"Differential expression analysis of genes encoding for heat shock proteins in different morphotypes of *Aspergillus flavus*"

A PROJECT

Submitted in fulfillment of the requirements for the award of the degree of

BACHELOR OF TECHNOLOGY

IN

Biotechnology

Under the supervision of

Asst. Prof. Dr. Jata Shankar

by

Sameeksha 141852

to

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN - 173234

HIMACHAL PRADESH, INDIA

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DECLARATION BY THE SCHOLAR

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

Signature of the Scholar

Sameeksha Sharma (141852)

Department of Biotechnology and Bioinformatics

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Date :

CERTIFICATE

This is to certify that the work which is being presented in the project title, **"Differential expression analysis of genes encoding for heat shock proteins in different morphotypes of *Aspergillus flavus*"** in fulfilment of the requirements for the award of the degree of Bachelor of technology and submitted in Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by Sameeksha (141852) during a period from July 2017 to May 2018 under the supervision of **Dr. Jata Shankar**, Assistant Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat.

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

Date :

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Assistant Professor

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ACKNOWLEDGEMENT

Words betray to express my heartfelt sentiment towards the two towering peaks of my life, my parents who have helped me to surpass all the academic pursuits and my life.

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I extend my cordial thanks to all those who directly or indirectly helped in the successful fulfilment of my work.

Date :

Place : JUIT, Waknaghat Sameeksha

LIST OF TABLES AND FIGURES

LIST OF ACRONMYS AND ABBREVIATIONS

A. flavus	*Aspergillus flavus*
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Conc.	Concentration
Mm	Millimeter
B1	Blue1
B2	Blue2
G1	Green1
G2	Green2
$_0$C	Degree Celsius
kDa	Kilodalton
(n)	Haploid
Hsps	Heat shock proteins
Rpm	Rotation per minute
Ppb	Parts per billion
aW	Water activity
mL	Millimeter
Ct	Cycle threshold
Ng	Nanogram

ABSTRACT

Aspergillus flavus is a lysotrophic and disease causing fungus distributed world-widely. It appears everywhere in nature, and is capable of affecting wide range of host. It colonizes on cereal grains, legumes and tree nuts. Many strains produce significant quantities of toxic compounds known as mycotoxins. These mycotoxins are toxic to mammals. *Aspergillus flavus* is also an opportunistic pathogen which causes aspergillosis in immune-system compromised individuals. Heat shock proteins (HSP) are a family of proteins that are produced by cells in response to exposure to stressful conditions or routine biological processes. It has been reported in several studies that reactive oxygen species production is increased in those cells that are under thermal stress which also activates Hsps . In current scenario to overcome various fungal related diseases in both plants and animals, research is being focused on role of various heat shock proteins in morphogenesis of *Aspergillus flavus* and screening of probable heat shock proteins which could be drug targets in coming future.

CHAPTER– 1

INTRODUCTION

1.1 *Aspergillus.flavus*:

Aspergillus flavus is an infective fungus which is saprotrophic in nature and is distributed worldwide. It is omnipresent in nature and affects a wide range of host. It mainly colonizes on cereal grains, legumes and tree nuts. *A.flavus* infections can occur while hosts are present pre-harvest phase. However it doesn't show any symptoms until post harvest storage and/ or transport. It not only causes pre-harvest and postharvest infections but several strains of this fungus also produce significant amount of virulent compounds termed as mycotoxins (vigorous carcinogen).

1.2 Mycotoxin and its classification:

Mycotoxin is a secondary metabolite which is produced by *Aspergillus flavus* that have potential to cause disease and its severity often led to even death in humans and animals as well. These mycotoxins becomes virulent to mammals upon consumption. These are classified into six groups inclusive of Aflatoxins, Ochratoxin, Citrinin, Patulin, Ergot alkaloids and Fusarium. *A.flavus* is a disease causing fungus which causes aspergillosis and hepatocellular carcinoma (liver cancer) in humans and ear rot (corns) in plants.

1.3 Scientific Classification of *Aspergillus.flavus* :

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

Table 1.1 Scientific classification of *A. flavus*

1.4 Morphological stages of fungi:

It exists in three morphological stages: Conidia, Hyphae and Mycelia.

Conidia: Spore of fungi which is immobile and reproduced asexually, produced at the tip of hyphae or on conidiophores (spore-producing structures) which differs in shape, size and colour; large ones are called macroconidia while small ones are called microconidia. As they are originated through cellular process of mitosis they often called mitospores. If conditions remains favourable; two new (n) cells genetically similar to the (n) parent can develop into new organism. In ascomycetes; asexual mode of reproduction happens because of the conidial formation, borne on conidiophores. The morphology of these specialized spore- producing conidiophores is often idiosyncratic and can be used in the identification of several species.

Hyphae: Hyphae are composed of long filamentous branches called hypha which is found in fungi and actinobacteria having average size of 4 to 6 microns. They are chief structures that are essential for growth in these species and jointly are cited to as mycelium. Each hypha is consist of one cell confined by a protective cell wall composed of chitin which contain internal septa which serve to divide cells. Septa are important because they allow cellular organelles like ribosomes to pass in between the cells through large pores. However all fungal species do not contain septa. It performs functions like nutrient transportation, nutrient absorption from soil and nutrient absorption from a host etc.

Mycelia: Mycelia is the vegetative part of fungus comprised of mass of branched and tubular filaments (hyphae). It makes up the thallus or undifferentiated body of a fungus. It can be minute in size or furthermore; can be developed into visible structures such as brackets, mushrooms, puffballs, rhizomorphs , sclerotia , stinkhorns, toadstools, truffles etc. At specific stage either through special fruiting bodies or directly it produces spores. It performs functions like nutrient absorption, decomposition phenomenon etc.

