus* conidia. Thus, current chapter includes proteomic approach to profile protein/ enzymes or biochemical pathway during geminating conidia stage of *A. flavus* using nLC-Q-TOF mass spectrometry. Present study indicated that most of the proteins/enzymes showed expression of translational processes, metabolism of carbohydrate and amino acid, MAPK signaling cascade, and biogenesis of cell wall.

3.2 MATERIAL AND METHODS

3.2.1 *Aspergillus flavus* strain and culture condition

As mentioned in the previous chapter, *Aspergillus flavus* strain (MTCC9367) [296] was maintained on SDA media (Himedia, India) for 1 week at 30°C, followed by conidia collection in PBS (pH 7.4) with 0.05% Tween 20. Conidial suspension was collected and filtered in order to separate hyphae as well as mycelia. Further the conidia samples was centrifuged at 10,000 rpm for 5 min and conidial pellets was washed using chilled PBS (pH 7.4). They were further stored in refrigeration (4ºC) for further use. Working conidial cells were 10^6 conidial cells/ml, used for further analysis [258].

3.2.2 Morphotypes of *Aspergillus flavus*

To check conidial purity light microscopic studies were carried out and also to check the morphological stage of *A. flavus*. To check the morphogenesis of *A. flavus* LCB staining (Himedia, India) was used. *A. flavus* 10^6 conidial cells/ml were grown at temperature of 30°C and 150 rpm. Sample collection were performed in regular time intervals from 2 hrs to 8 hrs until the identification of germ tube stage, 40× by Magnus MPS-USB (Olympus, India). Maximum cellular homogeneity was maintained by using two biological replicates. *A. flavus* showed germ tube formation at 7h time point, which were used further for global proteome analysis.

3.2.3 Large scale culture for protein extraction

A. flavus conidia (10^6 cells/ml) were cultured in SD broth and incubated for 7h at 30°C to obtain germ tube stage of *A. flavus* and subjected to centrifugation to remove extra media. Further the washing of collected cells was performed with PBS twice (pH 7.4) to remove ant medium residues Cells were collected followed by haemocytometer count. Counting was performed in triplicates. Then pellets (cells) were snapped chilled with liquid nitrogen and stored in ultra-freeze for protein extraction.

3.2.4. Protein extraction

Total proteins were extracted at 4°C in sodium phosphate buffer of pH 7.4 (50 mM) having 2 mM EDTA, 0.2 mM DTT and 1mM PMSF with continuous stirring for 3h [49]. Further, centrifugation of samples was performed for 20 min at 15,000 × g. Overnight precipitation using trichloroacetic acid (5%) was performed at -20ºC followed by washing with chilled acetone. Protein pellets were then dried and dissolved in rehydration buffer (2% CHAPS, 8 M Urea, 25 mM DTT). Further, Bradford analysis was performed to calculate protein concentration and stored at -80ºC until further use. [297].

3.2.5 SDS-PAGE analysis

The quality of extracted protein was checked with 12 % SDS-PAGE, and quality protein samples were used for protein analysis using nLC-Q-TOF [298]. In brief, extracted protein sample was precipitated with phenol: chloroform in the ratio of 400:100 µl followed by adding 300 µl distilled water. Centrifugation was performed at 10,000 × g at 25°C for 10 min. Pellet was dried and reconstituted in buffer (10% SDS, Tris-HCl (pH 68), glycine 20%, distilled water 2.8 ml, Bromophenol blue (0.02 w/v), β-mercaptoethanol). Proteins with sample buffer was boiled at 95°C for 5 min. SDS-PAGE gel (12%) were used for analysis followed by fixing in acetic acid, methanol and distilled water (25:100:125 ml) for 2h. Staining of gel was performed in colloidal commasie stain and kept for overnight.

3.2.6 Mass spectrometer analysis (nLC-Q-TOF)

3.2.6.1 Sample preparation

Sample preparation was performed using ammonium bicarbonate (50mM) upto 1 mg/ml of final concentration. Protein identification was performed using quadrapole Time of Flight-Liquid Chromatographic-MS/MS analysis. Precipitation of 100 µl of protein sample was performed using acetone followed by reduction at 56ºC using 10 mM dithiothreitol for 1 h. Further alkylation was performed for 45 min using 55 mM IDA at room temperature followed by trypsin digestion at 37ºC and kept overnight. Elution of digests was performed with formic acid (0.1%). Further SDS-PAGE was run to ensure complete digestion.

3.2.6.2 MS AND MSMS ANALYSIS

Separation of digested proteins was performed using Acquity Waters UPLC system coupled with qTOF-LC-MS/MS (Waters, Corporation) [299]. The reaction mechanism was of 60 min via BECH18 column (Size of column, 2.1 mm x 150 mm x 1.7 um) using buffer A and B that constitute 0.1% formic acid and acetonitrile or 0.1% formic acids respectively at flow rate of 200 µl per min. Subsequent to peptide separation, they were ionized using Electrospray Ionization (ESI) at 275ºC with a spray volume of 2 kV. Generated mass spectra of peptides were obtained using automated MS/MS mode. Then obtained mass spectra of peptides were analysed using Protein Linux Global Server software from Waters Corporation.

3.2.6.3 Identification of protein

SEQUEST search algorithm (90% identity) was performed for data analysis which includes Mass Lynx 4.1 WATERS. Following parameters were used to obtain data; one missed cleavage of enzyme trypsin, modification of cystine residue with carbamidomethyl and methionine oxidation having peptide and fragment tolerance of 100 and 200 ppm respectively. The obtained data was searched using UniProt database against *Aspergillus flavus* or *Aspergillus* species [300].

3.2.7 Protein-interaction network of identified proteins involved in various biological functions

STRING database (version 10) was used to develop protein network to identify protein and related biological pathways. Proteins specific to *A. flavus* was subjected to STRING database to predict their cellular interactions at confidence level of 0.400 [301]. The gene ontology annotation of predicted proteins were done using GO and KEGG (Kyoto encyclopaedia of gene and genome) of UniProt database.

3.2.8 Bioinformatic approach for analysis of cell wall biogenesis pathway

Total identified proteins were separated on the basis of biological functions and the proteins involved in carbohydrate metabolism pathway were analysed using FungiFun software (2.2.8) as morphogenesis is the key function in fungal development [302].

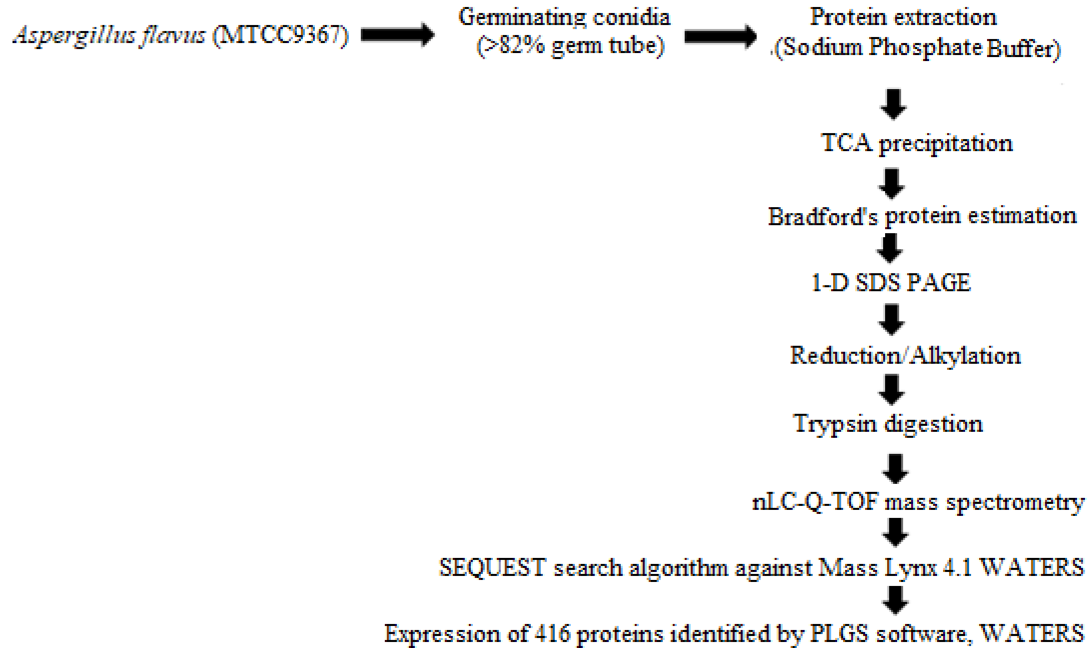


Figure 3.2 The overview of methodology used to accomplish current study

3.3 RESULTS

3.3.1 Conidial germination

Light microscopic studies revealed that *A. flavus* starts bearing germ tube at 7h time point as a result of polarized growth. Cells analysed after 2h of germination showed more than 80% of cells in swollen conidial stage (Figure 3A) which were found to be turned to germ tube satge after 7h of germination (Figure 3B). Counting was performed in triplicates using haemocytometer and the mean were taken, which suggests our data is significant. Figure 3.2 depicts workflow of our study.

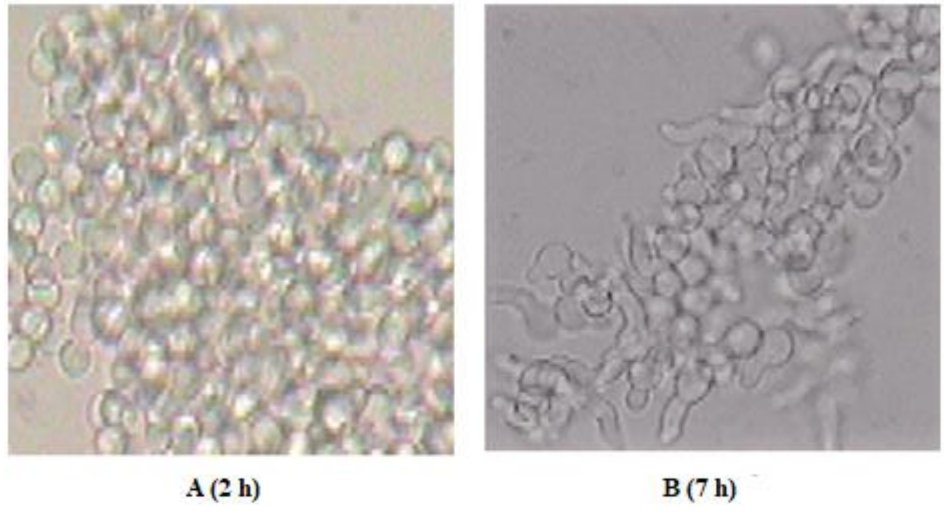


Figure 3.1: Morphological transition of *Aspergillus flavus* from conidia (2 h) to germinating stage (7h)

3.3.2 Global proteome analysis of *A. flavus* germinating conidial stage using nLC-Q-TOF mass spectrometer

nLC-Q-TOF analysis resulted into 416 cellular proteins of *A. flavus* which was identified using UniProt database specific to *Aspergillus* species. Identifies 416 proteins included *Aspergillus* orthologs (*A. awamori*, *A. niger*, *A. parasiticus*, *A. terrus*, A. *oryzae*, *A. clavatus Emericella nidulans*, *Neosartorya fumigate* and *N. fischeri*) based on the Protein Lynx Global Server (PLGS) score. The results also included sequence coverage, matched peptides, pI and molecular weight of the identified proteins. Further, identified proteins MW ranged between 2.5 to 25 kDa, and the pI value between 4-11. Furthermore, the sequence coverage lied between 4 to 97 %. The PLGS scores ranged from 2.3 to 2,004. Gel picture of SDS-PAGE showing separated peptides is represented in Figure 3.3. Global proteome of *A. flavus* was compared with the transcriptomic and proteomic studies of expressed protein/ mRNA of *A. fumigatus* & *A. niger* at dormant and geminating stage conidia (Table B.2).

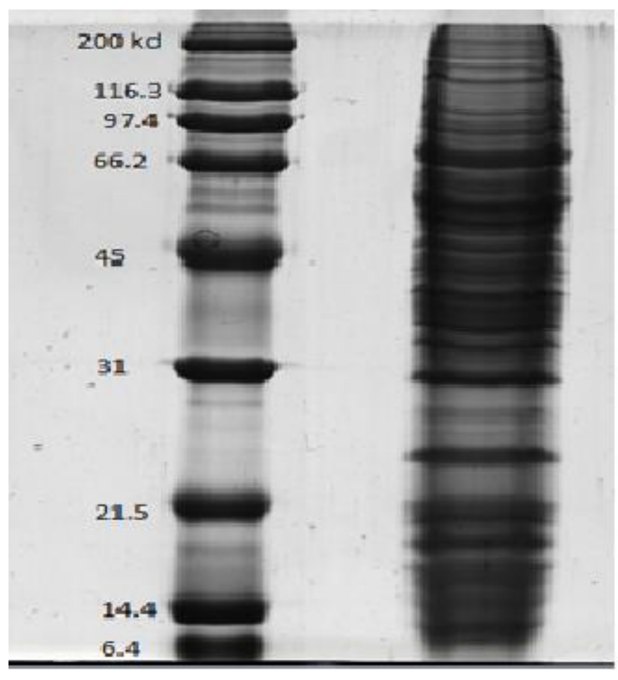


Figure 3.3 Representation of a gel picture showing separated protein bands using 12% SDS-PAGE of germinating stage of *Aspergillus flavus* (7h)

Gene ontology functions were attributed to 416 proteins using UniProt database. Expressed proteins were assigned single GO terms as molecular functions, cellular component and biological functions (Figure 3.4). Among the expressed proteins molecular functions includes proteins from hydrolase activity (36 %), transferase activity (14.6 %), nucleic acid binding activity (10.5%) and antioxidant activity (11.9%). Further cellular component analysis belonged to mitochondrial proteins (21%), nucleus (20.8%), secretory protein (13.72%), membrane proteins (12.8%) and others**.** Further the GO categorisation on the basis of biological functions showed proteins expressed from carbohydrate metabolism (25%), metabolism of nucleic acid (12.6%), secondary metabolism activity (9.2%), amino acid metabolism (7.1%), lipid metabolism (4.2%) and other protein functions.

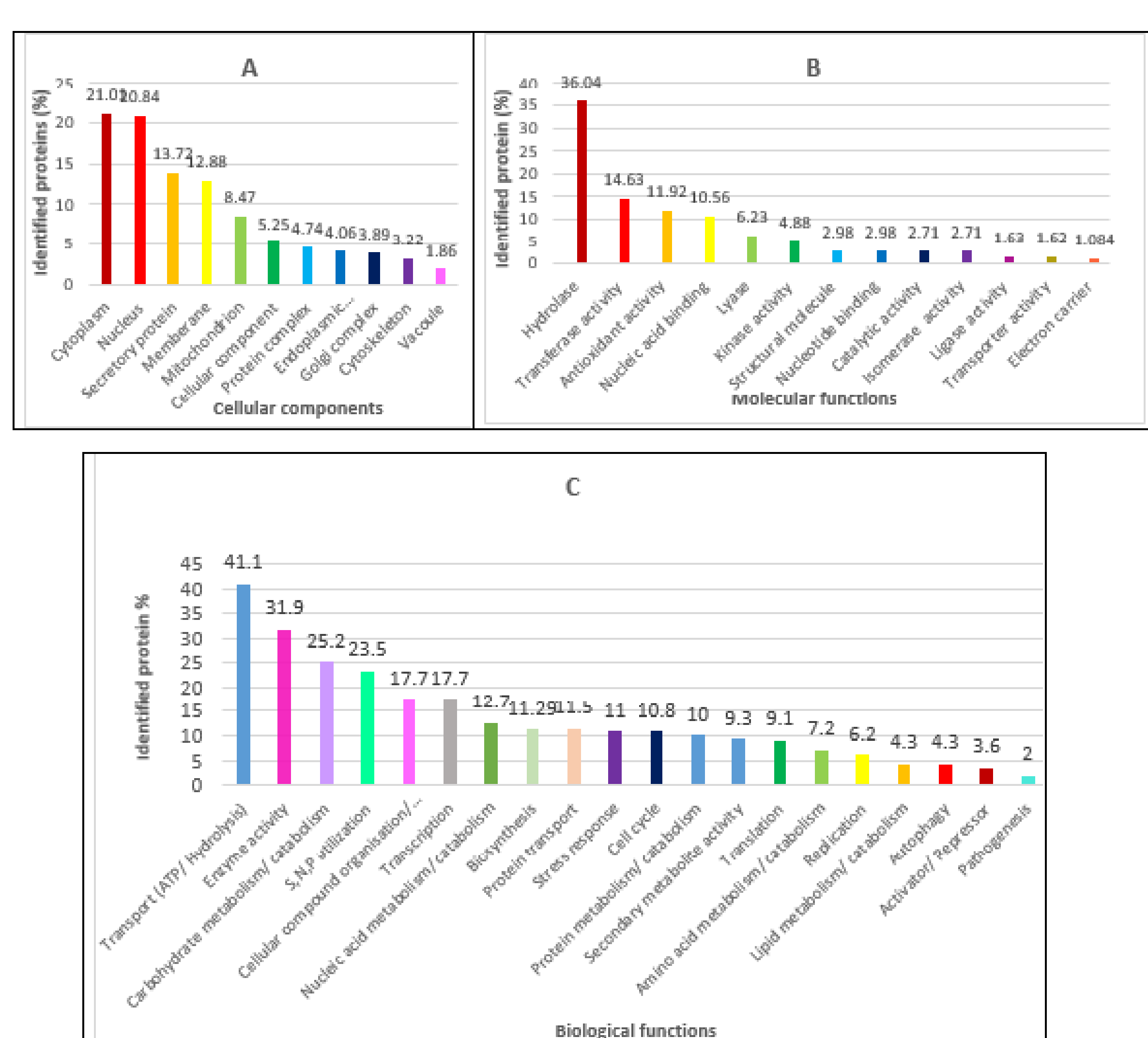


Figure 3.4 Cellular functions (A), Molecular functionas (B) and Biological functions (C) of identified proteins from GO interaction studies

Along with UniProt database 416 proteins were also searched against NCBI database for homologs or orthologs if reported in *A. flavus*. The analysis revealed out of 416 proteins in *Aspergillus* species, 389 proteins were reported in *A. flavus*, whereas 27 proteins were not recognized in *A. flavus* in both NCBI and UniProt database (Table 3.1).