CHAPTER– 2

REVIEW OF LITERATURE

2.1 Aflatoxins:

It produces vigorous carcinogen called aflatoxin upon infecting agricultural crops. Aflatoxins are those secondary metabolites which are produced predominatly by *A.flavus* and *A. parasiticus* species. Aflatoxins B1 and B2 are produced by*A. Flavus* species while aflatoxins G1, G2, B1 and B2 are produced by *A. parasiticus*. Aflatoxin B1 is considered to be virulent and vigorous hepatocarcinogenic natural compound eternally identified). *A. flavus* affects a wide range of agricultural crops including monocots and dicots as well .It affects pre-harvest crops in the field (maize ears, cotton balls, peanut pods and tree nuts)and post-harvest grains during storage after insect or mechanical damage has occurred. *A. flavus* is frequently linked with contamination of aflatoxins and renders non- consumable crops. Severe toxicity and everlasting carcinogenicity are interacted with consumption of aflatoxin-contaminated food and feed inhumans and animals as well. Aflatoxin content in food is examined and synchronized in countries across the world due to the health hazard of aflatoxins to humans and its severity on livestock and livestock productivity. 220ppb is the maximum licit limit imposed by the United States Food and Drug Administration (USFDA) for consumption and interstate shipment of food and feed(Robens 2001; Richard and Payne 2003; Robens and Cardwell 2005) [1].

2.2 Disease caused by *A.flavus* in both plants and animals:

A. flavus may cause disease in immune-system compromised individuals. It is considered to be the second leading cause of invasive and non-invasive aspergillosis after *A. fumigatus* . *A. flavus* causes diseases in many agricultural crops like in corn, cotton, peanuts, pistachio nuts, walnuts etc. It can attack seeds of monocots and dicots as well exhibit that this fungus has evolved a mechanism so to break the host resistance. Slight plant disease-causing fungi have such a wide host range. Comparitive to *A. fumigatus* and *A. nidulans, A. flavus* lacks specificity of the host[2].Once damage

caused either by mechanical or insects takes place it affects corn ears, cotton balls and peanut pods.[3]. Under favourable weather conditions,*A. flavus* can cause ear rot on maize. *A. flavus* have tendency to colonize grains seeds and oil crops becauseof its potential to grow at low a_w. Parameters correlated with *A. flavus* infections in plants are high temperature and plant stress. [4].

AFB1 has been found to be common contaminant of major crop such as maize, groundnuts, cotton and other pre-harvested/post-harvested crops among varied types of aflatoxins. [5,6]. Aflatoxins are genotoxic carcinogen in which AFB1 is the most toxic compound classified by the International Agency for Research on Cancer (IARC) in 1994[7,8]. Regular intake of aflatoxin having low level through food products that are contaminated can leads to aflatoxicosis which is distinguished by jaundice, quickly developing ascites, hypertension, vomiting, abdominal pain, pulmonary edema, fatty infiltration and necrosis of the liver [9].

2.3 Role of Heat shock proteins:

Stress plays a vital role in functional characterization of fungi. Protein denaturation causes native misfolding of protein and protein aggregation eventually leading to cell apoptosis and loss of biological functions[10]. The survival of organism is encouraged by response of set of proteins to changes related to stress.The family of these proteins is termed as Heat shock proteins. They belong to a protein family which are produced in response to exposure to stressful conditions or routine biological processesby cells. On the basis of their respective molecular mass they are of the following types: HSP60 (57-69 kDa), HSP70 (70 kDa), HSP90 (90 kDa), HSP98 families. They can be found everywhere in a cell like in mitochondria, cytosol, endoplasmic reticulum, cell membrane or nucleus[11].

Functions of Hsps involves replication, cell cycle progression, transcriptional and post translational processes such as protein folding, stability, degradation, and transportation. Hsps are even reported in the activation of key signal transducers in fungi [11,12]. They are highly conserved biomolecules which are constitutively expressed and upregulated in respond to varied stress conditions including both biotic and abiotic[13]. It is also suggested that they plays significant role in homeostasis stress response as well.

There are several problems which are caused by change in temperature and are linked with temperature dependent morphological transitions &protein folding [14]. Cells which are affected by thermal stress generally shows Heat-shock response. [12]. It has been reported that (ROS) production is increased in cells under thermal stress which also activates Hsps [15]. In dimorphic fungus, temperature stress may exhibit an effect on divergent phases of life cycle; thus protein expression can be heat-induced or phase-specific[16]. One such interesting area to focus on is Hsps, due to its very wide role in fungal survival during stress conditions. In recent years, Hsp 90 has been proposed as antifungal target [17,18,19]. So our research is focused on role of various heat shock proteins in morphogenesis of *Aspergillus flavus* and screening of probable heat shock proteins which could be drug targets in coming future.

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CHAPTER – 3

OBJECTIVES

Objective 1: To study the role of Hspsin different morphotypes of *Aspergillus flavus*.

Objective 2: Semi quantitative analysis of transcripts with respect to heat shock proteins (Hsp60, Hsp70, Hsp90, Hsp98) and regulatory gene (tubulin).

Objective 3: Quantitative analysis using Real time PCR.

A.flavus is a delicate disease causing fungus infecting plants, animals and humans as well. It is known to produce vigorous carcinogen called aflatoxin which is a secondary metabolite and can cause disease and its severity may led to even death in humans and animals both when it comes to affect agricultural crops.Aflatoxin B1 is considered to be virulent and vigorous hepatocarcinogenic natural compound characterized till now.Aflatoxin biosynthesis happens at 30°C and gets inhibited at 37°C. Hence, role of Hsps seem critical here.

The aim of my study is to analyse the differential expression of gene encoding for the Hsps in different morphotypes of *Aspergillus flavus* and to assess the role of Hsps using transcript profiling.

CHAPTER– 4

METHODOLOGY

4.1 Microorganism and Growth Conditions

4.1.1 Media preparation and Culturing of *A.flavus*:

1. 9.75 grams of PDA was dissolved in 250 ml flask containing distilled water followed by heating till boiling in microwave to get it properly mixed; media was then autoclaved.
2. Pouring was done in LAF on petri-plates; once media gets solidified on plates, streaking was done by using culture of *A.flavus* on plates.

4.2 Cell count by haemocytometer

Cell count was done by using Haemocytometer to check the viability of cells.

1. Spore suspension was prepared in 1 ml of PBS.
2. Clean haemocytometer and cover slip by using 70% ethanol.
3. 10 µL of cell suspension was pipette in the counting chambers.
4. Conidia was counted and viability and non-viabilty of the cells were seen.
5. Colony forming units was calculated using CFU formula:
   CFU= (No. of Colonies* dilution factor) / Volume of inoculum taken)

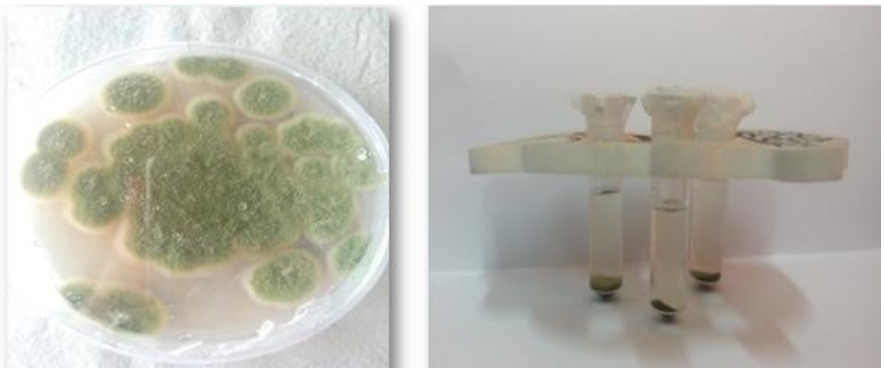

Fig 4.1: *A.flavus* (MTCCC11866) on PDA plate and harvested conidia

4.3 Large-Scale RNA extraction

1. 2.4 gm of PDB was dissolved in 100 ml flask each containing 100 ml distilled water to prepare broth and then media was autoclaved.
2. Innoculation was done by using *A.flavus* culture and poured it 1 ml in each flask.
3. Flasks were then kept in incubator at 37°C for shaking at different time intervals of 4 hrs, 7 hrs and 12 hrs respectively.

4.4 RNA extraction by TRIzol method

4.4.1 For 4 hour culture:

1. A. flavus germ cells were crushed by using liquid nitrogen followed by adding 1ml TRIzol reagent.
2. Incubation was done at room temperature for 5 minutes.
3. 500µL of chloroform was added, followed by mixing gently for phase separation.
4. Overagain incubation was done at room temperature for 2-3 minutes
5. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C.
6. After centrifugation process sample was clearly separated into 3 layers from which the clear upper phase was transferred in autoclaved micro centrifuge tube.
7. Precipitation of RNA was done by adding 250µL of isopropanol in each tube followed by mixing.
8. Overagain incubation was done at room temperature for 10-15 minutes.
9. Centrifugation of samples was done at 12,000rpm for 15 minutes at 4°C.
10. Supernatant was discarded and pellet of RNA was washed with 200µL-300µL of 70% ethanol.
11. Centrifugation was done at 7,000 rpm for 5 minutes at 4°C.
12. Washing step was repeated overagain.
13. After 2 turns of washing resulted pellet was left for 15-20 minutes to let it dried completely.
14. 30-40µL DEPC treated autoclaved water was added.

15. Incubation at 65°C for 10 minutes was carried out.
16. Atlast storage of samples were done at -20°C.
17. OD was checked in nano-drop spectrophotometer to check the conc. of RNA.

4.4.2 For 7 hour culture:

1. RNA extraction from the harvested cells of time interval 7 hr was carried out using the TRIzol method.
2. A. flavus germ cells were crushed by using liquid nitrogen followed by adding 1ml TRIzol reagent.
3. Incubation was done at room temperature for 5 minutes.
4. 500µL of chloroform was added, followed by mixing gently for phase separation.
5. Overagain incubation was done at room temperature for 2-3 minutes
6. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C.
7. After centrifugation process sample was clearly separated into 3 layers from which the clear upper phase was transferred in autoclaved micro centrifuge tube.
8. Precipitation of RNA was done by adding 250µL of isopropanol in each tube followed by mixing.
9. Overagain incubation was done at room temperature for 10-15 minutes.
10. Centrifugation of samples was done at 12,000rpm for 15 minutes at 4°C.
11. Supernatant was discarded and pellet of RNA was washed with 200µL-300µL of 70% ethanol.
12. Centrifugation was done at 7,000 rpm for 5 minutes at 4°C.
13. Washing step was repeated overagain.
14. After 2 turns of washing resulted pellet was left for 15-20 minutes to let it dried completely.
15. 30-40µL DEPC treated autoclaved water was added.
16. Incubation at 65°C for 10 minutes was carried out.
17. Atlast, storage of samples was done at -20°C.
18. OD was checked in nanodrop spectrophotometer to check the conc. of RNA.

4.4.3 For 12 hour culture:

1. RNA extraction from the harvested cellsof time interval 12 h was carried out using the TRIzol method.
2. A. flavus germ cells were crushed by using liquid nitrogen followed by adding 1ml TRIzol reagent.
3. Incubation was done at room temperature for 5 minutes.
4. 500µL of chloroform was added, followed by mixing gently for phase separation.
5. Overagain incubation was done at room temperature for 2-3 minutes
6. . Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C.
7. After centrifugation process sample was clearly separated into 3 layers from which the clear upper phase was transferred in autoclaved micro centrifuge tube.
8. Precipitation of RNA was done by adding 250µL of isopropanol in each tube followed by mixing.
9. Overagain incubation was done at room temperature for 10-15 minutes.
10. Centrifugation of samples was done at 12,000rpm for 15 minutes at 4°C.
11. Supernatant was discarded and pellet of RNA was washed with 200µL-300µL of 70% ethanol.
12. Centrifugation was done at 7,000 rpm for 5 minutes at 4°C.
13. Washing step was repeated overagain.
14. After 2 turns of washing resulted pellet was left for 15-20 minutes to let it dried completely.
15. 30-40µL DEPC treated autoclaved water was added.
16. Incubation at 65°C for 10 minutes was carried out.
17. Atlast, storage of samples was done at -20°C.
18. OD was checked in nanodrop spectrophotometer to check the conc. of RNA.