Table 3.1: List of 27 identified proteins which were not recognized in *A. flavus* present in NCBI protein database and UniProt database

Accession No.	Protein name	Reported organism	GO functions
Q078T0	3- hydroxyphenylacetate 6 hydroxilase	*E. nidulans*	Phenylacetate degradation, Aromatic compound metabolism
A2R180	Actin cytoskeleton regulatory complex protein pan1 (Pan1)	*A. niger*	Endocytosis, Calcium ion binding
Q12732	Averantin hydroxylase	*A.parasiticus*	Aflatoxin biosynthetic process Monooxygenase, Oxidoreductase
A2QM49	Lucopene cyclise phytoene synthase	*A.niger*	Carotenoid biosynthesis, Intramolecular lyase activity, Isomerase, Transferase
Q5BBL4	Class E vacuolar protein sorting machinery protein (Hse1)	*E.nidulans*	Ascospore-type prospore assembly, Membrane budding, Protein transport
Q03149	Conidial yellow pigment specific polyketide synthase	*E.nidulans*	Heptaketidenaphthopyrone YWA1biosynthesis, Polyketide biosynthesis, Pigment biosynthetic process, Conidiation, Sporulation
Q9Y7C8	Dihydromonacolin L monooxygenase (LovA)	*A.terreus*	Polyketide biosynthetic process, Lovastatin biosynthesis, Monooxygenase, Oxidoreductase
Q5B6U3	DNA damage binding protein (Cmr1)	*E.nidulans*	Cellular response to DNA damage stimulus
A1DA65	Fumitremorgin C monoxygenase	*N. fischeri*	Alkaloid metabolism, Monooxygenase, Oxidoreductase
A1CBF3	Increased rDNA silencing protein 4	*A. clavatus*	ATPase activity, Coupled to transmembrane movement of substances
A1CLY7	Ketocytochalasin monoxygenase	*A. clavatus*	Monooxygenase, Oxidoreductase
P24686	Negative regulator of mitosis	*E.nidulans*	Ubiquitin-protein transferase activity, Cell cycle, Cell division, Mitosis
A1C8C3	Ophiobilin-F synthase	*A. clavatus*	Isoprenoid biosynthetic process, Lyase, Transferase, Magnesium ion binding, Terpene synthase activity
A2R919	Cft1	*A. niger*	mRNA processing
Q9Y7B3	Protein dopey	*E. nidulans*	Protein transport, Cell morphogenesis, Cleistothecium development, Conidium formation, Endoplasmic reticulum organization
Q5BGR2	Protein mesA	*E. nidulans*	Establishment of cell polarity, Hyphal growth
Q5BDB9	Protein OS 9 homology	*E. nidulans*	ER-associated ubiquitin-dependent protein catabolic process, Retrograde

			protein transport, ER to cytosol
A1D3V8	Sds 23	*N. fischeri*	Serine/threonine phosphatase inhibitor activity, Cellular response to glucose starvation, cell cycle
P24817	Ribosomal inactivating β-momorchin	*M. charantia*	Antiviral protein, Hydrolase, Protein synthesis inhibitor, Toxin, rRNA N-glycosylase activity
Q2TWP5	Senstive to high expression protein 9 homolog mitochondria	*A. oryza*	Sterigmatocystin biosynthesis, Mycotoxin biosynthesis, RNA polymerase II transcription factor activity
Q9UUZ9	Thiamine thiazole synthase	*A. oryza*	Thiamine biosynthesis, Mitochondrial genome maintenance, Response to stress, Thiazole biosynthetic process
A2QFG8	Transcription activator of gluconeogenesis acuK	*A. niger*	Gluconeogenesis,Transcription, Transcription regulation, Activator
A2QJF9	Transcriptional activator of proteases prtT (prtT)	*A. niger*	Positive regulation of pyrimidine-containing compound salvage, transcription, Regulation of protein catabolic process
Q4WE58	tRNA adenine 58 N 1 methyltransferase non catalytic subunit (trm6)	*A. niger*	tRNA (adenine-N1-)-methyltransferase activity, tRNA processing
A1DA60	Tryprostatin B 6 hydroxylase	*N. fischeri*	Alkaloid biosynthesis, Monooxygenase, Oxidoreductase
Q5B288	AN5342	*E. nidulans*	Methylation, Stress response
A1CEE0	Vacuolar membrane associated protein (iml1)	*A. clavatus*	Intracellular signal transduction, Regulation of autophagosome assembly, GTPase activator activity

3.3.3 MAPK pathway; crucial signaling pathway for *Aspergillus flavus* germination

Morphogenesis of *A. flavus* involves germination of *A. flavus* conidia viz. carbohydrate metabolism process, protein synthesis, cell cycle and signal transduction proteins (Figure 3.5). Proteins identified related to carbohydrate metabolism were β-mannosidase, β-glucosidase, α-galactosidase, exopolygalactoneurase, enolase, hexokinase-I, mannitol 2 dehydrogenase, rhamanogalacturonase etc. Further proteins related to protein synthesis process involved 40S ribosomal protein (S1), pob3, FK506 binding protein 4, spt16, DnaJ, pepdyl prolyl cis trans isomerase, tRNA uracil-O-2, methyltransferase, trm82, trm6, eukaryotic translation initiation factor 3 subunit A etc. We have observed various proteins involved in cell cycle such as sds23, grrA, separin, chitin synthase C, flap endonuclease, DNA ligase 4, sepA, abaA, bimC, ficompl 1 mytochondrial specific, sec31 sconB, cell

division control protein etc. We have observed the expression of several proteins involved in the activation of signaling pathways such as mpkC, cdcA, MARK2, PKC etc.

Protein kinase C
Serine threonine protein kinase MAkk2
Serine threonine protein kinase ste20
Serine threonine protein kinase 3
Serine threonine protein kinaseNek3
Serine threonine protein phosphatase 2B catalytic subunit
Mitogen activated protein kinase mpkc
Tyrosine protein phosphatase cdcA
Increased rDNA silencing protein 4

MAPK pathway

Conidia having pre mRNA and ribosomes

Germinating conidia

Digestive enzymes
Enolase
Protein png1
Hexokinase1
Pectate lyase
Pectinestrase
Isocitrate lyase
Neutral trehalase
Chitin synthase
Alpha xylosidase
Feruloyl esterase
Beta glucosidase
D xylulose kinase
Beta mannosidase
Beta galactosidase
Alpha galactosidase
Phosphoglucomutase
Pyruvate carboxylase
Protein OS 9 homolog
Alpha glucosidase
Rhamanogalactoneurase
Exopolygalactoneurase
Endo 1,4 beta xylanase
Xanthin dehydrogenase
Putative urea carboxylase
Mannitol 2 dehydrogenase
Nitrate reductase NADPH
GPI mannosyl transferase
Aconitate hydratase mitochondria
Lactame utilizaztion protein lamB
Glucan endo 1, 6 beta glucosidase
GPI ethanoloamine phosphate transferase
Arabinan endo 1,5 alpha L arabinosidase B
Methylthioribulose 1 phosphate dehydrogenase
Glutathione dependent formaldehyde activating

Translation
Dicer like proteins
FKS06 binding protein 4
40S ribosomal protein S1
FACT complex subunit spt16
LFACT complex subunit pob3
Dnaj homology 1 mitochondria
Protein disulfide isomerase tigA
Mannose 6 phosphate isomerase
tRNA uracil O 2 methyltransferase
Peptidyl prolyl cis trans isomerase
Elongation factor G mitochondrial
ATP dependent RNA helicase eIFA4
Dipthamide biosynthesis protein
tRNA guanine 37 N1 methyltransferase
tRNA dihydrouridin 47 synthase NADP
Clustered mitochondria protein homolog
Eukaryotic translation initiation factor 3
Signal recognition particle 54 kDa protein
Polyadenylate binding protein cytoplasmic
Mitochondrial zinc maintainance protein 1
Eukaryotic translation initiation factor 3 subunit
trRNA guanine N 7 methyltransferase non catalytic
tRNA adenine 58 N1 methyltransferase non catalytic

Cell cycle (Mitosis)
Separin
Protein sds23
DNA ligase 4
F box protein greA
Chitin synthase C
SCF subunit sconC
Flap endonuclease 2
Autophagy protein 5
Transcription factor steA
Regulatory protein abaA
Cytokinesis protein sepA
Cytokinesis protein sepH
Kinesin like protein bimC
E3 ubiquitin ligase complex
Negative regulator of mitosis
Mitochondria fusion 1 protein
Nitrogen permease regulator 3
Transporter protein sec31
SCF subunit sconB protein ste1
Protein disulfide isomerase tigA
Sterol 3 beta glucosyl transferase
Nuclear disruption protein nudF
SCF E3 ubiquitin ligase complex
Tyrosin protein phosphatase cdcA
Orotidine 5 phosphate decarboxylase
DNA mismatch repair protein msh3
Topoisomerase 1 associated factor 1
E3 ubiquitin ligase complex
Pro apoptotic serine protease nma1111
MYND type zinc finger protein samB
Plasma membrane fusion protein prm1
Chromosome segregation protein
Phosphatidylinositol transferase protein sfh5
Mitochondrial disruption and morphology

Figure 3.5: Representation of the sequential event involved germination of *A. flavus*, important biological functions were attributed, diagram dipicted using coral draw software (x8)

3.3.4 *Aspergillus flavus* is accompained by modulation of cell wall

Categorisation of 416 proteins showed majority of proteins from cell wall modulation, revealed that modulation of cell may be a crucial step in *A. flavus* germination. Important

proteins were Fks 1, ArgJ (mitochondrial), pectate lyase A, exopolygalacturonase C, GPI ethanolamine phosphate transferase 1, chitin synthase C, endopolygalacturonase B etc. In addition, enzymes such as β-galactosidase, β-glucosidase (A, B and C), endo β-1, 4 gucosidase, agdC etc were observed and reported to be involved in glucan degradation.

3.3.5 Protein/enzymes expressed in biosynthesis of AF

Functional analysis of 416 proteins/enzymes revealed the expression of various proteins/enzymes related to pathway involved in AF biosynthetic. Example are AflR, AflN, averufin oxidase-A, sterigmatocystin-8-O-methyltransferase, norsolorinic acid reductase, VERB desaturase and O-methyl sterigmatocystin oxidoreductase. GO studies further showed proteins involved in secondary metabolism process such as, mep20, nonribosomal peptide synthetase (13, 10 and 11), psi producing oxygenase A and β-cyclopiazonate dehydrogenase.

3.3.6 Proteins associated with autophagy, dicer like and heat shock responses

Expressed proteins were also found to be involved in stress responses and defence activity. Major classes of proteins involved in above functions were related to dicer proteins (1, 2 and 22), Hsps (60 and 70 kDa) and autophagy proteins (sec16, autophagy protein 9, metacaspase 1B, sterol 3 β-glucosyltransferase, N-permease regulator 3 and COPII). Other proteins related to stress response were catalase peroxidase, grrA, DnaJ (mitochondria), catalase A and uba4

3.3.7 Protein-protein interactome of identified proteins of *Aspergillus flavus*

Protein-interaction function of a protein could be determined by its interaction with other proteins using STRING (10). Thus protein-protein interaction has been performed of *A. flavus* specific proteins (due to the specificity of the software). However outliers were removed and more than 2 interactions of proteins were again applied to STRING software (Figure 3.6). Results revealed that, at interaction these proteins were involved in biosynthesis of amino acids, biogenesis of cell wall, carbohydrate metabolism, transcription and protein synthesis given in Table 3.2.

3.3.8 Bioinformatical approach for analysis of carbohydrate metabolism pathway

Proteins identified as carbohydrate metabolizing proteins in *A. flavus* were further analyzed for their association in different pathways (cellular, molecular and biological). The analysis was done using FungiFun software (2.2.8). Results showed that proteins involved in

carbohydrate metabolism were also involved in various pathways such as cellular metabolic processes, metabolism of organic substrates and macromolecules and biogenesis of cell wall. The proteins selected in this study are listed in Table B.1.

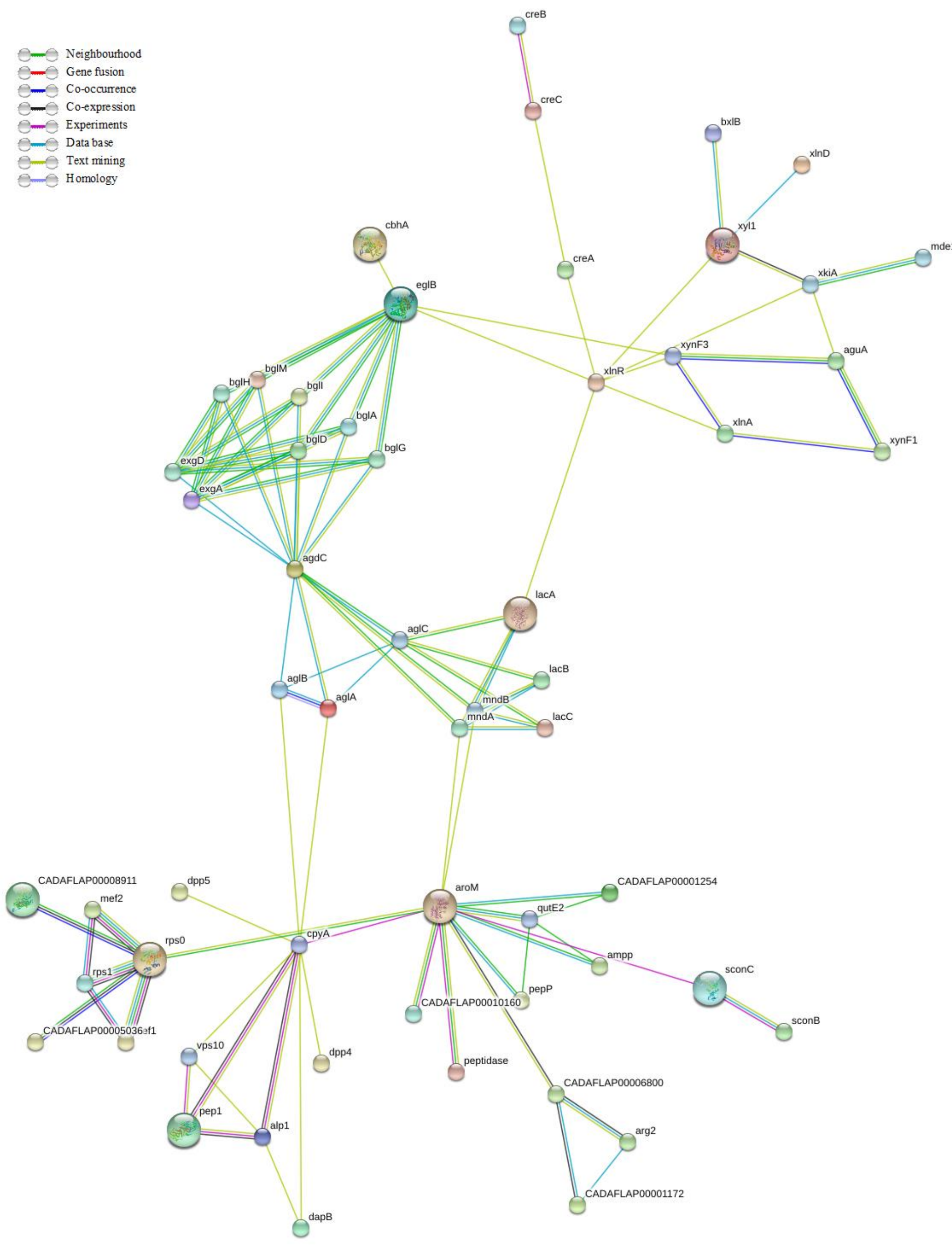


Figure 3.6: Protein-protein interaction network of expressed proteins from germinating *A. flavus* conidia using STRING software (version 10), interacting proteins are listed in table 3.2

Table 3.2 Proteins more than 2 interactions, used for construction of interactome network with the help of STRING database, listed in GO (biological functions)

Functional Category	String ID	Protein Name
Carbohydrate metabolism	xyl1	NADPH dependent D xylose reductase xyl1
	cbhA	1 4 beta D glucan cellobiohydrolase A
	xkiA	D xylulose kinase A
	agalA	A- galactosidase A
	xlnD	Exo 1 4 beta xylosidasexlnd
	agdC	A- beta glucosidase agdc
	eglB	Endo beta 1 4 glucanase B
	aglB	A- galactosidase B
	exgA	Glucan 1 3 beta glucosidase A
	lacC	β- galactosidase C
	bglM	β- glucosidase M
	lacA	β-galactosidase A
	bglI	β-glucosidase I
	aguA	α-glucuronidase A
	bglD	β-glucosidase D
	bglG	β-glucosidase G
	mndA	β-mannosidase A
	exgD	Glucan 1 3 beta glucosidase D
	bglH	β- glucosidase H
	bglA	β-glucosidase A
	mndB	β- mannosidase B
	aglC	A-galactosidase C
	xynF1	Endo 1 4 beta xylanase F1
	xlnA	Endo 1 4 beta xylanase A

	lacB	β-galactosidase B
	xynF3	Endo 14 beta xylanase F3
	bxlB	Exo 14 beta xylosidasebxlb
Amino acid Biosynthesis	aroM	Pentafunctional AROM polypeptide
	arg2	Amino acid acetyltransferase mitochondrial
	CADAFLA P00006800	Arginine biosynthesis bifunctional protein argj mitochondrial
	mde1	Methylthioribulose 1 phosphate dehydratase
Amino acid metabolism	cpyA	Carboxypeptidase Y homolog A
	CADAFLA P00010160	Carboxypeptidase
	peptidase	Carboxypeptidase
	dapB	Dipeptidyl aminopeptidase B
	dpp4	Dipeptidyl peptidase 4
	dpp5	Dipeptidyl peptidase 5
	pepP	Xaa Pro aminopeptidase pepp
	CADAFLA P00001254	Xaa Pro aminopeptidase
Transcription	creC	Catabolite repression protein crec
	xlnR	Xylanolytic transcriptional activator xlnr
	ampp	Xaa Pro aminopeptidase P
	creA	DNA binding protein crea
	sconB	E3 ubiquitin ligase complex SCF subunit sconb
	creB	Ubiquitin carboxyl terminal hydrolase creb
Translation	rsp0	40S ribosomal protein S0
	mef1	Elongation factor G mitochondrial
	mef2	Ribosome releasing factor 2 mitochondrial
	rps1	40S ribosomal protein S1
	CADAFLA P00006800	Arginine biosynthesis bifunctional protein argj mitochondrial

Cell wall biogenesis	agdC	α,β glucosidase agdc
	exgA	Glucan 1 3 beta glucosidase A
	exgD	Glucan 1 3 beta glucosidase D
Transport	vps10	Vacuolar protein sorting targeting protein 10
	xlnD	Exo 1 4 beta xylosidasexlnd
Proteolysis	alp1	Alkaline protease 1
Nucleic acid Biosynthesis	CADAFLA P00001172	Adenylosuccinatesynthetase
Cell cycle	sconC	E3 ubiquitin ligase complex SCF subunit sconc
Aromatic compound metabolism	qutE2	Catabolic 3 dehydroquinase 2

3.4 DISCUSSION

This chapter includes *A. flavus* proteomic profile at germinating conidial stages. To our understanding, this is the primary record describing global proteome analysis of *A. flavus* at germinating conidial stage. Previously, proteome analysis of *A. flavus* at mycelia stage has been conducted by Olga Pechanova et al., which exhibited protein from metabolic process and enzymes involved in AF biosynthesis [52]. Recently, *A.niger* transcriptomic studies has been carried out by Leeuwen et al., at germinating conidial stage (8 h) that showed polarized growth is involved in germ tube which includes transcription, cell wall biogenesis, carbohydrate metabolism and DNA processing [291]. In the present study global proteome profile of *A. flavus* at germinating conidial stage confirmed expression of carbohydrate metabolism proteins, cell wall biogenesis proteins, and AF biosynthesis proteins. MAPK signalling pathways was found to be active during morphogenesis. Heat shock proteins, autophagy proteins, dicer like proteins, proteins involved in pathogesis and allergen proteins were also observed.