4.5 Gel electrophoresis

4.5.1 Visualisation of RNA bands of 4 hour sample:

1. 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose in 100 ml of 1X TAE buffer.
2. Solution was boiled until agarose gets dissolved properly and makes a clear solution.
3. It was left over for 15 minutes to get cool down.
4. Once it gets cool down, 5µL of EtBr was added and shake gently and poured in gel casting tray; gel comb was placed then to make wells and it was left for 15-20 minutes to get completely solidified.
5. After gel gets solidified; casting tray was put in electrophoresis tank containing 1X TAE buffer.
6. Comb was removed.
7. 4µL of ladder was loaded.
8. 4 hour sample was added along with dye to wells in 2:2 ratio.
9. Gel was run at 150W for 45 minutes.
10. It was visualized in Gel doc system under ultra-violet light using Alphaimager.
11. RNA bands of good intensity were observed.

4.5.2 Visualisation of RNA bands of 7 hour sample:

1. 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose in 100 ml of 1X TAE buffer.
2. Solution was boiled until agarose gets dissolved properly and makes a clear solution.
3. It was left over for 15 minutes to get cool down.
4. Once it gets cool down, 5µL of EtBr was added and shake gently and poured in gel casting tray; gel comb was placed then to make wells and it was left for 15-20 minutes to get completely solidified.
5. After gel gets solidified; casting tray was put in electrophoresis tank containing 1X TAE buffer.
6. Comb was removed.

7. 4μL of ladder was loaded.
8. 7 hour sample was added along with dye to wells in 2:2 ratio.
9. Gel was run at 150W for 45 minutes.
10. It was visualized in Gel doc system under ultra-violet light using Alphaimager.
11. RNA bands of good intensity were observed.

4.5.3 Visualisation of RNA bands of 12 hour sample:

1. 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose in 100 ml of 1X TAE buffer.
2. Solution was boiled until agarose gets dissolved properly and makes a clear solution.
3. It was left over for 15 minutes to get cool down.
4. Once it gets cool down, 5μL of EtBr was added and shake gently and poured in gel casting tray; gel comb was placed then to make wells and it was left for 15-20 minutes to get completely solidified.
5. After gel gets solidified; casting tray was put in electrophoresis tank containing 1X TAE buffer.
6. Comb was removed.
7. 4μLof ladder was loaded.
8. Sample was added along with dye to wells in 2:2 ratio.
9. Gel was run at 150W for 45 minutes.
10. It was visualized in Gel doc system under ultra-violet light using Alphaimager.
11. RNA bands of good intensity were observed.

4.6 cDNA preparation for RNA samples (4hr, 7hr and 12hr):

1. TAKARA BIO INC. cDNA synthesis kit was used.
2. A total of 20μL of sample was prepared.
3. Nanodrop spectrophotometer reading was taken to obtain that vol. which would contain equal conc. of RNA in the samples.

COMPONENTS	QUANTITY
cDNA synthesis buffer	4μL
Dntp	2μL
Oligo dT primer	1μL
Reverse transcriptase enhancer	1μL
Reverse transcriptase enzyme	1μL
RNA template	1μL
Water (DEPC treated)	10μL

Table 4.1: cDNA reaction mixture components

4. Total volume of 20 μL reaction mixture was prepared.
5. To carry out a single cycle of for cDNA synthesis PCR vials were mixed gently and put into thermocycler using following time-temp. profile.
   i. 30℃ for 10 min.
   ii. 42℃ for 1 hour.
   iii. 95℃ for 5 min.
6. We obtained cDNA product whose amplification had to be carried out at the end of the cycle.
7. Amplification of cDNA product was carried out.

4.7 Semi Quantitative Analysis of Transcripts with respect to tubulin; (regulatory gene) and Hsp60,70 and 98:

1. Reaction mixture of 15µL was prepared for each sample.
2. Composition of reaction mixture are as follows:

COMPONENTS	QUANTITY
Buffer	2µL
Dntp	0.5µL
Tubulin/Hsp60/Hsp70 and Hsp98forward primer	0.5µL
Tubulin/hsp60/Hsp70 and Hsp98 reverse Primer	0.5µL
Template	1µL
Taq. Polymerase	0.25µL
Water	10.25µL

Table 4.2 : PCR reaction mixture components

3. PCR reaction was set in thermocycler.
4. In thermocycler PCR vials were put and reaction was set according to following time-temperature profiling:
    i. Initial denaturation: 95°C for 5min.
    ii. Denaturation: 95°C for 30 sec.
    iii. Annealing: 53.6 °C for 45 sec.
    iv. Extension: 72 °C for 30 sec.
    v. Final extension: 70°C for 7min.

vi. Hold at 4°C

5. 35 cycles of PCR were carried out.
6. PCR product was then stored at -20°C.

NAME	Tm(°C)	SEQUENCES
Tubulin FP	55.9	F 5'-GGAATGGATCTGACGGCAAG-3'
Tubulin RP	56.8	R 5'-GGTCAGGAGTTGCAAAGCG-3'
Hsp60 FP	53.5	F 5'-GGTTTGACAGCTCCAAGG-3'
Hsp60 RP	54.2	R 5'-GTGGTACCAAGGAGAGAGG-3'
Hsp70 FP	54.5	F 5'-CCTACTCCCTCAAGAACACC-3'
Hsp70 RP	53.8	R 5'-GAGACTCGTACTCCTCCTTG-3'
Hsp90 FP	55.7	F 5'-CGTCAAGTCCATCACTCAGC-3'
Hsp90 RP	62.5	R 5'-GCTTGTGGATGCGCTCGGC-3'
Hsp98 FP	52.4	F 5'-GAGAGATGAGGCAGAACG-3'
Hsp98 RP	53.9	R 5'-TCCACCTCGAGTCTTTCG-3'

Table 4.3 : List of primers with their Tm and sequence

7. By performing gel electrophoresis on the amplified cDNA sample; preparation of cDNA was confirmed.

4.8 Gel electrophoresis of cDNA samples:

1. 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose in 100 ml of 1X TAE buffer.
2. Solution was boiled until agarose gets dissolved properly and makes a clear solution.
3. It was left over for 15 minutes to get cool down.
4. Once it gets cool down, 5µL of EtBr was added and shake gently and poured in gel casting tray; gel comb was placed then to make wells and it was left for 15-20 minutes to get completely solidified.
5. After gel gets solidified; casting tray was put in electrophoresis tank containing 1X TAE buffer.
6. Comb was removed.
7. 1Kb of DNA ladder was loaded to check the amplification results.

8. Amplified cDNA samples were loaded.
9. Gel was run at 150W for 45 minutes.
10. It was visualized in Gel doc system under ultra-violet light using Alphaimager.
11. Bands were observed.

4.9 Real-time PCR:

1. Real-time PCR was carried out in a thermocycler using CFX96 BIO-RAD Real-Time System.
2. Cyber-green dye was used to illuminate each sample with a beam of light of specified wavelength.
3. It detects the fluorescence emitted by the excited fluorophore.

COMPONENTS	MIXTURE
SYBR-green Dye	6μL
Reverse Primer	0.5μL
Forward Primer	0.5μL
cDNA	0.5-1μL(200ng)
Water	4.5μL

Table 4.4 : PCR reaction mixture components for Real-Time PCR

4. PCR cycles consist of three stages:
    i. 95°C (seperates double chain of nucleic acids).
    ii. 50-60°C (binds primers with template of DNA).
    iii. 68-72 °C (polymerization done by using DNA polymerase).

5. Results were observed in the form of amplification curves & Ct values which reveals differential expression of genes.

CHAPTER – 5

RESULTS AND DISCUSSIONS

5.1 RNA Isolation

1. The RNA samples were run on 1.5% agarose gel stained with 5µL of EtBr in gel electrophoresis unit. The bands were observed in gel doc system.