Fungal cell wall has been known prime target for antifungal drugs in recent studies. So it is vital to understand the pathway of cell wall biosynthesis and the singal transduction pathway involved in early germination stage, using global proteome analysis. β-1, 3-glucansynthase (Fks1), a plasma memberane protein was observed in our studies. According

to several studies, deletion of *fks1* makes the fungal species lethal [303, 304], however, characterization of mutant *fks1* has been successfully accomplished in *A. fumigatus* Δ*fks1* [305], which contradicted the statement. Second component most aboundently found in fungal cell wall is chitin. *csmA* and *csmB* are the chitin synthase genes involved in decreasing A. *fumigatus* growth rate [294, 306]. In our study, we found two proteins (GPI monnositol transferase 3 and GPI ethanolamine phosphate transferase 1) which act as anchor proteins and play important role in formation of cell wall can be crucial for drug targets. FungiFun analysis also revealed that cell wall organization is also mediated by proteins involved in carbohydrate metabolism, which also includes glucosidase and hydrolase activity.

Total proteome analysis of mycelia stage of *A. flavus* showed AF biosynthesis proteins such as AflK, VERB synthase, O-methyltransferase A and AFB1-aldehyde reductase [52]. Furthermore, it has been observed that proteins/enzymes involved in a biosynthesis of AF may starts at early stage, as we found enzymes such as AflN, averantin hydroxylase, p450 monooxygenase, versicolorin B desaturase etc. in our studies. These findings reveal that biosynthesis of AF may starts at early germination stages of *A. flavus*.

Previously it has been reported that MAPK pathway is predominant pathway in *A. nidulans* which executes development of fungi and production of secondary metabolites [307]. Our studies also co related with the previous studies as we observed various signaling proteins such as mpkC, Pkc, serine threonine protein kinases atg1, tyrosine protein kinases cdcA etc. which suggests MAPK pathway is active in germinating conidial stage of *A. flavus*. Also, studies on filamentous fungi by Dirr et al., and Valinate et al., showed MAPK pathway is the active pathway, which needs Pkc for activation [308, 309]. So, our finding revealed that in early germination stage MAPK pathway plays crucial role in biogenesis of cell wall and morphogenetic transition from one form to other. Also, our studies suggested that MAPK pathway may involve in biosynthesis of AF in *A. flavus* at germinating conidial stage. Previously it has been shown that expression of *aflR* and *laeA* (transcriptional factors in secondary metabolite gene clusters) is directly proportional to presence of MAPK module [307].

Studies related to development of antifungal drug targets showed that, in recent years, fungai have developed resistant strains against various classes of drugs such as, azoles, echinocandins, polyenes etc. [310]. Previously, *A. fumigatus* paradoxical effect was studies and the findings revealed that, in response to echinocandin, expression of *pkcA* was found to be upregulated, which may had triggered calcineurin pathway and synthesis of chitin [311]. Another study on *A. fumigatus* showed ergosterol biosynthesis proteins, AflN, p450 monooxygenase and cytochrome P450 reductase, which were suggested as proficient drug targets [294]. The same is also found in our results. Cell wall proteome analysis of *A. fumigatus* by Camper J et al., showed various conserved fungal proteins such as Gel1-4, Ecm33 Crf1, EglC and Bgt1 and showed no coherence with human proteins Gel1and Crf1 were further used as antifungal targets to develop vaccine candidates [312]. Our study showed two proteins specific to fungi viz. EglB and Ecm14 involved in metabolism of carbohydrates and may be studied for drug targets against *A. flavus*. Aspf12 is another class of proteins specific to fungi having no homology with humans, involved in the regulation of immune responses in *A. fumigatus* [313]. We also found Aspf1 2 in our study, which could be studied for development of vaccine candidate against *A. flavus* in early germination stages.

Hsps are widely known to express in different stress conditions, which are also reported to be involved in morphogenesis [314]. In our studies we found the expression of Hsp60 and Hsp70, which may also be involved in morphogenesis of *A. flavus.* Previous transcriptomic studies on *A. flavus* (aflatoxigenic and non-aflatoxigenic strain) have shown the expression of *hsp70* at 30ºC [315]. However, *A.fumigatus* proteome profile showed Hsp90 protein is involved in morphogenesis and conida formation, whose scarcity causes susceptibility for antifungal drugs [314, 316, 317]. We did not find Hsp90 in our studies.

Autophagy proteins in fungi are known to involve in transportation process and nutrient recycling, which provides cell survivability for prolonged time and also important in germination of conidia. Global proteome analysis of *A. flavus* (germinating stage) provided proteins related to autophagy process such as sec16, autophagy related protein 2, 9, 13, 22 etc. (discussed in results), which suggests autophagy process is active at early germination stages of *A. flavus*. Previous studies showed that autophagy genes *(atg1* and *atg8*) are

directly linked with growth and development of fungi [318]. Post transcriptional regulation in fungi is mediated by dicer like proteins [319], which could be involved in the fungal morphogenesis. In our study, we observed Dicer like proteins (1, 2 and 21), which can be an interesting area to explore further for identificant of anti-morphogenetic fungal targets. Further, STRING analysis for interactome study of *A. flavus* proteins (157) were carried out. Proteins showing maximum interaction were AROM polypeptide, which is known to be involved in biosynthesis of amino acids followed by agdC, a cell wall biogenesis protein. Further, protein related to activation of transcriptional processes (xlnR) was also found to show significant interactions.

Global proteome analysis also showed several pathogenic proteins such as β-cyclopiazonate dehydrogenase, mep20, NRPS (10, 11, 13, 3), sphingomyelinase D and psi producing oxygenase A expressed at early germination stage of *A. flavus* (7h). In previous studies, β-cyclopenazole dehydrogenase has been found to be involved in the synthesis of α-cyclopiazonic acid (fungal neurotoxin) [320]. Psi producing oxygenase A is known to oxidize linoleic acid and also involved in secondary metabolite regulation and also triggers the activation of human immune system in response to fungal development [321]. Also, sphingomyelinase D is the reported pathogen in *A. flavus*, as it mediates lysophospholipid cleavage by hydrolase activity [322]. So this chapter concluded that *A. flavus* germination could activate MAPK pathway to client cell wall modulation and biosynthesis of secondary metabolites.

SUMMARY

Proteomic studies at germinating stage of conidia showed that *A. flavus* involves MAPK signaling pathay, cell wall modulation and production of aflatoxin biosynthesis enzyme.

CHAPTER 4

To Understand the Mechanism of Action of Quercetin Mediated Inhibition of Aflatoxin Biosynthesis in *Aspergillus flavus*

4.1 INTRODUCTION

As discussed in previous chapters *A. flavus* produces most potent mycotoxin, AF (AFB1 and AFB2) [323, 324]. Around 4.5 billion population of developing countries are present to the uncheck measures of AF which brings about severe aflatoxicosis [325, 326]. The WHO has adviced that there is an increase in threat of hepatocellular carcinoma due to AF intake from food products in lower doses [327]. Along with this *A. flavus* is also responsible to cause aspergillosis in humans [328]. To withstand the AF exposure, EU has set the limit of AF consumption, which ranges between 10-30 ppb in most of the countries [329, 330]. These limitations results in the economic loss in the agricultural industries [331].

Zea mays L. (maize) is a primary food crop after rice and wheat [332]. Maize is a most susceptible crop for *A. flavus* colonization. Also, maize contaminated with AF, if consumed by animals/humans causes biotransformation of AF into carcinogenic AFB1-exo-8, 9 epoxide [333]. Analysis of corn flour for AF contamination showed 80% sample was contaminated with AFB1, which was higher than EU regulation limits [334]. Recently, transcriptomic studies on interaction of maizen and *A. flavus* showed increase in the expression of genes involved in AF biosynthesis pathway [335]. Various studies have been conducted onmaize-pathogen interaction till now [242]. Recently, genotypic and phenotypic interaction studies (48h) of *A. flavus* and maize was performed in different conditions such as sterilized maize kernals, injured maize kernals and *in-vivo* conditions which resulted in the expression of a total of 819 unique genes [336]. Until now, there are limited proteomic studies on *A. flavus* and corn interaction. Proteome studies are important over transcriptomics, as post-transcriptional regulations may be the alteration in gene products [55]. To restrict AF from food crops requires an economical, efficient and sustainable source, to overcome contamination and food-crop losses. Phytochemicals (thiols, phenolic, flavonoids, tocopherol, anthocyanin and carotenoids) found in different plant parts (fruits,

vegetables, seeds, leave, etc.) involves an extensive range of biological effects (antimicrobial, anti-inflammatory and antioxidant) [249, 250]. Studies conducted by Zhou et al., (2015) on quercetin mediated inhibition of AFB1 at 800 µg/ml concentration produced by *A. flavus* [228]. However, molecular mechanism behind quercetin mediated inhibition of F biosynthesis is unknown. Other studies on quercetin inhibitory mechanism in AF mediated damage of hepatic cells of mice (HepG2) showed that quercetin mediates production of reactive oxygen species, responsible to cause cytotoxicity and lipid peroxidation [337].

Thus, proteomic approaches (nLC-Q-TOF mass spectrometry) were undertaken to understand the mechanism of quercetin mediated inhibition of *A. flavus* AF biosynthesis pathway. To further understand the role of substrate in *A. flavus* germination, two different substrates were considered, viz. *A. flavus* cultured in corn flour media (CF) and *A. flavus* cultured in corn flour media with quercetin (CF_Q) for the current study. Results of current chapter demonstrated that trans-membrane transporter proteins were highly expressed in response to CF_Q in comparison to CF. In addition, cAMP/PKA signaling pathway was observed in CF_Q in comparison to CF. Also, AFB1 at different time point (7h, 12h, 24h and 48h) using quantitative High Performance Liquid Chromatography (HPLC) was determined. Overall, we also demonstrated the inhibition of AF biosynthesis in the presence of quercetin in *A. flavus*.

4.2. MATERIAL AND METHODS

4.2.1 *Aspergillus flavus* culture conditions and quercetin treatment

Aspergillus flavus conidia (MTCC9367) were harvested and working conida concentration of 10^6 cells/ml were prepared, also germ tube stage was achieved as described in previous chapters (chapter 2 and chapter 3) [338]. Large-scale culture for protein extraction was performed at 7h time point (germ tube stage). Results from previous chapter-2 showed that MIC_{50} value of quercetin against *A. flavus* is 113µg/ml. Hence, in the current chapter, to understand the inhibitory action of quercetin against *A. flavus*, previously determined MIC_{50} value has been used for the proteomic analysis [229]. Working solution of MIC_{50} (113µg/ml) of quercetin (HiMedia, India) was freshly prepared in 90 % methanol of HPLC grade (Sigma Aldrich, India). A total of 10^6cells/ml were inoculated in both the substrates (CF and CF_Q) at pH 5.7 for 7h and incubated at 30°C. Germinated conidia of *A. flavus* were

filtered, washed thrice (to remove additional media) and subjected to protein extraction. For the quantitative estimation of AFB1, HPLC analysis was conducted and extraction of culture filtrate was performed at four different time points (7h, 12h, 24h and 48h). Pellets were further stored at 4°C for further use. Figure 4.1 depicts workflow of study**.**

4.2.2 Total protein extraction and identification by nLC-qTOF mass spectrometric analysis

As described in previosuely reported studies, media (substrate) play a critical role in morphogenesis of fungi and expression of proteins and transcripts [339, 340, 341]. Hence, we have selected corn flour supplemented media in the current chapter whereas in the chapter-3 (SD broth, a labatory media for fungus culture). Corn flour supplemented media was selected to mimic the natural host/crop such as corn to understand substrate dependent expression of proteins and biochemical pathways. In the current study proteins were extracted from *A. flavus* cultured in corn flour with quercetin (CF_Q) and without quercetin (CF) following the protocol described in chapter-3 using sodium-phosphate extraction buffer method [49]. Also, the extracted proteins were estimated by Bradford's method [297]. Further subjected to SDS-PAGE protein analysis as described in chapter 3 [298]. Protein samples were subjected to ammonium bicarbonate buffer treatment, precipitation and trypsin digestion as described previously. The digested peptides were separated using C18 nano-LC column. Protein separation and digestion studies was performed at Sandor Life sciences Pvt. Ltd., Hyderabad, India (sandorlifesciences.co.in), which included Synapt G2 (Waters, India Inc.), electrospray ionization technique, quadruple time of flight analyzer as described in previous chapter [338]. PLGS software was used to match MS/MS spectra [299]. Overlaier were removed and the final data showed 843 *A. flavus* proteins in CF and 705 *A. flavus* proteins in CF_Q. To understand the quantitative expression difference between identified proteins from CF and CF_Q, both the proteins were compared on the basis of matching peptides. The software normalized the ratio of spectral intensity ratio were normalized using software followed by calculation of ion count based relative difference in differentially expressed proteins [339].

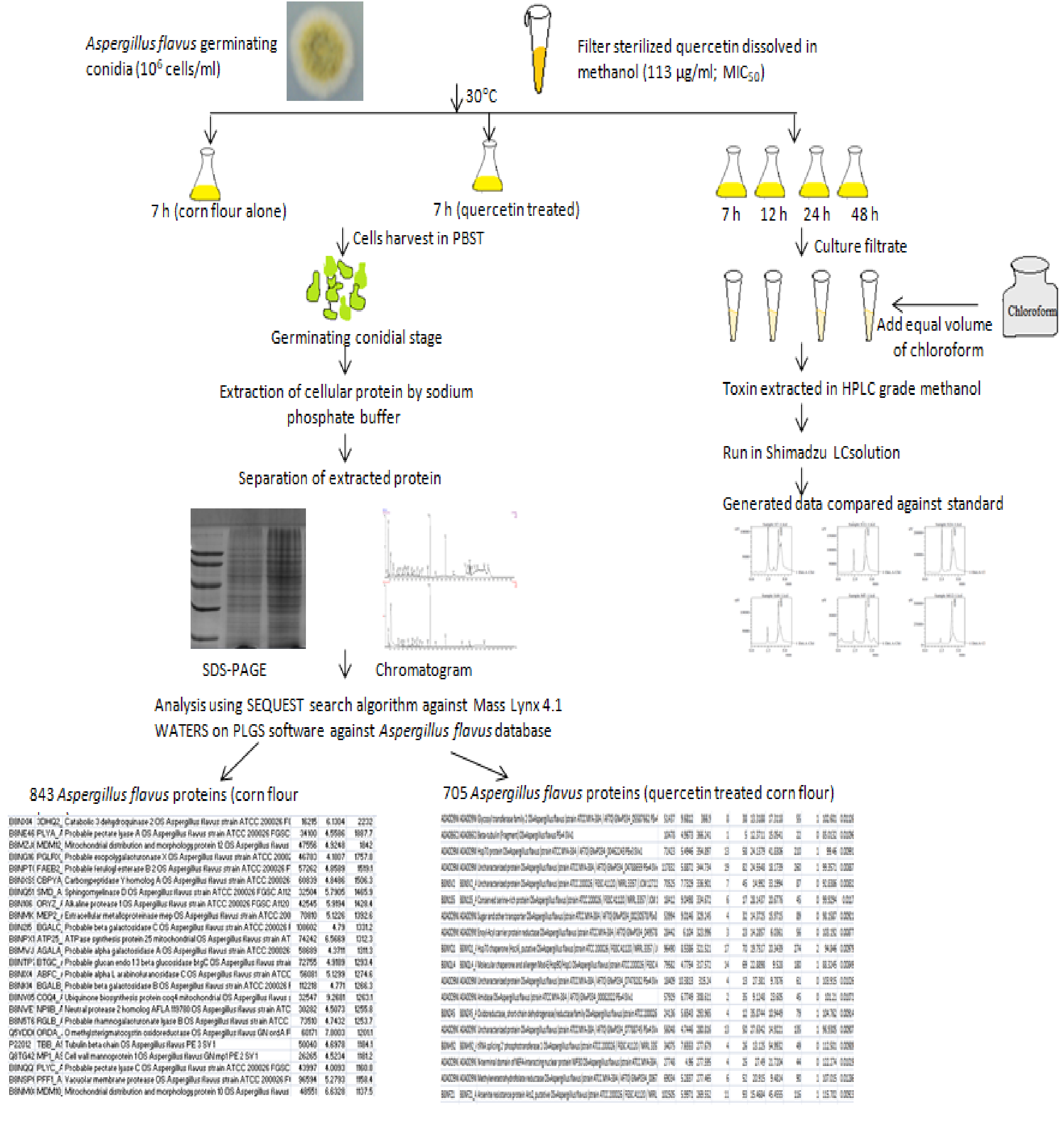


Figure 4.1 Experimental designs to obtain protein data and HPLC analysis of *A. flavus* cultured on corn flour with and without quercetin (113 µg/ml) at 30°C, followed by protein profiling form PLGS software using UniProt database

4.2.3 Scanning electron microscopy

As swelling of conidia is the key step in germination of *A. flavus*. Hence, to understand the inhibitory mechanism of quercetin on swelling of *A. flavus* conidia, SEM analysis was performed. For SEM analysis, 10^6 conidia of *A. flavus* were subcultured on CF and CF_Q for 4 hours and were harvested by centrifugation at 2700 rpm and washed with sterile distilled

water. Conidia were then fixed in 4% gluteraldehyde in PBS under vacuum for 24hours. The cells were washed with distilled water and were post-fixed with 1% osmium tetroxide for 1h and dehydrated by passage through ethanol solutions of increasing concentration. The sample were then mounted on aluminium sheet and coated with gold-palladium alloy. SEM analysis was carried out at Amity University, NOIDA, India. The observations were made on a Zeiss SEM (MA EVO-18 Special Edition).

4.2.4 Isolation and HPLC analysis of culture filtrate for AFB1 detection

To understand the effect of quercetin on AFB1production in CF and CF_Q HPLC analysis was performed at 7h, 12h, 24h and 48h. A total of 10^6 conidia were aseptically inoculated in each flask at 30°C at kept for shaking at 250 rpm. Chloroform mediated extraction of supernatant (1:1 v/v) was performed at 25°C. Separating funnel was used to separate organic phase [340]. AF was separated from chloroform using whatmann no. 1 filter paper followed by air-circulated drying at 37°C in oven. The residues then reconstituted in eppendorf, re-dissolved in methanol (500 µl) of HPLC grade (Merck, India) followed by filteration using 0.22 µm syringe filter. AFB1 standard (Sigma, USA) was also dissolved in HPLC grade methanol (1 mg/ml), and stored for further use at 4°C and filtered using 0.22 µm microporous membrane. Filtered samples (standard and unknown) of 10 µl volume were injected in Shimadzu LC solution HPLC system. The mobile phase used in our analysis was acetone: methanl: water in the ratio of 1:1:2 v/v and 1ml/min of flow rate. UV detection was set at 365 nm. Qualitative HPLC analysis was carried out in Institute of Bioengineering and Biological sciences, Varanasi, India. Further, quantitation of AFB1 in unkonown (anylate) was calculated using following formula [341].

$$\text{Response factor} = \frac{\text{Peak Area of standard AFB1}}{\text{Standard amount}}$$

$$\text{Amount of Anylate } (\mu g/mL) = \frac{\text{Peak Area of unknown}}{\text{Response factor}}$$

To calculate the concentration of AFB1 in unknown samples, all the required values were kept in the above formula and tabulated for further analysis.