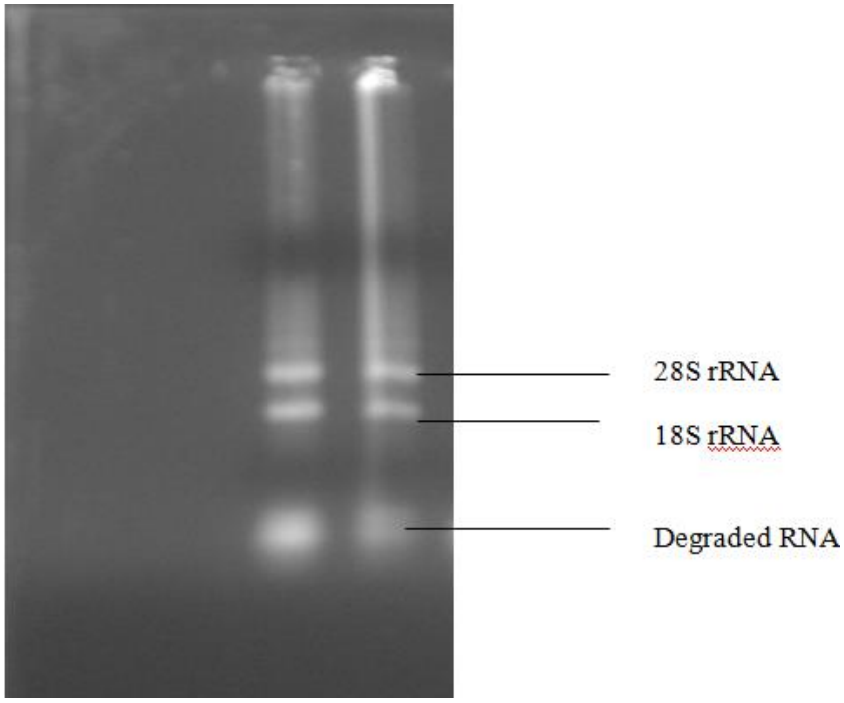

Fig.5.1 Gel doc image depicting RNA bands of 4 hours sample

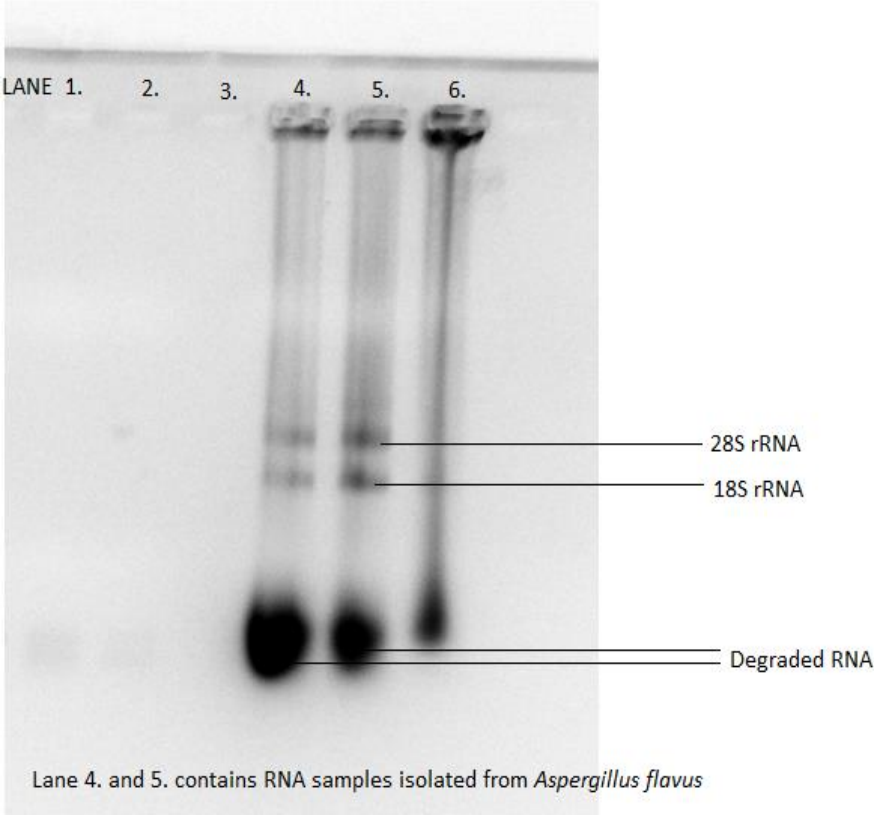

Fig.5.2 Gel doc image depicting RNA bands of 7 hour sample

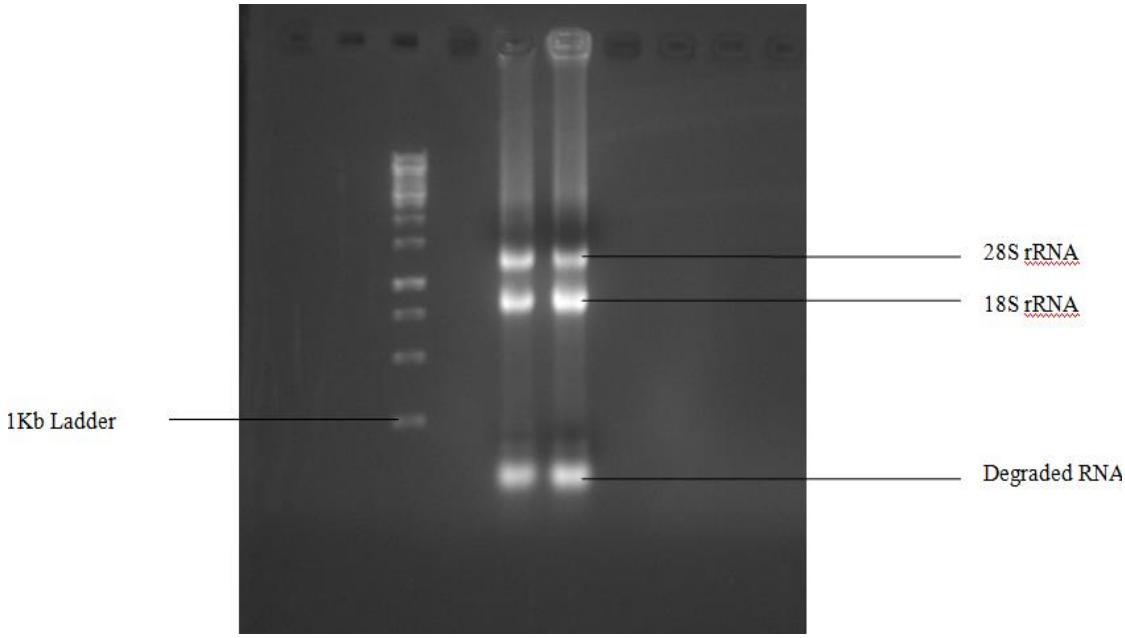

Fig.5.3 Gel doc image depicting RNA bands of 12 hour sample

RESULT: RNA was successfully extracted from the samples.

DISCUSSION: RNA bands were observed after the samples were run on 1.5% agarose gel. 28S rRNA and 18S rRNA bands were observed. In (fig.3.); The band intensity of 28S rRNA and 18S rRNA of both samples were good which implies RNA was of good quality. However; some of the sample was also degraded. In (fig.4.); The band intensity of 28S rRNA and 18S rRNA of first sample was not so good which implies that RNA was not of good quality while band intensity of 28S rRNA and 18S rRNA of second sample was good which implies that RNA was of good quality. However; some of the sample was also degraded. In (fig.5.); The band intensity of 28S rRNA and 18S rRNA of both samples were good which implies RNA was of good quality. However; some of the sample was also degraded.

5.2 Semi Quantitative Analysis

i. The PCR product after cDNA synthesis, with tubulin as control was run on 1.5% agarose gel, stained with 5µL of EtBr and the bands were observed in the gel doc system.