4.3 RESULTS

4.3.1 Identification of cellular proteins expressed in CF and CF_Q

Cellular protein identification was performed by nLC-qTOF analysis for global proteome analysis in CF and CF_Q at 7h time point (Figure A.1). Results showed the expression of 843 and 705 proteins respectively. Molecular weight of proteins expressed in CF ranged between 2.4 kDa to 248 kDa whereas in CF_Q between 10.5 kDa to 249 kDa. PLGS score was found to be 9.17 to 2231.95 and 1.13 to 2409.65 respectively, with the sequence coverage ranged between 7% to 100% for CF and 2% to 61% for CF_Q. In differential expression studies a total of 163 proteins were identified. Overall expressed proteins are represented in Figure 4.2. Gene ontology functions were attributed to 843 and 705 proteins was assigned single GO terms such as molecular functions, biological process and cellular components (Figure A.2, A.3). Among the expressed proteins of CF majority of proteins were involved in hydrolase activity (1,4-β-xylosidase XlnD, exopolygalacturonase B, feruloyl esterase B 1, glucan 1,3-β-glucosidase A etc.) followed by nucleic acid binding activity (exosome complex exonuclease Rrp6, sacI domain and endonuclease exonuclease phosphatase, exonuclease etc.). Further, cellular component analysis showed cytoplasmic protein followed by membrane proteins. Further, biological functions of CF proteins showed transcriptional process (fungal specific transcription factor, AF biosynthesis regulatory protein, BZIP transcription factor, apoptosis antagonizing transcription factor, C2H2 transcription factor amdX, swi5, spt6 etc.) and carbohydrate metabolism (α-xylosidase, xlnD, α-galactosidase B, α-L arabinofuranosidase A, β-galactosidase A etc.)

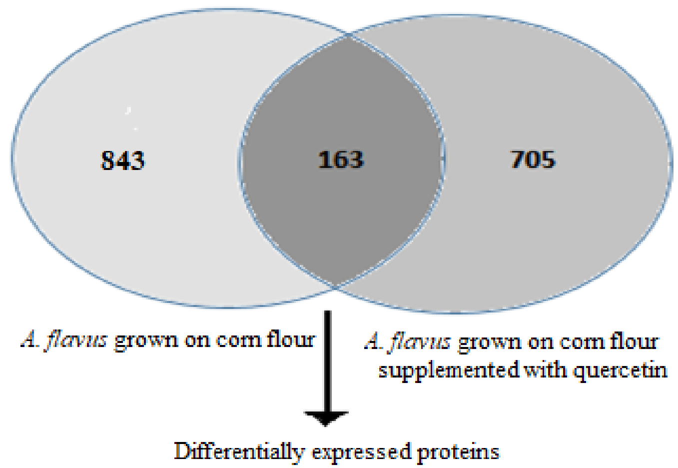


Figure 4.2: Venn diagram representing the comparative analysis of expressed proteins based on similarity and differentially expressed proteins in CF and CF_Q

Also, protein transport activity (sec23, sec10, sterol-3-β glucosyltransferase etc.) was also observed. However, GO studies on CF_Q proteins majorly showed transferase activity (1,3-β-glucanosyltransferase, acetyltransferase GNAT domain, AICARFTIMPCHase bienzyme, amino-acid N-acetyltransferase subunit Mak10 etc.) and protein binding activity (26S proteasome regulatory subunit rpn2, 39S mitochondrial ribosomal protein L46, 5-AMP-activated protein kinase). Further, cellular component analysis showed protein integral components of membrane proteins. GO categorization of biological functions showed organic substrate transport proteins and stress response proteins. Expression of quercetin 2, 3-dioxygenase was also reported in CF_Q which was not observed in CF.

4.3.2 Comparative analysis of differentially expressed proteins in *A. flavus* based on fold change revealed transportation as a major biological function in response to quercetin stress

A total of 163 proteins were found to be differentially expressed in CF and CF_Q from 705 and 843 proteins of CF_Q and CF respectively. Results were analyzed based on fold change. Results (CF_Q/CF) revealed that 105 proteins from a total of 163 proteins were down-regulated (0.027 to 0.93 folds) whereas, 58 proteins were up-regulated (1 to 22.87 folds). Some major categories of proteins (AF biosynthesis, transport activity and oxidative stress response) showing differential expression is represented in Figure 4.3.

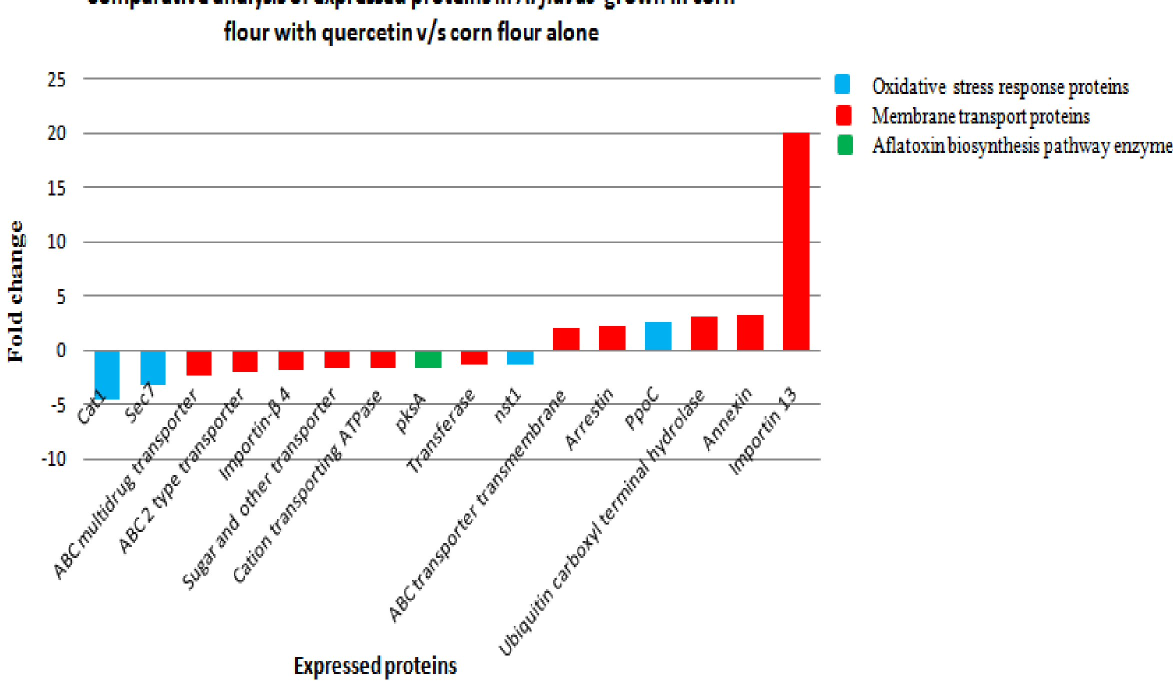


Figure 4.3 Graphical representation of differentially expressed proteins of *A. flavus* that includes oxidative stress response, membrane transport and AF biosynthesis pathway enzymes indicating the up regulation or down regulation of proteins based on fold change in CF_Q v/s CF

Further, 163 proteins (differentially expressed) were subjected to GO studies to identify quercetin mediated activation or inhibition of biological processes. Among the 163 expressed proteins Blast2GO analysis revealed majority of proteins from transport activity (importin 13, hex B, importin-β- 4, sugar and other transporter etc.). ABC transporter proteins showed down-regulation in CF_Q which suggested the inhibition of efflux process in *A. flavus*. Several stress response proteins (sec7, TORC1 growth control complex subunit kog1, acyA, dishevelled Egl-10, pleckstrin domain protein) found down-regulated whereas some of the stress response proteins (Ras-GEF domain protein, leucyl-tRNA synthetase) found up-regulated in CF_Q. These findings revealed that *A. flavus* activates trans-membrane transport protein in responsed to quercetin mediated oxidative stress.

4.3.3 Comparative analysis of trans-membrane proteins expressed in *A. flavus* grown on CF and CF_Q

A total of 120 proteins were found to be expressed in CF_Q, which were involved in organic compound transport activity on the basis of GO studies. Out of these 120 proteins, 34 proteins showed trans-membrane transport activity for example, ABC multidrug transporter, ABC bile acid transporter, ABC transporter trans-membrane, integral plasma membrane protein, Na^+/H^+ antiporter nha1, adaptin, allantoate permease, ankyrin repeat protein, importin-β-N-terminal domain protein, E1-E2 ATPase etc. In addition, amino acid transport proteins were also observed for example, amino acid permeases, aspartate aminotransferase and intracellular protein transporter usoA, OPT oligopeptide transporter protein. Transport proteins also involved a category of carbohydrate trans-membrane transporter proteins for example, MFS sugar transport, monocarboxylate permease, sugar and other transporter, sterol-3-β-glucosyltransferase. Along with above major classes ion transporter trans-membrane transporter proteins were also observed (calcium transporting ATPase, cation chloride co-transporter, phosphate transporter, plasma membrane zinc ion transporter, and siderophore ion transporter). These findings offer an insight into the quercetin mediated transportation mechanism in *A. flavus*. Expression analysis of CF proteins related to trans-membrane transport activity showed less expression in comparison with CF_Q. Current data was compare with different previous studies conducted on *A. flavus* is listed in Table 4.1.

Table 4.1: Comparative analysis of expressed proteins if *A. flavus* for their presence /absence in different studies on the basis of different biological functions

Identified proteins	***A. flavus* grown on CF_Q**	***A. flavus* grown on CF**	***A. flavus* grown on SD broth [338]**	***A. flavus*-maize interaction, mycelia stage [336]**	***A. flavus* mycelia stage proteins [52]**
Trans-membrane Transporter proteins					
ABC bile acid transporter	+	-	-	+	-
ABC multidrug transporter	+	+	-	-	-
ABC transporter transmembrane	+	+	-	+	+
ABC-2 type transporter	+		-	-	-
Adaptin N terminal region	+	-	-	+	-
Allantoate permease	+	-	-	-	-
Amino acid permease	+	-	-	+	+
Ankyrin repeat protein	+	+	-	-	+
Aspartate aminotransferase	+	+	-	+	-
Ferric reductase	+	-	-	-	-
ATP-binding cassette transporter	+	-	-	-	-
Calcium-transporting ATPas	+	-	-	-	-
Cation chloride cotransporter	+	-	-	-	-
Importin-β N-terminal domain protein	+	+	-	+	-
Importin 13	+	+	-	-	-
Major facilitator super family protein	+	-	-	-	-
Meiotically up-regulated protein 113	+	-	-	-	-
MFS sugar transporter	+	-	-	+	-
Sugar and other transporter	+	+	-	+	-
MIT microtubule interacting and transport domain protein	+	-	-	-	-
Monocarboxylate permease	+	-	-	-	-

Nitrogen permease regulator of amino acid transport activity 3	+	-	-	-	-
OPT oligopeptide transporter	+	-	-	-	-
Phosphate transporter	+	-	-	-	+
Plasma membrane zinc ion transporte	+	-	-	+	-
Siderophore iron transporter	+	-	-	-	-
Sugar and other transporter	+	-	-	+	-
VHS domain protein	+	-	-	-	-
Vitamin H transporter	+	-	-	-	-
V-type proton ATPase proteolipid	+	-	-	-	-
Nitrate transporter	-	+	-	+	-
Get1	-	+	+	-	-
Mch1	-	+	-	-	-
Oxidative stress response					
Catalase A	+	+	-	+	-
Catalase-peroxidase	+	+	+	-	-
PpoA	+	-	-	-	-
PpoC	+	-	-	-	-
Fatty acid oxygenase	+	-	+	-	-
Cat1	+	+	-	-	+
Pgh2/cox2	+	-	-	-	-
RTA1	+	-	-	-	-
Xyl1	-	+	+	-	-
Psi producing oxygenase A	-	+	+	-	-
Nst1	+	+	+	-	-
Erp38	-	+	-	-	+
TigA	+	-	+	-	-
Uba4	+	+	+	-	-

4.3.4 Comparative analysis of proteins related to oxidative stress

Toxigenic and harmful effects of AFB1 produced by *A. flavus* rely on various stress factors which includes oxidative stress conditions [342]. Hence, somewhere secondary metabolite biosynthesis is linked with oxidative stress. Quercetin is one of the phytochemical, which is known for the production of ROS, creating oxidative stress in microorganisms. In the current study, proteome analysis of CF_Q showed expression of antioxidant proteins/enzymes such as, nst, cycloxygenase cox2, mycelia catalase cat1, catalase A, catalase peroxidase, fatty acid oxygenase ppoA and ppoC, uba4, hsp70, and RTA1 like protein, involved in oxidative stress response. Results showed that quercetin mediates production of oxidative stress related proteins in *A. flavus*, which was found to be highly expressed in CF_Q in comparison to CF. Oxidative stress related proteins expressed in CF were xyl1, nst1, ppoC, hsp60, hsp 70 etc. However, differential expression studies on CFQ and CF does not give a clear idea of quercetin mediated stress response. For example, ppoC (2.48 folds) was found to be up-regulated and nst1 (0.74 folds), cat1 (0.22 folds) was found to be down-regulated in CF_Q v/s CF. Current data was also compared with previous studies on *A. flavus* which is tabulated in Table 4.1.

4.3.5 Signaling pathway

We observed MAPK signaling pathway in CF utilizing corn flour as carbon source. Proteins identified were Mpkc, PKC, serine threonine protein kinase (ste20, sepH, kcc4 and sky1), acyA. Notably, a switch in the signaling pathway was reported in response to quercetin treatment in CF_Q which is represented in Figure 4.4. Gene ontology studies on 705 proteins of CF_Q revealed expression of 24 proteins involved in GPCR mediated cAMP/PKA signaling pathway and PKC signaling pathway. Proteins identified were acyA, cAMP-dependent protein kinase catalytic subunit PKAC1, cAMP-specific phosphodiesterase, GTPase-activator protein for ras-like GTPas, guanyl-nucleotide exchange factor (sec7), phosphoinositide phospholipase C, PKC, Ras GTPase activating protein, Ras guanine-nucleotide exchange protein, Rho-GTPase activator (Bem2) etc. Absence of MAPK pathway in CF_Q may involve role of quercetin as a stress factor, which mediate of suppression of Raf by cAMP. Overall results may be further validated for identification of drug targets against *A. flavus* signaling.

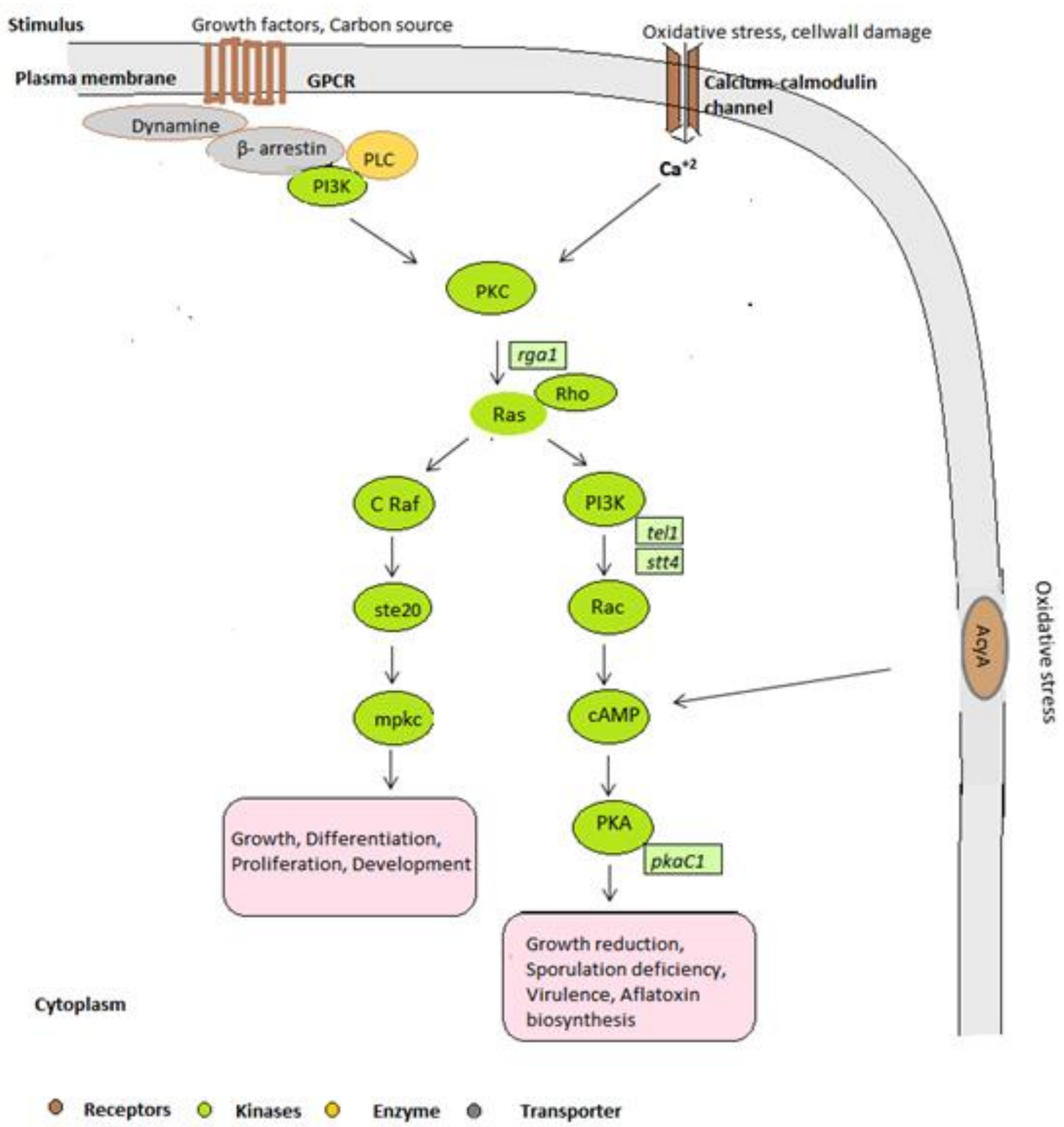


Figure 4.4 Hypothesized signal transduction pathway at germinating *A. flavus* conidia with and without quercetin (stress). *A. flavus* grown on corn flour showed MAPK pathway, a switch of signaling pathway to cAMP/PKA in response to quercetin

4.3.6 Quercetin treatment inhibits several important aflatoxin biosynthesis intermediates and decreased expression of PksA in aflatoxin gene cluster

Mojority of enzymes/proteins involved in AF biosynthesis was found to be inhibited in CF_Q in comparison with CF. A total of 705 expressed proteins of CF_Q showed polyketide synthase AflC/ PksA/ PksL1, fatty acid synthase subunit-α and β which may be involve in

AF biosynthesis pathway. However, expressed proteome of CF showed almost all the enzymes of AF biosynthesis pathway [343] such as aflR (regulatory protein), nonribosomal peptide synthetase 10, subunit α and β of sterigmatocystin fatty acid synthase, sterigmatocystin biosynthesis polykretide synthase, polyketide synthase, noranthrone synthase, noranthrone monooxygenase etc. (Figure 4.5). ACoA synthetase was also observed in CF but showed no expression in CF_Q, which suggested quercetin mediated inhibition of Acetyl coenzyme A synthesis which is a precursor molecule in AF biosynthesis. CF_Q showed inability to produce enzymes of AF biosynthesis pathway aflter PksA. Moreover, differential study showed the downregulation of PksA upto 0.6 folds in CF_Q in comparison with CF. Overall analysis suggests quercetin mediated downregulation/inhibition of PksA enzyme of *A. flavus*.

4.3.7 SEM analysis revealed anti-*Aspergillus* properties of quercetin

To understand the effect of quercetin on the conidial cell wall of *A. flavus*, Scanning Electron Microscopy (SEM) analysis was performed from 4 h germinated conidia in CF and CF_Q. Our results swelling of conidia was more in CF (4 µm) when compared with CF_Q (3.6 µm). CF_Q has shown to exhibit smooth surface of conidia which was found to be rough in CF. Also, CF_Q exhibited protuberance which was not observed in CF. Comparative SEM analysis of CF and CF_Q is presented in Figure 4.6. Overall findings showed delay of isotropic growth in *A. flavus* when provided with quercetin.

Sequential representation of enzymes involved in Aflatoxin Biosynthesis Pathway in *Aspergillus flavus* and the comparative analysis of expressed proteins/enzymes in two different growth conditions

CF *Aspergillus flavus* cultured on corn flour
CFQ *Aspergillus flavus* cultured on CF with quercerin

Actetate
CF Acetyl CoA synthetase
Acetyl CoA
CF CFQ Non ribosomal peptide synthase
CF CFQ Fatty acid synthase α, β
CF CFQ Polyketide synthase PksA
Polyketide
CF Noranthrone synthase
Noranthrone
CF Noranthrone monoxygenase
Norsolinic acid
CF CFQ Dehydrogenase
CF NOR reductase
Averantin
CF CFQ P450 monoxygenase
5'- hydroxy averantin
CF CFQ Alcohal dehydrogenase
CF Averantin hydrolase
Oxyaverantine
Averufanin
Averufin
CF CFQ Oxidase
Versicolorin hemiacetal acetate
Esterase CF
Versicoronal
CF VERB synthase
Versicolorin B
CF Desaturase
CF Sterigmatocystein biosynthesis P450 monoxygenase
Versicolorin A
Dihydrodimethyl sterigmatocystein
CF CFQ Dehydrogenase
CF CFQ Monoxygenase
Dimethylsterigmatocystein
O-methyltransferase B
Sterigmatocystein
Dihydrosterigmatocystein
CF O-methyltransferase A
O-methyl sterigmatocystein
Dihydro-O-methylsterigmetocystein
Oxidoreductase
CF CFQ Oxidoreductase
Oxidoreductase
AFB1 AFB2
AFG1 AFG2

Figure 4.5 Enzymes from AF pathway expressed in *A. flavus* grown on CF and CF_Q

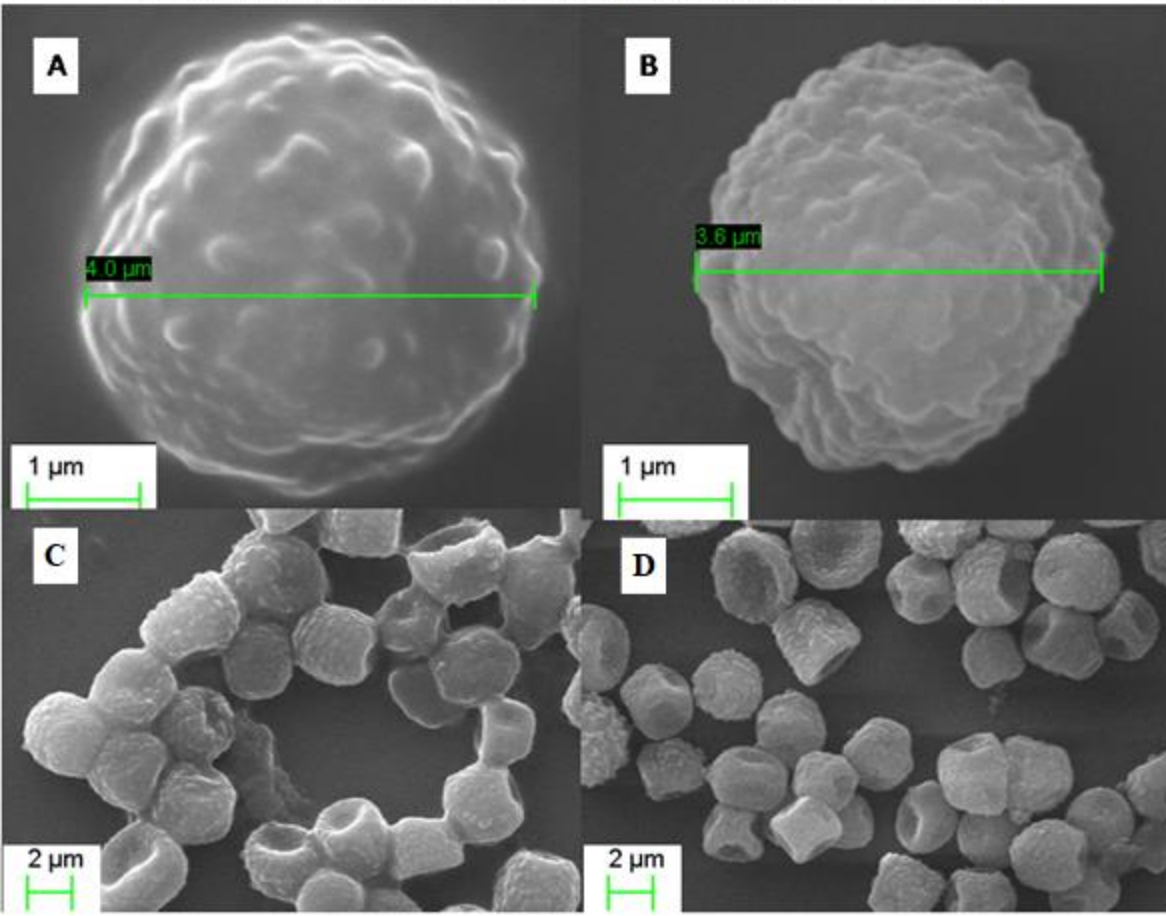


Figure 4.6 Cell wall architecture of *Aspergillus flavus* (4h), without queretin treatment (A and C) and with queretin treatment (B and D)

4.**3.8 Quantitative HPLC analysis revealed quercetin mediated aflatoxin B1 inhibition after 24h of germination**

HPLC analysis was conducted to evaluate quercetin mediated inhibiton of AF biosynthesis in *A. flavus*.Comparative analysis was performed between CF_Q and CF. Obtained AFB1peaks from HPLC analysis were efficiently resolved. Results showed the presence of similar peak in each sample when compared with standatd AFB1 (Figure A.3). Also, the quantitative analysis showed 1% decrease in AFB1 at 7h to 24h in CF_Q when compared with CF. However, significant decrease upto 51% was observed at 48h time point. Overall HPLC analysis suggested that AF biosynthesis in *A. flavus* is efficiently inhibited by quercetin. quercetin as a potent inhibitor of AF production. Also, substrate (CF) is one of the important

factors which mediate AF biosynthesis in *A. flavus*. The bar diagram on HPLC data representing comparative studies in CF and CFQ is presented in Figure 4.7.

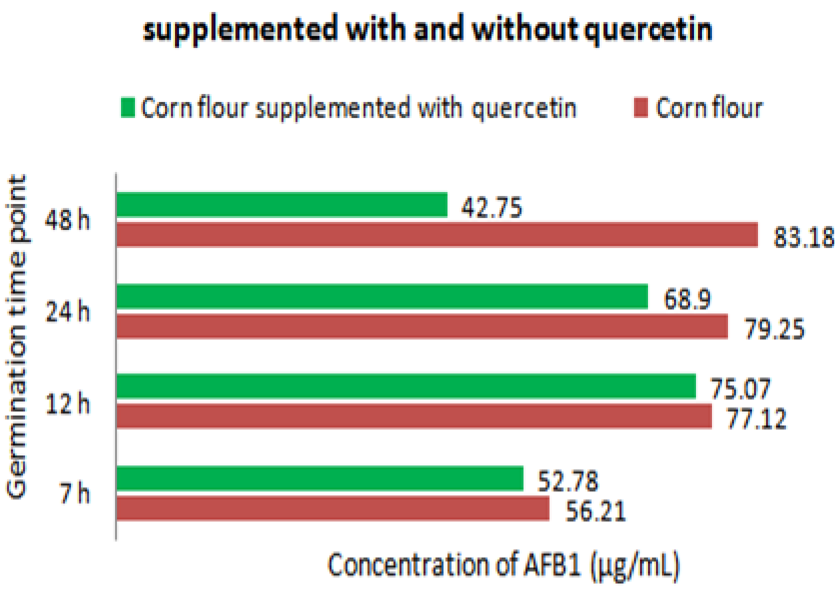


Figure 4.7 Comparative analysis of AFB1 production measured by HPLC in culture filtrate of *A. flavus* grown on CF and CF_Q at different time (7h, 12h, 24h and 48h)

4.4 DISCUSSION

Proteome profile of CF and CF_Q showed expression of stress response proteins and proteins involved in transferase activity as major protein categories. CF_Q showed expression of transportation proteins as a major biological function. Transportation majorly involves influx and efflux of proteins, which involves two major super families. Former involves solute carrier family protein and later involves ATP binding cassette (ABC) and MFS superfamily proteins [344]. In the current study expression of ABC multidrug transporter showed the inhibition mechanism of quercetin intake in *A. flavus*. In previous studies solute carrier proteins such as organic ion transporting peptide, organic cation transporter and ATP independent organic ion transporters have been reported [345]. In the current study, various proteins involved in influx transportation activity in *A. flavus* was observed which includes aspartate aminotransferase, sodium-hydrogen exchanger family (nha1), importin-β (N-terminal domain), cation chloride co-transporter, plasma-membrane zinc ion transporter, phosphate transporter and siderophore iron transporter. Various *in-silico* approaches have

shown that influx of quercetin across the plasma membrane is mediated by glucose transporters [346]. In the current study, we have found the expression of various proteins which are involved in glucose transportation for example, monocarboxylate permease, glycoprotein glucosyltransferase, UDP-glucose, MFS sugar transporter, etc. The identified proteins may involve in the quercetin influx across plasma membrane of *A. flavus*. The protein data of CF_Q when compared with CF data showed limited transportation proteins, which majorly included ABC transporters but lacked influx proteins [338]. The difference in the expression of proteins in CF and CF_Q indicated that substrate composition and stress factors plays major role in expression of transport proteins in *A. flavus*. These finding correlated with the studies of Gautam et al., (2008) in the transcriptomic studies on *A. fumigatus* in response to amphotericin B. Resuls showed that *A. fumigatus* increased the expression of transport proteins and cell stress proteins in response to amphotericin B [49]. In the coherence with the previous studies we have identified the expression of quercetin 2,3-di-oxygenase in the current study, which mediates the inhibition of jasmonic acid pathway as a result of carboxylic acid formation against carbon monoxide [347, 348]. Overall findings showed that *A. flavus* trans-membrane transporter proteins expression highly depends on substrate and stress factors.

Transcriptional and translational processes in *Aspergillus* are highly dependent on various stress factors. One the the class of proteins which are found to be expressed in response to stress are heat shock proteins [314]. In the current study, the expression of Hsp70 and Hsp60 revealed that Hsps are key proteins involved in stress response. Sevetal studies showed, two types of pathways are predominant in *Aspergillus* species viz. MAPK pathway and cAMP/PKA pathway which mediates morphogenesis, cell wall biogenesis, metabolism, regulation of germination, sporulation, mycotoxin production, and stress tolerance [349]. Cell wall integrity and signaling pathway is known to be first line of defense against oxidative stress. In the favorable growth conditions and oxidative stress conditions *A. flavus* have shown the expression MAPK signaling pathway [338, 350]. However, in the current study in MAPK pathway was found to be completely inhibites, as none related proteins were observed in CFQ, which suggested quercetin mediated inhibtition of MAPK pathway at early germination time points in *A. flavus*. Also, we have observed the expression of cAMP/PKA signaling pathway in response to quercetin treatment in *A. flavus*. This finding correlated

with the studies of Chen and Dickman (2005), which showed MAPK pathway inhibition by cAMP pathway, hence inhibiting sclerotium formation. In addition, they revealed cAMP independent PKA mediates Ras inhibition (activator of MAPK pathway), hence involved cAMP mediated inhibition of Ras dependent MAPK pathway [351]. Also, other studies by Nicolas et al., (2002) showed the cAMP mediated inhibition of Raf1by blocking its activity hence inhibiting NIH3T3 cells growth [352]. Previous studies showed the expression of PKC signaling pathway in *C. albicans* and *S. cereviciae* when treated with caspofungin. Results showed activation of calcineurin followed by downstream MAPK pathway, which mediates resistance from drug [353]. Hence, it can be suggested that signaling transduction pathway specificity depends on different fungal species and also on different stress condition. Our findings from SEM analysis showed the inhibition of conidial swelling, which is known to be a key step in *A. flavus* germination [291].

Previous studies have shown that AF production is inhibited by quercetin [337] which is also involved in anti-*Aspergillus* responses [229]. In the current study CF_Q has shown the expression of PksA and limited sets of enzymes involved in AF biosynthesis. Non expression of several AF biosynthetic pathway enzymes in CF_Q showed quercetin mediated inhibition of AF biosynthesis. Also, results revealed that expression of enzymes involved in AF bisoynthesis starts at germination or post germination stages. In coherence with our finding, A. *terrus* protein profile at germination stage showed expression of 10 enzymes involved in geodin (mycotoxin) production [354]. HPLC analysis in the current study showed the decrease in AFB1 production in CF_Q in comparison to CF. In support of this result, another study showed the reduced expression of AF pathway genes at 24h to 72h in response to 2-phenylethanol [355]. However, in the current study CF showed expression of enzymes in AF biosynthesis pathway including transcriptional factors AflR. Zhou et al., (2015) showed similar results of quercetin mediated inhibition of AFB1 production by *A. flavus* at 800 µg/ml using HPLC analysis [228]. Hence, quercetin can be taken into consideration as a promising antifungal compound against *Aspergillus* species.

SUMMARY

PksA has been downregulated in response to quercetin treatment in *A.flavus*, and majority of the enzymes following PksA were not expressed in CF_Q.

CHAPTER 5

Structural-Functional Analysis of Interaction of Quercetin and Hexanoic Acid with Polyketide Synthase A (PksA) Domains (Seven) of *Aspergillus flavus* To Predict the Susceptibility of Quercetin Mediated Inhibition of PksA

5.1 INTRODUCTION

As discussed earlier, *A. flavus* is known to produce AF (AFB1 and AFB2) therefore causing contamination in food-crops [356]. IARC have caterorised these toxins as carcinogenic compounds (http://monographs.iarc.fr/). Various safety administrations have undertaken measures to withstand AF contamination, as for the protection of mankind [357].

Taking insight in AF biosynthesis mechanism, *A. flavus* mediated AF biosynthesis is a sequential event which involves polyketide biosynthesis pathway [356, 358]. Environmental factors such as substrate, pH, temperature, morphological stages etc. plays major role in AF production [51, 359]. AF biosynthesis pathway involves multi-domain enzyme polyketide snthases (PKSs), which is known to be one of the crucial enzymes. PKS are known to utilize acyl group for the production of complex natural products [360]. There are mainly three different types of PKSs, among which fungi bears PKS-type I. Fungal PKS-type I is a multifunctional protein of 180-250 kDa. Domains of PKS-type I includes major domains such as acyl carrier proteins (ACP), acyl transferase (AT) and ketoacylsynthase (KS) and few optional domains such as methyltransferase (MT), dehydratase (DH), keto reductase (KR), cyclase (CYC) and enol reductase (ER) [361-365]. Gene count of AF pathway in *A. flavus* is approximately 30 genes along with regulatory genes (*aflr* and *afls*) and fatty acid synthases [366]. Six-carbon compound (hexanoyl-CoA) is known to be the starter unit or precursor molecule in AF biosynthesis produced from fatty acyl synthases. Molecular study on fatty acid synthase mutated strain *A. nidulans* revealed the active production of secondary metabolite when supplied with hexanoic acid [367-369]. Hence, hexanoic acid can act as a substrate molecule for PksA in AF biosynthesis pathway. In another study by Newman et al., 2012 showed that polyketides are novel molecules to target for therapeutics in response to AF contamination towards fungal contamination and diseases such as aspergillosis [370].

As discussed in chapter 2 and chapter 4, phytochemicals are the promising source to out-compete harmful activities of AF in food-crops [371]. Also, quercetin (plant flavanoid) which is commonly present in vegetables flower, seeds, fruit, tea, wine, honey, nuts and propiols [372]. Quercetin has also exhibited anti-cancerous properties by causing apoptosis in tumor cells [373]. AFB1 biosynthesis in *A. flavus* has also found to be inhibited by quercetin treatment [228]. Also in chapter 4, quercetin was found to downregulate AFB1 production by decreasing the PksA expression upto 0.6 fold at MIC_{50} (113 µg/ml). Since the mechanism of quercetin mediated AF biosynthesis inhibition in *A. flavus* is still unclear, *in-silico* approach may help in understanding quercetin mediated AF inhibition mechanism. Thus, to understand the inhibition mechanism, protein sequence of PksA (*A. flavus*) was retrieved from UniProt database followed by homology modelling. Further, docking studies were undertaken using two different ligands viz. hexanoic acid and quercetin. The overall analysis was performed on the basis of H- bonding binding energy, bond length, hydrophobic interaction and electrostatic energy which showed stronger binding potential of quercetin whith PksA domains with respect to hexanoic acid.

5.2 MATERIAL AND METHODS

5.2.1 Selection of biological data, sequence retrival and phylogenetic analysis

Polyketide synthase of *A. flavus* (strain ATCC 200026 / FGSC A1120 / NRRL 3357 / JCM 12722 / SRRC 167) expressed in response to quercetin treatment mediated proteomic studies conducted in chapter 4 was used in current study. Aminoacid sequence of A. flavus PksA was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/) with the NCBI Gene ID of 7914331, gene symbol: AFLA_139410, gene description: aflC/PksA/pksL1/polyketide synthase and UniProt accession number B8NI04_ASPFN. Homologous sequence search was performed using FASTA sequence obtained through pBLAST in NCBI database (http://www.ncbi.nlm.nih.gov/Blast.cgi). Similar sequences (90%) were aligned and identified using Muscle analysis in MEGA 6.06 (http://www.megasoftware.net/) and further converted into MEGA format [374]. To understand the evolutionary history of *A. flavus* PksA, Maximum Likelihood method was used which involves JTT matrix-based model [375].

5.2.2 Ligand preparation

Two different ligands viz. quercetin and hexanoic acid were used in current study. The 3D structure of both the ligands was retrieved from PubChem Structure search (https://pubchem.ncbi.nlm.nih.gov/) followed by modification in PyMOL molecular graphic system 1.3 (http://www.pymol.org/funding.html). Qualitative analysis of modified ligands was further validated by Lipinsky rule of five (http://www.scfbio-iitd.res.in/). The parameters were: input ligand file; PDB format, pH; 7.