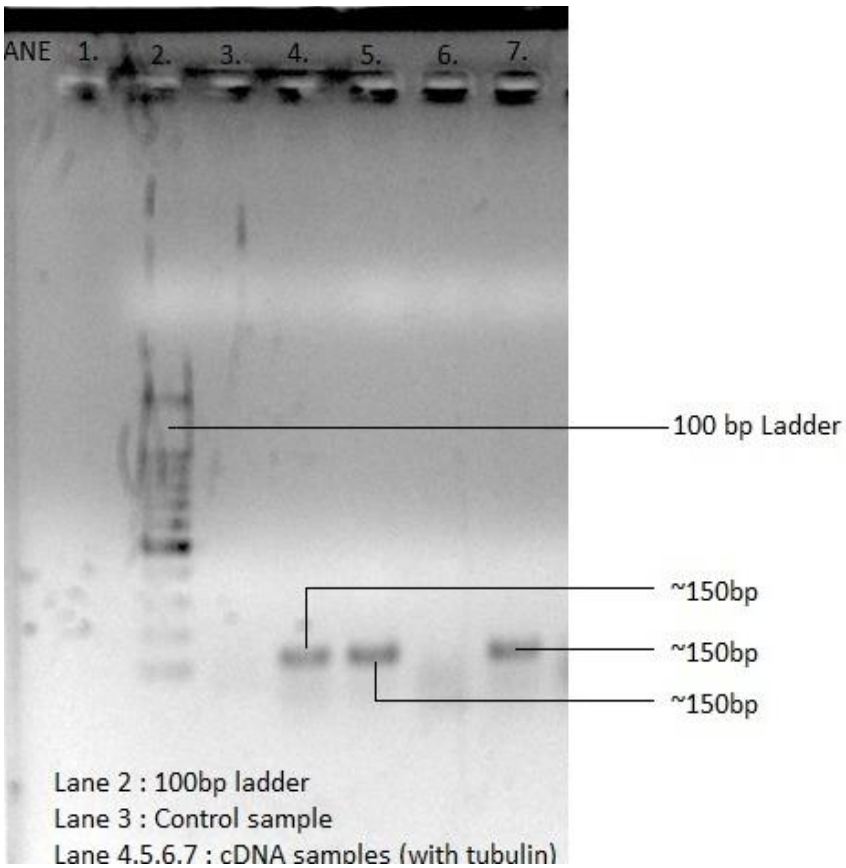

Fig.5.4 Gel doc image depicting expression of tubulin on cDNA samples

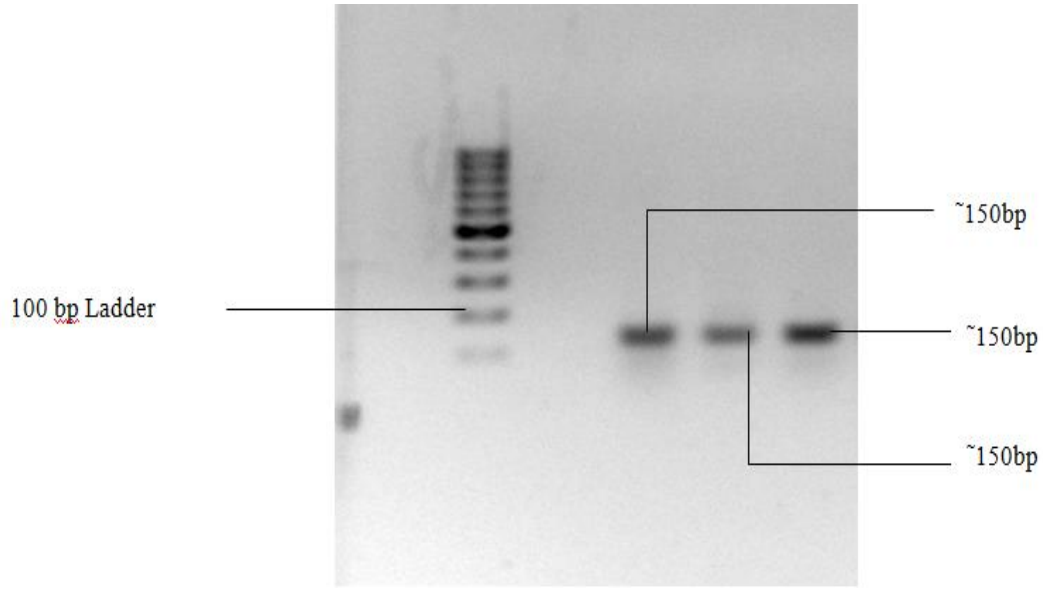

Fig.5.5 Gel doc image depicting expression of tubulin on cDNA samples (4,7 and 12hr)

RESULT: Visualization of the bands confirms the expression of tubulin in the cDNA samples extracted from *Aspergillus flavus***.**

DISCUSSION: In (fig.6.); Bands were observed in lane 4,5,7 but not in lane 6 which implies that there must have been some handling error as the sample was same. Size of the bands observed was approximately 150bp. The bands were intense, confirming the expression of tubulin in *Aspergillus flavus*. In (fig.7); Bands were observed in lane 4,5,6. Size of the bands observed was approximately 150bp. The bands were intense, confirming the expression of tubulin in *Aspergillus flavus*.

5.2.1 PCR product after cDNA synthesis was run on 1.5% agarose gel stained with 5µL EtBr to check the expression of Hsp60, Hsp70 and Hsp90:

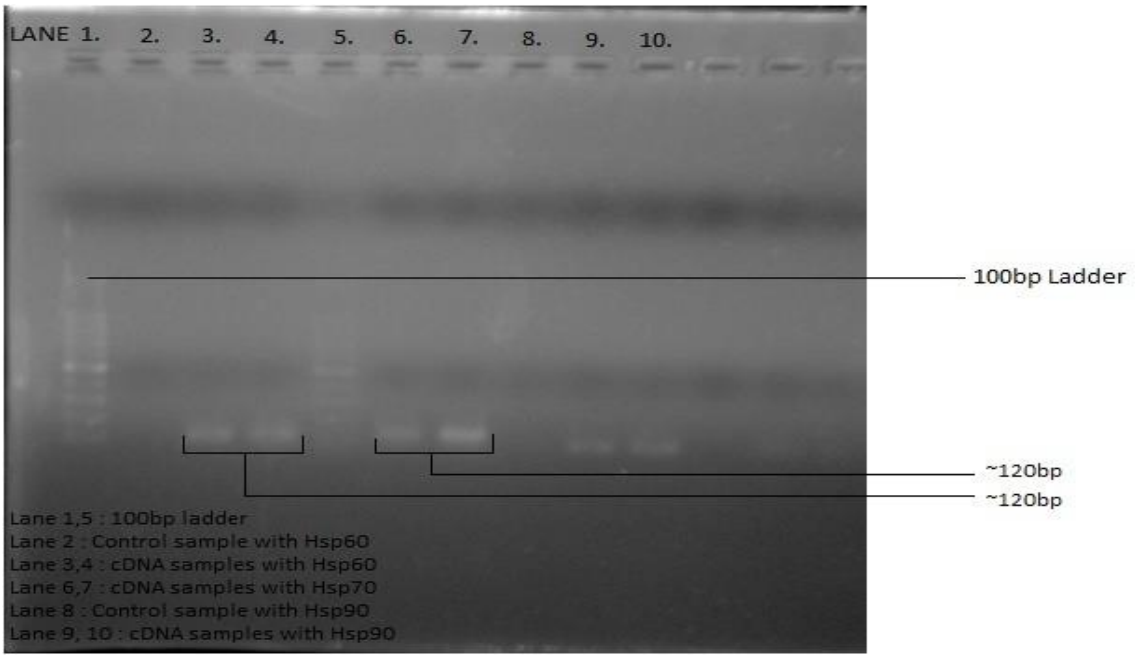

Fig.5.6 Gel doc image depicting expression of Hsp60, Hsp70 and Hsp90 on cDNA samples

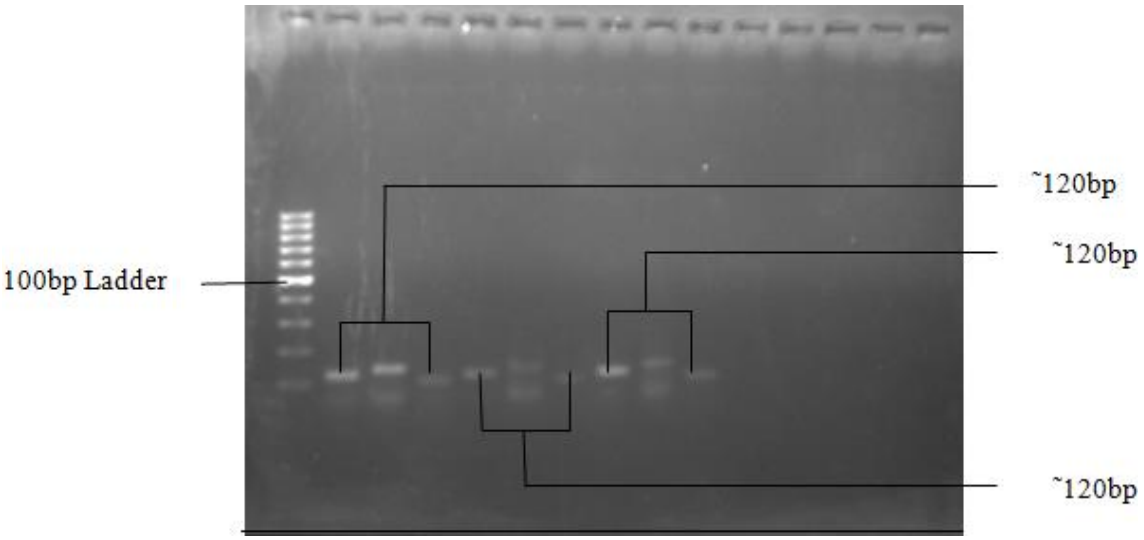

Fig.5.7 Gel doc image depicting expression of Hsp60, Hsp70 and Hsp98 on cDNA sample

RESULT: Bands were observed in the samples with Hsp60, Hsp70, and Hsp 98 confirming their expression in cDNA samples from *Aspergillus flavus*. The size of the bands was approximately 120bp.

DISCUSSION: In (fig.8.); Bands were observed in lanes 3, 4, 6, 7, 8, 9. The size of bands in lanes 3, 4, 5 and 6 was 120bp confirming the expression of Hsp60 and Hsp70. The bands observed in lanes 9 and 10 were less than 100bp, implying the formation of primer dimer in the sample with Hsp90. In (fig.9.); Bands were observed in lanes 2, 3, 4, 5, 6 , 7, 8, 9 and 10 and was approximately 120bp confirming the expression of Hsp60, Hsp70 and Hsp98.

5.3 Real-Time PCR:

Time intervals	Tubulin	Hsp60	Hsp70	Hsp98
4hr	21.39	29.06	26.36	24.87
7hr	20.75	23.29	23.07	22.98
12hr	20.46	32.80	29.04	35.05

Table 5.1 Ct values of Hsp60, Hsp70 and Hsp98 genes

Time intervals	Hsp60	Hsp70	Hsp98
4hr	7.67	4.97	3.48
7hr	2.54	2.32	2.23
12hr	12.34	8.58	14.59

Table 5.2 Normalised Ct values of Hsp60, Hsp70 and Hsp98 genes

RESULT: Ct values were observed revealing expression of Hsp60, Hsp70 and Hsp98 genes.

DISCUSSION: Less the Ct value of gene after normalisation, more will be its expression. RT-PCR results showed the amplification of Hsp60, Hsp70 and Hsp98

genes. However; normalised low Ct value of Hsp60, Hsp70 and Hsp98 at 7 hour time point depicts high expression of genes encoding for heat shock proteins.

CHAPTER - 6

CONCLUSION

The entire idea of the project was to isolate, identify and characterize the fungi at different morphotypes : swollen conidia at 4 hr interval, germinating conidia at 7 hr interval, formation of hyphae growth at 12 hr interval. Also, to check the expression of three selected heat shock proteins genes (Hsp60, Hsp70 and Hsp98) during the morphogenesis of *Aspergillus flavus* (strain Af67); (MTCC118866) at three different time points of 4 hr, 7 hr and 12 hr obtained. It was observed that swollen conidia were obtained at 4 hours, followed by germinating conidia at 7 hours by undergoing differentiation. Further differentiation leads to formation of hyphae growth at 12 hours, staining by Lactophenol Cotton Blue. RT-PCR results showed the amplification of Hsp60, Hsp70 and Hsp98 genes. However, normalised low Ct value of Hsp60, Hsp70 and Hsp98 at 7 hour time point depicts high expression of genes encoding for heat shock proteins. This also suggests exiting from conidial dormancy and for germinating conidia heat shock proteins are required. From recent report by Shraddha et al; 2015[21] germinating conidia of *Aspergillus flavus* showed high abundance of heat shock proteins which evident our results. Several inhibitors are available for heat shock proteins; future studies could be performed to validate the role of heat shock proteins in morphogenesis of *Aspergillus flavus.*

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