5.2.3 Domain Investigation, Homology modelling and validation

To identify different domains of *A. flavus* AflC/PksA/pksL1/polyketide synthase (UniProt accession; B8NI04_ASPFN) proteins, the query was subjected to NCBI database. Results showed seven PksA domains which is mentioned in Figure 5.1.

smart00825	PKS_KS; Beta-ketoacyl synthase	Location:375 to 803
smart00827	PKS_AT; Acyl transferase domain	Location:905 to 1202
cd00833	PKS; polyketide synthases	Location:375 to 801
TIGR04532	PT_Product template domain	Location:1309 to 1635
pfam00550	PP_Phosphopanthotenic attachment site	Location:1716 to 1783
pfam16073	SAT_ACP transacylase starter unit	Location:1867 to 2106
pfam16197	KAsynt_C Ketoacyl-synthase C	Location:772 to 853

Figure 5.1 List of *A. flavus* Polyketide synthase A (UniProt accession; B8NI04_ASPFN) domains retrieved from NCBI database

Protein sequence of seven different conserved domains of PksA was obtained from online conversion tool (http://insilico.ehu.es/translate/) which mediates the translation into amino acid sequence further referred SWISS-MODEL for homology modelling (http://swissmodel.expasy.org/) [376]. Models which were generated were ordered on the basis of sequence identity. All the proteins structures were saved in PDB format and validated by PROCHECK (http://www.ebi.ac.uk/thornton-srv/) for quality proteins. Identification of modeled structure for two domains were less than 20%. To validate the

structure, results were generated in the form of Ramachandran plot for all the models. All the domains were observed to be in favorable region, hence considered for analysis.

5.2.4 Molecular docking studies

Autodock tools-1.5.6 was used for docking studies and calculations in interaction studies. Proteins were modified by addition of kolmann charges, merging of H-atoms, AD4 assignment of atoms and removal of water molecules followed by documenting in pdbqt format. For preparation of ligand gasteger charges was added including addition of polar H-bonds and defining rotatable bonds. Ligand parameters (initial position, torsions and orientation) were set as random followed by rotatable torsions release. Lamarckian genetic algorithm was applied for search parameters which exhibited 50 runs. Autogrid program mediated establishment of 0.375 Å Affinity (grid) maps. Electrostatic and van der wall interactions were calculated using autodock parameter set and distance-dependent dielectric functions respectively. 250000 energy evaluations were set for termination. Population size was set at 150, translational step at 0.2 Å and torsion steps of 5 of quaternion [377, 378]. The results were obtained in the form of inhibition constant, binding free energy, final intermolecular energy and internal energy. Good results were attained from 50 runs which were further compared. Further, Ligplot studies were conducted to understand the interaction studies (hydrophobic and H- bonding) between ligand and receptor protein and analyzed 3d in PyMOL. Interactions of both the ligands were compared with all the domains of *A. flavus* PksA for determining the efficient binding.

5.3 RESULTS

5.3.1 Sequence alignment, phylogenetic analysis and homology modelling

pBLAST search for homologues identification resulted into12 similar sequences of >90% identity for protein sequence (PksA) which were further analyzed for phylogenetic relationship of *A. flavus* PksA. The evolutionary tree results obtained from MEGA tool suggested that *A. flavus* PksA showed similarity in sequences with other *Aspergillus* species such as *A.oryzae*, *A.sojae*, *A. nomius* and *A. bombycis*. Results are shown in Figure 5.2.

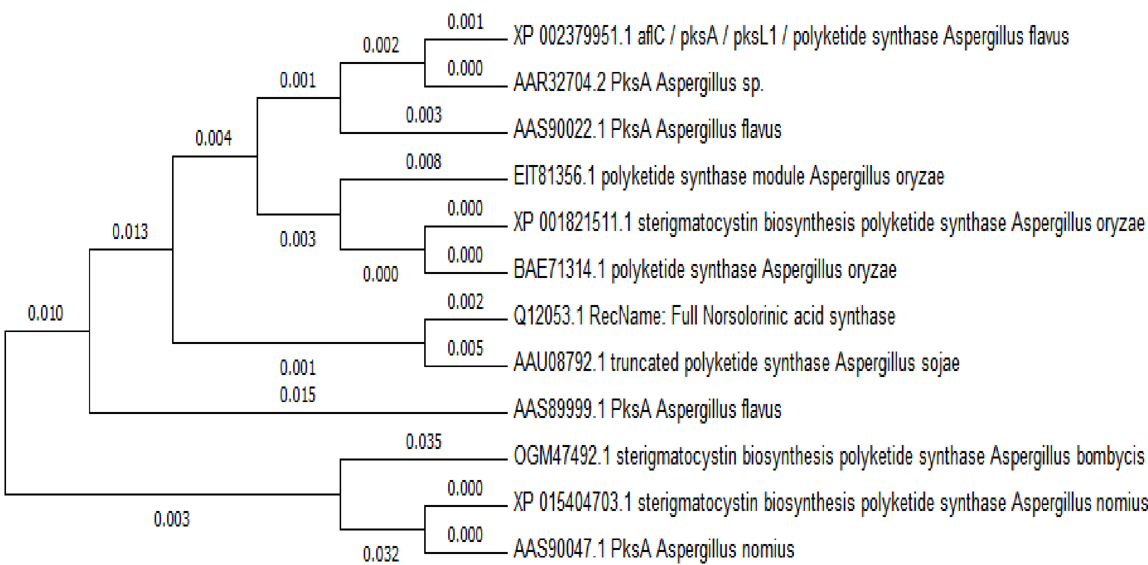


Figure 5.2 Phylogenetic analysis of *A. flavus pksA* gene by Maximum Likelihood method using JTT matrix-based model from MEGA 6.06 software

PyMol software was used to obtain 3d structure of quercetin and hexanoic acid and is mentioned in Figure 5.3. Further, the structures were validated by lipinsky rule of five, represented in Table 5.1. Validation results showed the docked structures exhibits drug like properties and are appropriate for doking studies. *A. flavus* PksA domains obtained from NCBI conserved domain sequence database were subjected to SWISS_MODEL server for retrievel of best possible model. The best model was selected on the basis of sequence identified and further subjected to homology modelling (Figure 5.4). The SWISS-MODEL Template Library was searched with BLAST and HHblits which involves HMM-HMM alignment for template identification as well as to attain target-template alignments.

Quercetin

Hexanoic acid

Figure 5.3 Three dimentional structure of quercetin (Id: 5280343) and hexanoic acid (Id: 8892) obtained from Pubchem

Table 5.1 Molecular properties of ligands on the basis of lipinsky rule of 5

Compound	Molecular weight	H-bond donar	H-bond acceptor	Log P	Molar refractivity	Pubchem CId	Molecular formula
Quercetin	302 g/mol	5	7	2.010899	74.050484	5280343	$C_5H_{10}O_7$
Hexanoic acid	116.16 g/mol	1	2	0.316600	29.148993	8892	$C_6H_{12}O_2$

These approaches resulted in good alignments in both sequence identity levels (high and low) leads to experimental approach, resolution (if applicable), sequence identity to the target, coverage, etc. which suggests the best available template for our structure. The sequence of alligned and selected template is given in Table B.3. As indicated by Ramachandran plot (Table 5.2) 81.1% - 89.6% of all the domains were ranged in most favored region, 6% - 13.5% in the additional allowed region, 1.4% - 6.1% in generously allowed region and 0% - 1.5% in disallowed region. The overall goodness factor (G-factor) were lied within 0.11 - 0.36, distribution of main chain bond length of domains ranged between 96.5% - 100% and the range of covalent bond angle lied between 89.9% - 92%. Based on these validations, homology models were further used in molecular docking studies.

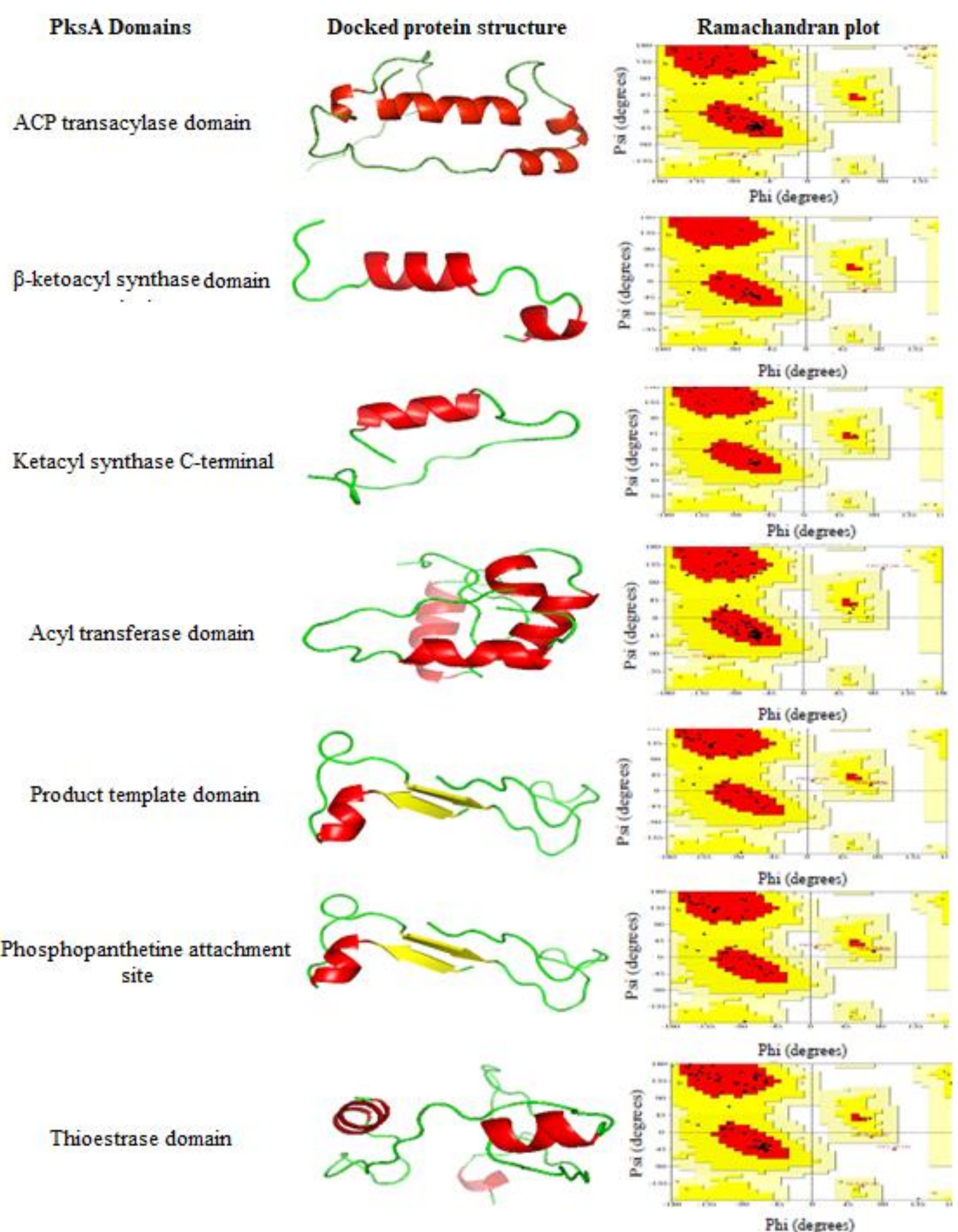


Figure 5.4 Docking studies on *A. flavus* PksA domains using Autodock software. The red color shows the α-helix and the yellow color shows the β-sheets. Structure stability is validated by Ramachandran plot by Procheck software. Red color region denotes residues of respective domain in most favourable region, yellow color denotes residues in additional allowed region, and light shade indicates residues in generously allowed region

Table 5.2 Parameters used for protein structural assessment by PROCHECK analysis

Domains	Ramachandran Plot (%)	G-factor	MCBL (%)	CBA (%)	Residues
ACP transacylase	81.8[a]; 12.1[b]; 6.1[c]; 0.0[d]	0.21[e]; 0.50[f]; 0.29[g]	96.6	86.3	72
β-ketoacyl synthase	70.8[a]; 25.0[b]; 4.2[c]; 0.0[d]	0.25[e]; 0.62[f]; 0.36[g]	97.8	85.9	28
Ketoacyl-synthetase C-terminal	88.5[a]; 11.5[b]; 0.0[c]; 0.0[d]	0.16[e]; 0.29[f]; 0.19[g]	96.5	90.6	33
Acyl transferase	77.0[a]; 9.6[b]; 1.4[c]; 1.4[d]	0.24[e]; 0.31[f]; 0.24[g]	97.8	89.9	83
Product template	81.1[a]; 13.5[b]; 5.4[c]; 0.0[d]	0.26[e]; 0.08[f]; 0.17[g]	100	92.0	48
Phosphopantetheine attachment site	81.1[a]; 13.5[b]; 5.4[c]; 0.0[d]	0.33[e]; 0.08[f]; 0.21[g]	100	92.3	48
Thioesterase	89.6[a]; 6.0[b]; 3.0[c]; 1.5[d]	0.11[e]; 0.13[f]; 0.11[g]	98.7	94.6	79

MCBL: distribution of main chain bond length; CBA: distribution of covalent bond angle; [a]residue in favored regions; [b]residue in allowed regions; [c]residue in generally allowed regions; [d]residue in disallowed regions; [e]G-factor score of dihedral bond; [f]G-factor score of covalent bond; [g]overall G-factor score

5.3.2 Docking scores

Interaction and structural studies between protein and ligand paved a way for drug designing and development. Docking was performed for quercetin and hexanoic acid with seven different domains of *A. flavus* PksA, which mediates AF biosynthesis. Table 5.3 accounts for binding free energy, inhibition constant, intermolecular energy and electrostatic energy values which shows the docking interactions of both the ligands.

Table 5.3 Comparative analysis of PksA domains and ligands (qurecetin and hexanoic acid)

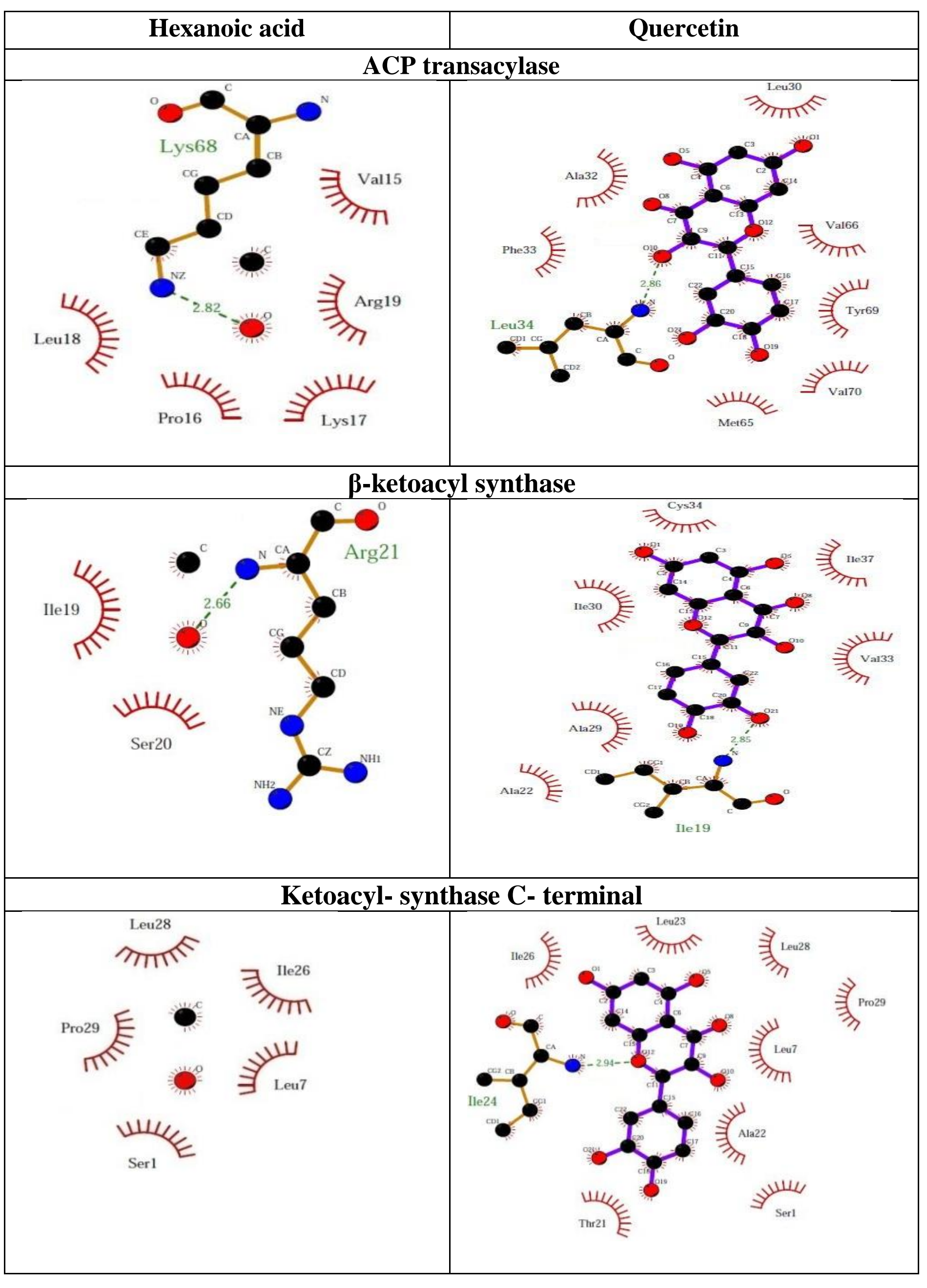
Ligands & Domains		Binding energy	Inhibition constant (nM)	Intermolecular energy	Electrostatic energy	Total Internal energy
Quercetin v/s Hexanoic acid	ACP transacylase	−6.33/ −4.74	23.08/ 327.21	−6.62/ −6.25	−0.14/−2.62	−0.39/ −0.09
	β-ketoacyl synthase	−5.6/ −4.41	78.19/ 585.52	−5.9/ −5.9	−0.24/ −2.75	−0.26/ −0.07
	Ketoacyl-synthetase C-terminal	−7.1/ −3.54	6.3/ 2.55	−7.39/ −5.03	−0.29/ −1.05	−0.39/ −0.11
	Acyl transferase	−6.15/ −3.79	31.17/ 1.67	−6.45/ −5.28	−0.1/ −2.02	−0.35/ −0.05
	Product template	−5.78/ −4.63	58.45/ 406.11	−6.07/ −6.12	−0.18/ −2.38	−0.37/ −0.08
	Phosphopantetheine attachment site	−5.8/−4.57	56.08/ 446.44	−6.1/ −6.06	−0.19/ −2.54	−0.37/ −0.05
	Thioesterase	−5.25/ −3.86	141.61/ 1.49	−5.55/ −5.35	−0.11/ −2.93	−0.38/ −0.07

The results in terms of binding energy revealed that quercetin (-7.1kcal/Mol to -5.25 kcal/Mol) requires less binding energy in comparison to hexanoic acid (-4.74 kcal/Mol to -3.54 kcal/Mol) for active binding with different substrates. Also, electrostatic energy required for domains-quercetin interaction was less (-0.29 kcal/Mol to -0.1 kcal/mol) in comparision to hexanoic acid (-2.93 kcal/Mol to -1.05kcal/Mol). Intermolecular energy of both the ligands was found to ranging between -6.62 kcal/Mol to -5.55 kcal/Mol for quercetin and -6.25 kcal/Mol to -5.03 kcal/mol for hexanoic acid. The overall finding suggested more efficient binding of quercetin in comparison to hexanoic acid.

Table 5.4 Comparative analysis based on Hydrophobic and hydrogen bonding of quercetin and hexanoic acid with different PksA domains of *A. flavus*

Domains		No. of H-bond	H- bonding	Bond length (Å)	Hydrophobic bonding
Hexanoic acid v/s Quercetin	ACP transacylase	1	Lys68	2.82	Leu18, Pro16, Lys17, Arg19, Val15,
		1	Leu34	2.86	Met65, Val70, Tyr69, Val66, Leu30, Ala32, Phe33, Leu34
	β-ketoacyl synthase	1	Arg21	2.66	Ile19, Ser20
		1	Ile19	2.85	Val33, Ile37, Cys34, Ile30, Ala29, Ala22
	Ketoacyl-synthetase C-terminal	0	-	-	Leu28, pro29, Ser1,Leu7, Ile26
		1	Ile24	2.94	Thr21, Ser1, Ala22, Leu7, Pro29, Leu28, Leu23, Ile26,
	Acyl transferase	1	Arg63	2.88	Lys66, Tyr17, Pro16
		1	Arg63	2.82	Pro16, Lys66, Tyr17, Val71, Ile70, Pro72, Met81, Val52, Leu56
	Product template	1	Arg15	2.72	Ala39, Leu41
		4	Arg15, Leu41, Ser37	2.63, 2.83, 2.49, 2.49	Ala39, Ser40, Met16, Ile18, Gln38
	Phosphopantetheine attachment site	1	Arg15	2.63	Ala39, Ser40, Leu41
		2	Trp35, Lys22	2.86, 2.78	Arg15, Trp17, Pro19, Met16
	Thioesterase	3	Asp5, Leu4, Gly3	2.90, 2.89, 2.69	Arg28, Leu10
		2	Lys17, Gly25	3.10, 2.85	Cys16, Pro18, Tyr26, Phe9, Asp24, Ala23

Ligplot analysis was further used to compare the more effective interactions of domains and ligands in terms of H-bonding, bond length and hydrophobic interaction. Results revealed that 12h-bonds was formed by quercetin interaction with seven domains of PksA whereas only 8 H-bonds was formed by hydroxylic acid with all PksA domains. Hydrophobic interaction comparison studies showed 46 interaction amino acids in quercetin whereas only 22 interacting amino acids in hexanoic acid. The overall result is listed in Table 5.4 and represented in Figure 5.5.



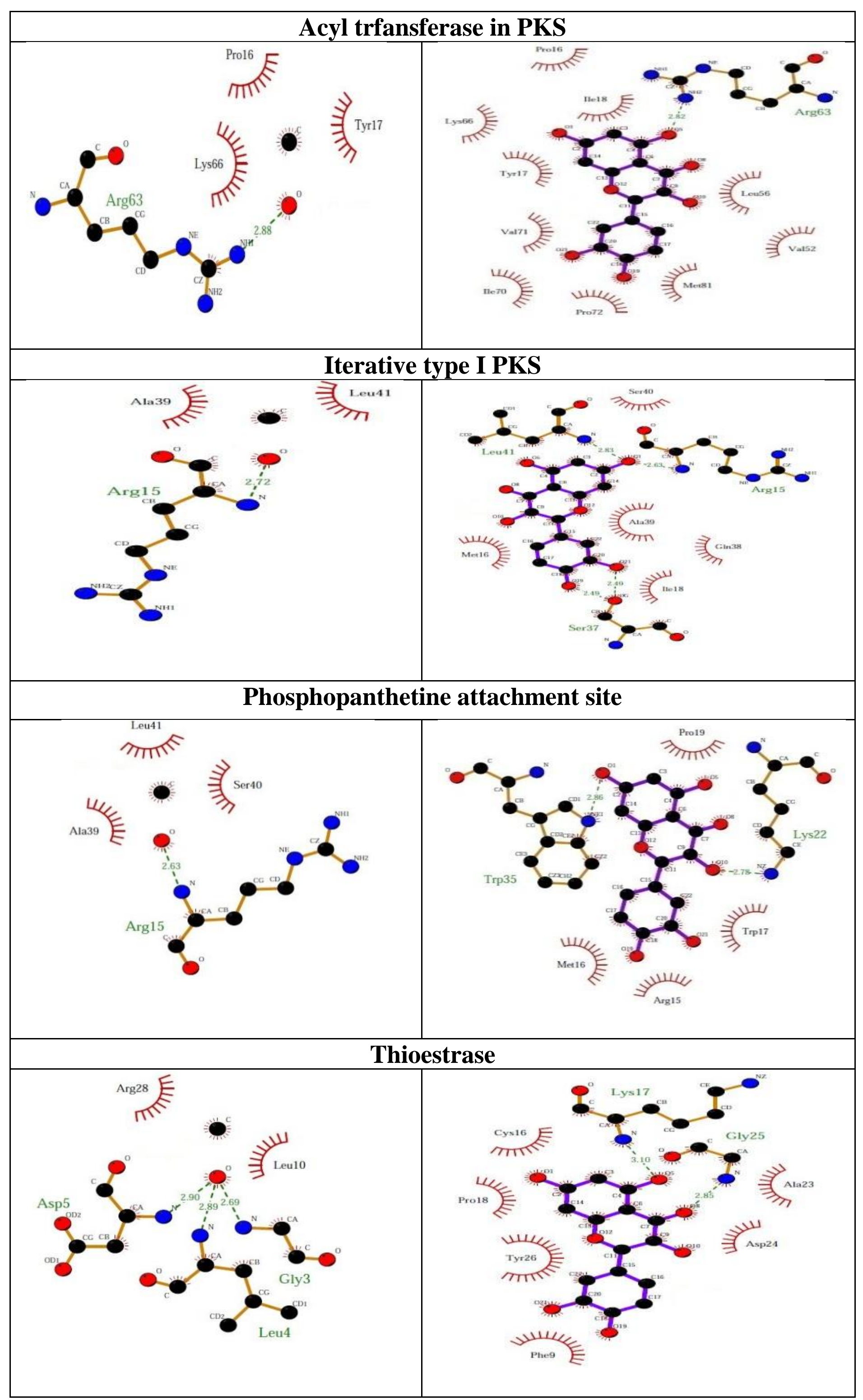


Figure 5.5 Post docking interactions between active site residues of seven different domains of PksA of *A. flavus* with two different ligands, quercetin and hexanoic acid showing schematic diagram of ligands interaction using Ligplot.

Some of the interaction studies showed similar results for both the ligands such as, Ligand interaction with AT domain leads to formation of H-bond at Arg63 position. Also, the bond length of quercetin was 2.82 whereas for hexanoic acid was 2.88, which showed similar binding patterns for AT domain. In PT domain quercetin showed stronger binding in comparison to hexanoic acid as former creates four H-bonds and later creates one H-bond. Quercetin showed stronger binding towards phosphopanthetene attachment site by forming two H- bonds whereas hexanoic acid created only single H-bond. On a whole molecular docking and Ligplot studies based on electrostatic energy, binding energy, H- bonding, hydrophobic interaction and bond length revealed that PksA domain have more binding potential for quercetin in comparison to hexanoic acid.

5.4 DISCUSSION

It is evident that the quercetin exhibits anti-AF properties [228, 337]. Quercetin inhibition studies have been shown in the previous chapter 2 and 4, which included assays, proteomic studies, SEM analysis and HPLC analysis. These finding concluded that quercetin is a potent inhibitor of AF biosynthesis and also exhibits antiaspergillus properties. PksA, a initial enzyme in AF biosynthesis containing active binding sites for hexanoyl CoA [366]. Novel compound identification as *A. flavus* mediated AF biosynthesis inhibitor requires step wise and multi-pronged advances.

AT domain of PksA is involved in substrate translocation from coenzyme-A to SAT [379]. In the current study, same binding patteren was observed in quercetin and hexanoic acid with AT domains which involved single H-bond at Arg-63 position. Binding affinity showed stronger affinity of quercetin in comparison to hexanoic acid in terms of binding energy viz. -6.15 kcal/Mol (quercetin) and -3.79 kcal/Mol (hexanoic acid). Hydrophobic interactions were also found to be stronger in quercetin (nine amino acids) in comparison to hexanoic acid (three amino acids). ACP transacylase domain is the starter unit of fungal Pks type-I which accepts six carbon acyl group for AF precursor initiation that is norsolinic acid [380]. In the earlier on hexanoyl acceptors it has been shown that it bears a conseved alanine group in ACP domain [381]. In the current study the docking results of ACP domain showed the presence of Ala32 in the hydrophobic region when trated with hexanoic acid, whereas it was found to be absent in quercetin treatment. This finding showed that binding pattern of quercetin and hexanoic acid is different in ACP domain of PksA. However, the

binding affinity of quercetin (-6.33 kcal/Mol) was more in comparison to hexanoic acid (-4.74 kcal/Mol) similar to hydrophobic interactions.

Another class of PksA domain is β-ketoacyl synthase domain, which is involved in enzyme bound substrate extension which also needs the involvement of ketoacyl synthetase domain present in C-terminous [382]. In the current study, quercetin showed stronger interaction in comparison to hexanoic acid for both the domains in terms of binding energy (−5.6 kcal/mol; (−7.1kcal/mol for quercetin and −4.41 kcal/Mol; −3.54 kcal/Mol for hexanoic acid). Another class of fungal PksA domain is PT domain which is presented beside ACP domain and mediates poly-β-keto intermediates stabilization by cyclic and aeromatic activities [383, 384]**.** The substrate binding regions of PksA is further divided into three sections viz. the phosphopantetheine localization channel, the hexyl-binding region and cyclization chamber [362]. Till now, crystal structure of PT domain of *A. parasiticus* PksA is available [384]. In the current study, we found the more prone bindig of quercetin in comparison to hexanoic acid towards PT domain. Both the ligands have shown the formation of H-bond at Arg15 position. In addition, quercetin forms three more H-bonds in different residues (Leu41 and two at Ser37). Also, in terms of hydrophobic interactions again quercetin showed stronger interaction with respect to hexanoic acid for PT domain. Interaction studies of both the ligands with phosphopantetheine attachment domain of PksA showed effective binding of quercetin in comparison to hexanoic acid.

Moreover, TE domain is involved in the release of product (norsolinic acid) from PksA with the help of PT domain which involves nucleophylic reactions on ACP damoin [385, 386]. Previous studies have showed that PT domain whit the help of KS and TE domain mediates hexanoyl starter unit formation [387]. Our study showed stronger interaction of quercetin towards TE domain in comparison to hexanoic acid interactions suggesting quercetin may be involved in the inhibition of norsolinic acid, hence inhibiting AF biosynthesis in *A. flavus*.

Summary

To summarize the study in current chapter we can conclude that quercetin may be an active binder and inhibitor of PksA hence inhibiting the formation of norsolinic acid.

CHAPTER 6

Summary and Future Prospects

Aspergillus flavus is an ubiquotus fungus, produce carcinogenic compound AF (AFB1) in pre- and post-harvested food crops. AF consumed by animals/humans from plant products causes a severe disease known as aflatoxicosis. Due to the lack of awareness among peoples of developing and under-developed countries, majorly Asia and Africa, the extent of contamination and co-occurance of mycotoxin is not known. So, it becomes important to understand the mechanism of a germaination of *A. flavus* conidia and AF production in early developmental stages, which can be helpful in providing early diagnostic markers. Also, due to development of *A. flavus* resistant strains against various antifungals such as, azoles, it has also become important to replace conventional chemical pesticides with natural, environment safe antimicrobial compound.

Current work has provided the global proteome analysis of *A. flavus* germinating conidia as well as the effective phytochemical (quercetin) as anti-Aspergillus and antiaflatoxigenic compound against *A. flavus* on common substrate (corn flour). Further the effect of quercetin on germination stage of *A. flavus* was observed and mechanism was hypothesised using in-silico approaches. The result from *A. flavus* conidia germination suggests that the morphogenesis from conidia to germ tube requires carbohydrate metabolism, translation, cell cycle, cell wall remodelling and transport proteins. Few enzymes from AF biosynthesis pathways were expressed at *A. flavus* early germination stages. MAPK pathway could be the crucial in modulation of cell wall and secondary metabolite biosynthetic pathway. Dicer like proteins, autophagy proteins, Hsps and MAPK pathway can be further explored for antifungal targets at early germination stages of *A. flavus*. Further, antifungal assay using four different phytochemicals (gallic acid, ascorbic acid, caffeine, and quercetin) against *A. flavus* suggested quercetin as a potential as antifungal natural product against *A. parasiticus* and *A. flavus* as its showed least MIC_{50} value of 113 µg/ml and 36 µg/ml respectively. Hence, could be implemented in pre and post harvested food crops as a bio pesticide. This finding brought up the issue to comprehend mechanism behind quercetin mediated inhibition of *A. flavus* and AF biosynthesis. Again, using nLC-QTOF and quantitative

HPLC analysis, AFB1 inhibition by quercetin, proteins/ enzymes expression at *A. flavus* germination stage and the possible mechanism behind AFB1 biosynthesis inhibition by quercetin was studied. Results revealed the inability of *A. flavus* in forming AF biosynthesis enzymes within the site of quercetin. PksA was down regulated against control, and other important enzymes involved in pathway such as, noranthrone synthase, norsolinic acid, NOR reductase etc. were found to be inhibited. Comparative proteomics of CF_Q v/s CF showed switch of cAMP/PKA from MAPK pathway, activation of various transport proteins involved in influx of quercetin and efflux ABC transporter in CF_Q at *A. flavus* germination stage. Therefore quercetin was suggested as effective anti-aflatoxigenic agent. As, results suggested the downregulation of PksA and inhibition of later enzymes at MIC_{50} value of quercetin, it again brought up the issue of quercetin competitive binding with the substrate of PksA and inhibition of further biosynthesis intermediates. To understand this, we had undertaken docking and ligplot studied using 3D structures of seven different domains of PksA enzyme of *A. flavus* against the substrates viz, quercetin and hexanoic acid. The result in terms of binding energy, electrostatic energy, H-bonding, bond length and hydrophobic interaction revealed that computational analysis added insight into the quercetin based inhibition of AF biosynthesis in *A. flavus* and could serve as a resource for experimental studies. For future prospects, to establish new early diagnostic, markers against *A. flavus* contaminantion and aflatoxicosis *in-vivo* studies are important using mice model and clinical studies. Also to develop new antiaflatoxigenic compound, plant-pathogen and quercetin interaction should be conducted to check the effects on AF production in pre harvested crops. Furthermore Quercetin from different plant sources for large scale production can be implemented.

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APPENDIX - A

Figure A.1 Comparative analysis of SDS-PAGE of cellular protein extract obtained by Sodium: Phosphate extraction buffer in CF and CFQ

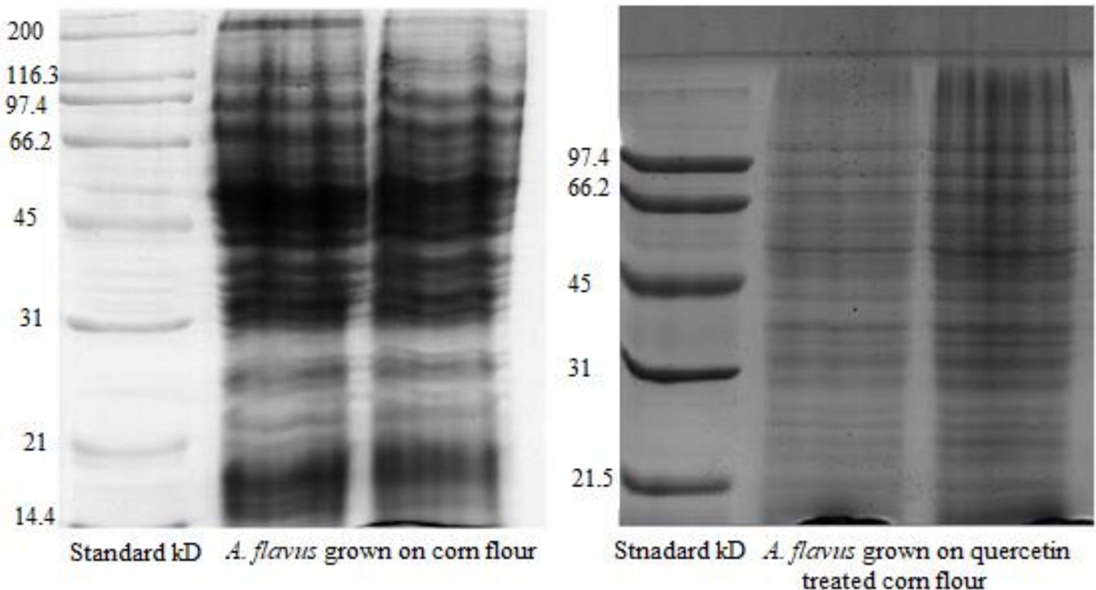
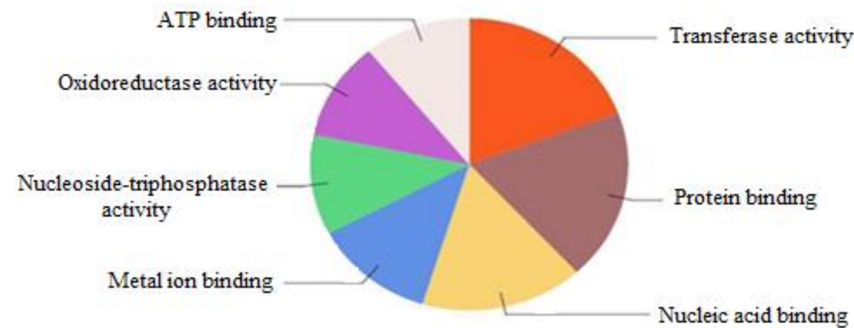
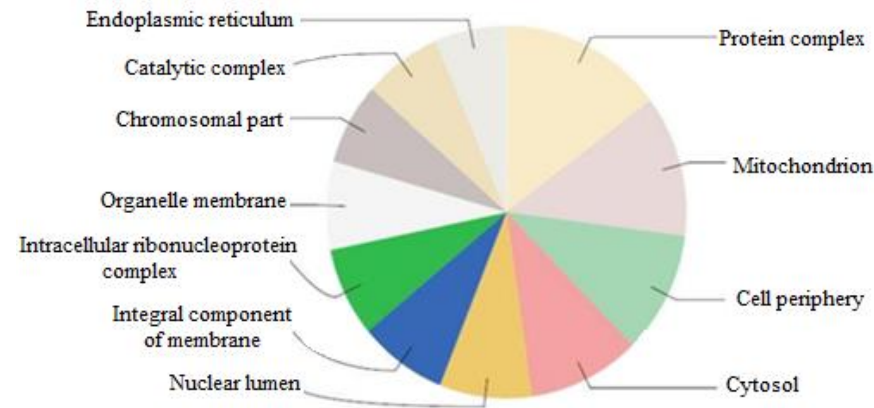


Figure A.2 Molecular functions, cellular localization, and biological processes of proteins enriched during CFQ





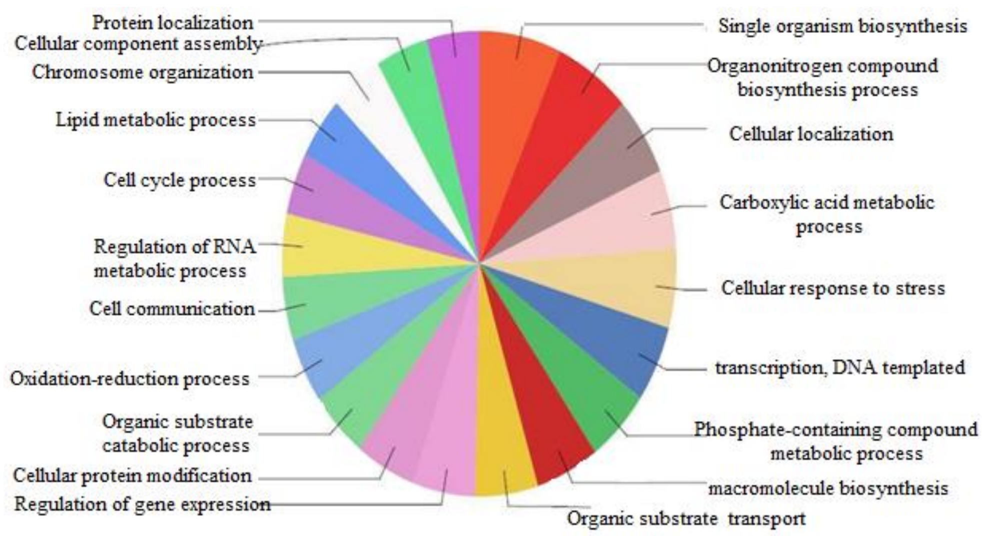


Figure A.3 Molecular functions, cellular localization, and biological processes of proteins enriched during CF

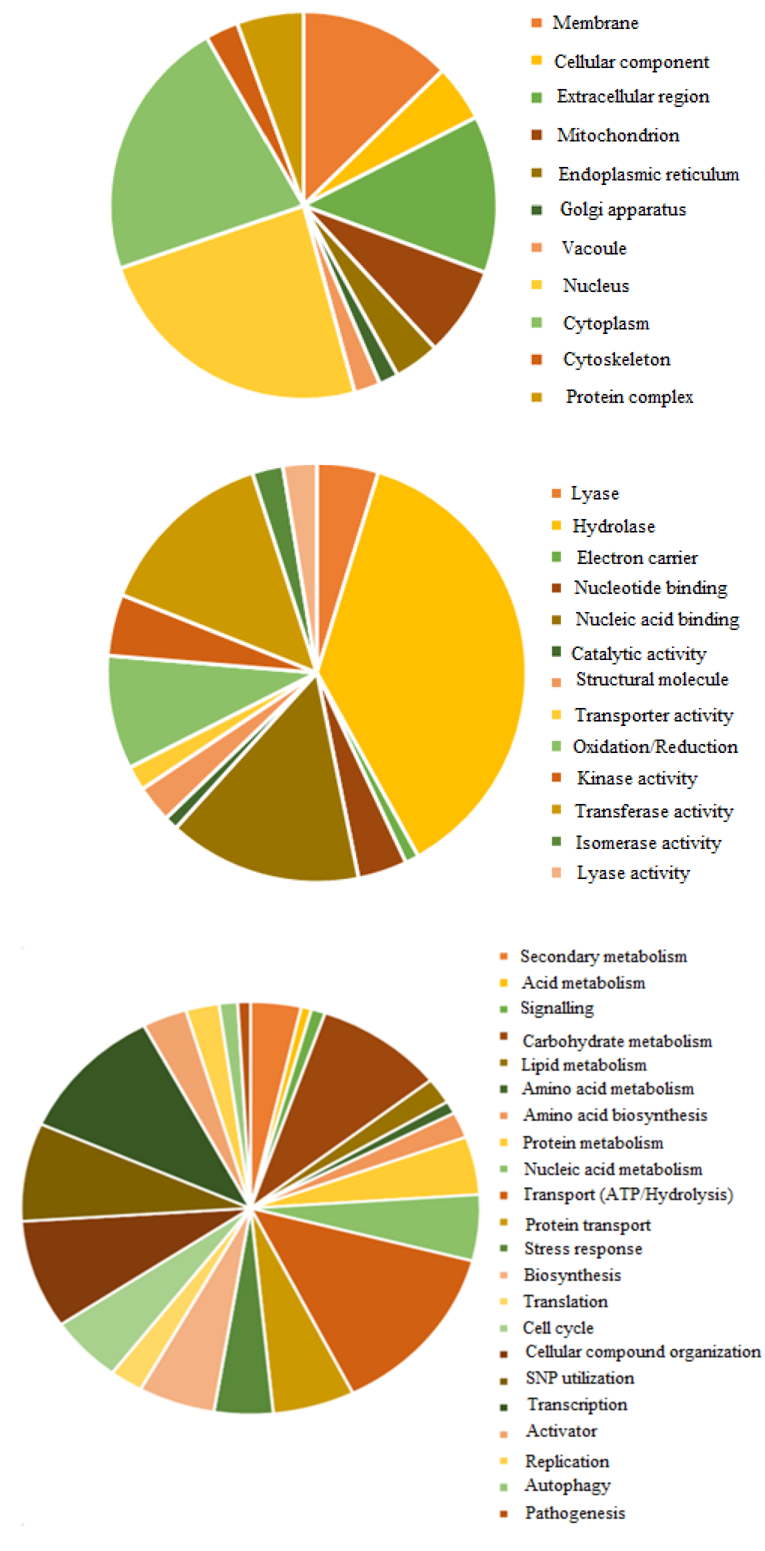
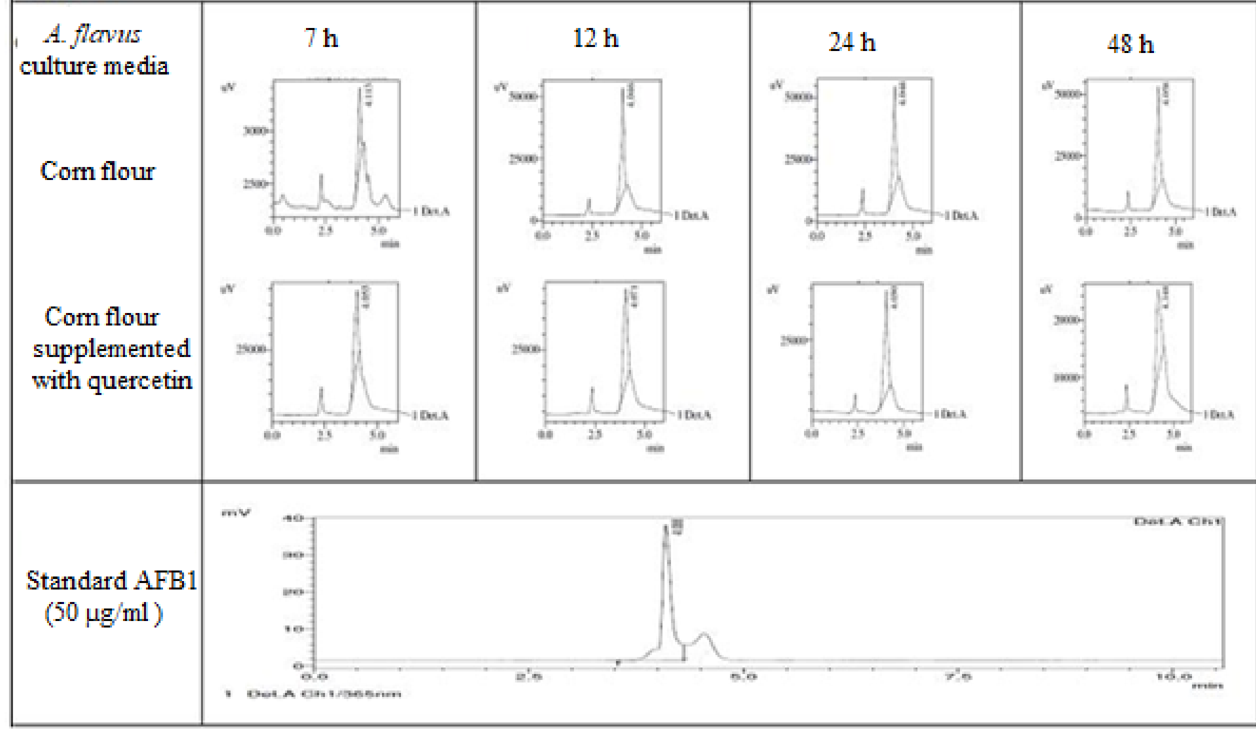


Figure A.4 Chromatogram of AF (AFB1) of *A. flavus* grown on two different culture conditions compared against each other and standard AFB1



APPENDIX B

Table B.1 Details of selected protein of carbohydrate metabolism involved in various biological, molecular and cellular processes. The data represents the significance in relation to p-values and the protein is represented by Gene Ontology IDs obtained from FungiFun 2.2.8 **B-** software.

GO ID	**GO name**	**Exact p-value**	**Adjusted p-value**	**# genes / category**	**# genes / input**
GO:0005576	extracellular region	3.5396e-88	3.1502e-86	49 / 108	49 / 88
GO:0000272	polysaccharide catabolic process	8.3886e-74	3.7329e-72	41 / 80	41 / 88
GO:0005975	carbohydrate metabolic process	3.6066e-72	1.07e-70	53 / 303	53 / 88
GO:0016798	hydrolase activity, acting on glycosyl bonds	6.828e-67	1.5192e-65	43 / 134	43 / 88
GO:0016787	hydrolase activity	2.269e-41	4.0389e-40	48 / 719	48 / 88
GO:0071555	cell wall organization	8.2287e-37	1.2206e-35	21 / 38	21 / 88
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	1.2833e-35	1.6316e-34	29 / 149	29 / 88
GO:0030245	cellulose catabolic process	9.981e-24	1.1104e-22	13 / 21	13 / 88
GO:0045490	pectin catabolic process	6.6053e-20	6.5319e-19	10 / 13	10 / 88
GO:0008422	beta-glucosidase activity	3.7158e-19	3.3071e-18	9 / 10	9 / 88
GO:0008152	metabolic process	6.0163e-18	4.8677e-17	44 / 1781	44 / 88
GO:0004650	polygalacturonase activity	5.8968e-15	4.3735e-14	9 / 20	9 / 88
GO:0045493	xylan catabolic process	2.6201e-12	1.7937e-11	8 / 23	8 / 88
GO:0047911	galacturan 1,4-alpha-galacturonidase activity	1.951e-11	1.2403e-10	5 / 5	5 / 88
GO:0052692	raffinose alpha-galactosidase activity	2.8076e-9	1.6658e-8	4 / 4	4 / 88
GO:0004557	alpha-galactosidase activity	1.396e-8	7.7653e-8	4 / 5	4 / 88

GO:0004565	beta-galactosidase activity	7.8E-06	4.09E-05	3 / 6	3 / 88
GO:0031176	endo-1,4-beta-xylanase activity	2.16E-05	0.000107	3 / 8	3 / 88
GO:0008810	cellulase activity	3.23E-05	0.000151	3 / 9	3 / 88
GO:0042732	D-xylose metabolic process	5.5E-05	0.000222	2 / 2	2 / 88
GO:0019566	arabinose metabolic process	5.5E-05	0.000222	2 / 2	2 / 88
GO:0009044	xylan 1,4-beta-xylosidase activity	5.5E-05	0.000222	2 / 2	2 / 88
GO:0030246	carbohydrate binding	8.64E-05	0.000334	4 / 32	4 / 88
GO:0016985	mannan endo-1,4-beta-mannosidase activity	0.000164	0.000608	2 / 3	2 / 88
GO:0030600	feruloyl esterase activity	0.000327	0.001163	2 / 4	2 / 88
GO:0030248	cellulose binding	0.000542	0.001854	2 / 5	2 / 88
GO:0016829	lyase activity	0.000585	0.001929	5 / 92	5 / 88
GO:0030570	pectate lyase activity	0.000809	0.00257	2 / 6	2 / 88
GO:0031222	arabinan catabolic process	0.001127	0.003457	2 / 7	2 / 88
GO:0052689	carboxylic ester hydrolase activity	0.001913	0.00532	2 / 9	2 / 88
GO:0046556	alpha-L-arabinofuranosidase activity	0.001913	0.00532	2 / 9	2 / 88
GO:0046373	L-arabinose metabolic process	0.001913	0.00532	2 / 9	2 / 88
GO:0005618	cell wall	0.00238	0.006417	2 / 10	2 / 88
GO:0005886	plasma membrane	0.00472	0.012356	2 / 14	2 / 88
GO:0006879	cellular iron ion homeostasis	0.00748	0.013585	1 / 1	1 / 88
GO:0004856	xylulokinase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0031225	anchored component of membrane	0.00748	0.013585	1 / 1	1 / 88
GO:0031221	arabinan metabolic process	0.00748	0.013585	1 / 1	1 / 88
GO:0006826	iron ion transport	0.00748	0.013585	1 / 1	1 / 88
GO:0004558	alpha-1,4-glucosidase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0016162	cellulose 1,4-beta-cellobiosidase	0.00748	0.013585	1 / 1	1 / 88

	activity				
GO:0016837	carbon-oxygen lyase activity, acting on polysaccharides	0.00748	0.013585	1 / 1	1 / 88
GO:0046557	glucan endo-1,6-beta-glucosidase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0004358	glutamate N-acetyltransferase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0042843	D-xylose catabolic process	0.00748	0.013585	1 / 1	1 / 88
GO:0042973	glucan endo-1,3-beta-D-glucosidase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0046576	rhamnogalacturonan alpha-L-rhamnopyranosyl-(1->4)-alpha-D-galactopyranosyluronide lyase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0031218	arabinogalactan endo-1,4-beta-galactosidase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0032450	maltose alpha-glucosidase activity	0.00748	0.013585	1 / 1	1 / 88
GO:0004338	glucan exo-1,3-beta-glucosidase activity	0.014904	0.024565	1 / 2	1 / 88
GO:0004042	acetyl-CoA:L-glutamate N-acetyltransferase activity	0.014904	0.024565	1 / 2	1 / 88
GO:0052861	glucan endo-1,3-beta-glucanase activity, C-3 substituted reducing group	0.014904	0.024565	1 / 2	1 / 88
GO:0052862	glucan endo-1,4-beta-glucanase activity, C-3 substituted reducing group	0.014904	0.024565	1 / 2	1 / 88
GO:0015926	glucosidase activity	0.014904	0.024565	1 / 2	1 / 88
GO:0047490	pectin lyase activity	0.022274	0.0354	1 / 3	1 / 88
GO:0004181	metallocarboxypeptidase activity	0.022274	0.0354	1 / 3	1 / 88
GO:0006526	arginine biosynthetic process	0.02959	0.044636	1 / 4	1 / 88
GO:0031410	cytoplasmic vesicle	0.02959	0.044636	1 / 4	1 / 88
GO:0005773	vacuole	0.02959	0.044636	1 / 4	1 / 88

Table B.2 Comparative analysis of *A. flavus* proteome with the expressed protein/ mRNA of *A. fumigatus* & *A. niger* at dormant and geminating stage conidia

Identified proteins (*A. flavus* conidia 7h)	**Dormant conidia (0 h)**		**Germinating conidia (6 h)**	
	***A. fumigatus* (protein profile)**	***A. niger* (mRNA profile)**	***A. fumigatus* (protein profile)**	***A. niger* (mRNA profile)**
Mannitol 1 phosphate 5 dehydrogenase	-	+	-	+
Heat shock 70 kDa protein	+	+	+	+
Heat shock protein 60	+	+	+	+
Catalase A	-	+	-	+
Catalase peroxidase	-	+	-	+
Superoxide dismutase Cu, Zn	-	+	-	+
Chitin synthase A	-	+	-	+
Chitin synthase C	-	+	-	+
1, 3-β glucan synthase component Fks-1	-	+	-	+
Endo β 1,4- glucanase B	-	+	-	
Cytochrome c oxidase subunit 1	-	-	-	+
Eukaryotic translation initiation factor 3 subunit A	+	-	+	-
Polyadenylate binding protein	+	-	+	-
40S ribosomal protein S1	+	-	+	-
40S ribosomal protein S0	+	-	+	-
Tubulin **β-** chain	-	-	+	-
Histone H1	-	-	-	-
NADP specific glutamate dehydrogenase	+	-	+	-
Pyruvate carboxylase	+	-	+	-
Actin	+	-	+	-
Aconitate hydratase	+	-	+	-
Alcohol dehydrogenase 1	+	-	+	-

Table B.3 Protein modeling parameters for homology modeling of seven domains of PksA of *A. flavus* from SWISS-MODEL accessible via ExPASy server

PksA domains	SMTL Id	Peptides	Coverage	% Identity
ACP transacylase domain	4ro5.1	Crystal structure of the SAT domain from the non-reducing fungal polyketide synthase CazM	0.84	18.18
β-ketoacyl synthase domain	4v58.1.G	Crystal structure of fatty acid synthase from thermomyces lanuginosus at 3.1 angstrom resolution.	0.77	20.73
Ketoacyl-synthetase C-terminal domain	4ro5.1.A	Crystal structure of the SAT domain from the non-reducing fungal polyketide synthase CazM	0.86	18.75
Acyl transferase domain	3rgi.1.A	Trans-acting transferase from Disorazole synthase	0.74	21.92
Product template domain	2zp2.1.A	C-terminal domain of KipI from Bacillus subtilis	0.62	32.50
Phosphopantethein e attachment site	5erb.1.A	Ketosynthase from module 5 of the bacillaene synthase from Bacillus amyloliquefaciens FZB42	1	65.71
Thioesterase domain	4na2.1.A	Crystal Structure of the second ketosynthase from the bacillaene polyketide synthase bound to its natural intermediate	0.98	40.51

LIST OF PUBLICATIONS

Research Articles

[1] **Shraddha Tiwari**, Nupur Gupta, Udayabanu Malairaman and Jata Shankar. (2017). Anti-*Aspergillus* properties of phytochemicals against aflatoxin producing *Aspergillus flavus* and *Aspergillus* parasiticus. **National Academy Science Letters**, vol. 40, no. 4, pp. 267-271, 2017. doi: 10.1007//s40009-0170569-y

[2] **Shraddha Tiwari**, Raman Thakur, Gunjan Goel and Jata Shankar. (2016). Nano LC-Q-TOF analysis of proteome revealed germination of *Aspergillus flavus* conidia is accompanied by MAPK signalling and cell wall modulation. **Mycopathologia**, vol. 181, no. 11-12, pp. 769-786, 2016. doi: 10.1007/s11046-016-0056-x

[3] **Shraddha Tiwari** and Jata Shankar. (2018). Integrated Proteome and HPLC analysis revealed Quercetin mediated inhibition of Aflatoxin B1 Biosynthesis in *Aspergillus flavus*. **3 biotech**, vol. 8, no. 1, pp. 47, 2018. doi: 10.1007/s13205-017-1067-0

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[1] **Shraddha Tiwari**, Nupur Gupta, Udaybanu M and Jata Shankar (2015) Participated in National conference on Public Health; Issues, Challenges, Opportunities, prevention, awareness organized by Krishi Sanskriti at Daulat Ram College, January- 15th- 16th 2016, New Delhi, India and oral presentation entitled "Anti-*Aspergillus* properties of phytochemicals against aflatoxin producing *Aspergillus flavus* and *Aspergillus* parasiticus"

[2] **Shraddha Tiwari** and Jata Shankar (2016) Participated in National conference on Fungal Biotechnology and 43rd Annual meeting of Mycology Society of India organized by Birla Institute of Scientific Research, November-16th-18th, 2016, Jaipur, India and poster presentation entitled "Proteome profile of germinating *Aspergillus flavus* conidia on corn flour supplement."

[3] **Shraddha Tiwari**, Raman Thakur, Gunjan Geol and Jata Shankar (2016) Internation conference on 7^{th}Advances against Aspergillosis organized by University of Manchester, March-3rd -5th, 2016, Manchester, United Kingdom and poster entitled "Identification and functional characterization of protein involved in germination of *Aspergillus flavus* conidia."