Evaluation of transcripts encoding transcriptional regulator gene (*aflR*) in Aspergillus flavus at 30°C and 37°C

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CERTIFICATE

This is to certify that the work titled **"Evaluation of transcripts encoding transcriptional regulator in *Aspergillus flavus* at 30°C and 37°C"**submitted by **Namgay Dema** in partial fulfillment for the award of degree of **Bachelor of Technology in Biotechnology** of **Jaypee University of Information Technology, Waknaghat, Solan** has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree.

Supervisor – **Dr. Jata Shankar**
Assistant professor
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Date: 27/05/15

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Last but not the least many thanks to my friends and other research scholars for their immense help.

SUMMARY

Aflatoxin is synthesized at 30°C and inhibited at 37°C and higher temperature. In order to understand the role of *aflR* gene in aflatoxin production two strains of *Aspergillus flavus* was chosen in which one strain is aflatoxin producing and other one is atoxigenic (aflatoxin non-producer). These isolates of Aspergillus flavus were MTCC AF9367 and MTCC 11580 of which later are atoxigenic and former is toxigenic. *aflR* gene expression level for both the strains were checked and compared by performing reverse transcriptase PCR. Temperature plays significant role as the gene expression level for the isolates grown at 30°C was higher than the isolates grown at 37°C. MTCC AF9367 was highly expressed among all the isolates. This study can be carried out further to elucidate the aflatoxin biosynthetic pathway and devise strategies to control aflatoxin contamination of pre-harvest agricultural crops and post-harvest grains during storage through genomics program. It can also be used to identify the all genes involved in aflatoxin biosynthesis and regulation as gaining a better understanding of the mechanism of aflatoxin formation.

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OBEJECTIVE

- Culturing of *Aspergillus flavus* strains MTCC-AF9367 and MTCC-11580

- Growing of *Aspergillus flavus* strains MTCC-AF9367 at different temperatures 30°C and 37°C.

- To check aflatoxin production at 30°C and 37°C using Potato Dextrose Agar media and β-cyclodextrin

- Isolation of genomic DNA from *Aspergillus flavus* MTCC-AF9367 and MTCC-11580.

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- Total RNA isolation from *Aspergillus flavus* MTCC-AF9367 and MTCC-11580 cultured at different temperatures 30°C and 37°C. cDNA synthesis and PCR using housekeeping genes

- Amplification of *aflR* gene

- Reverse transcriptase PCR of *aflR* gene to check the expression level at two different temperatures (30°C and 37°C)

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

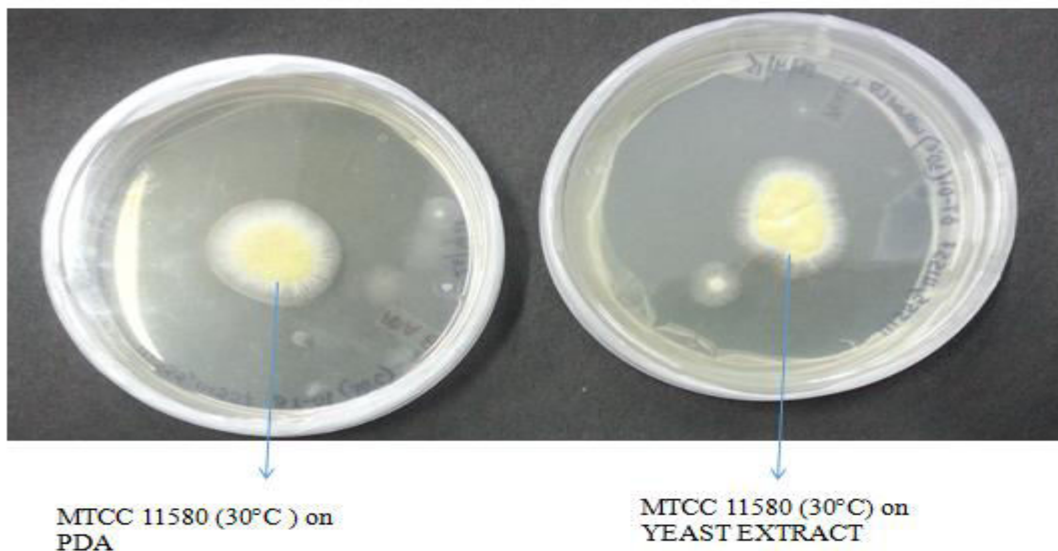
Aspergillus flavus has a world-wide distribution and normally occurs as a saprophyte in soil and on many kinds of decaying organic matter. It is a large genus composed of more than 180 accepted species. The genus is divided in seven sub genera and further divided into sections. As with fungi in general, *Aspergillus flavus's* taxonomy is complex and ever evolving (Chang et al., 1995). This particular genus can be identified by its characteristic conidiophores, but species identification and differentiation is rather complex because it is based on the range of morphological features. Some of the morphological features include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Its shape and size of vesicle, conidia and conidiospores are considered in micromorpology of aspergillus flavus. A. flavus is known as a velvety, yellow to green or brown mould with a goldish to red-brown reverse, basically yellowish to green color. The conidiophores are variable in length, rough, pitted and spiny. They may be either uniseriate or biseriate. They cover the entire vesicle, and phialides point out in all directions.

1.2 Growth of *Aspergillus flavus*:

It grows by producing thread like branching filaments known as hyphae. Filamentous fungi such as *Aspergillus flavus* are sometimes called molds. A network of hyphae known as the mycelium secretes enzymes that break down complex food sources. The resulting small molecules are absorbed by the mycelium to fuel additional fungal growth. The individual hyphae cannot be seen with naked or unaided eyes and only the dense mycelium can be seen. Normally the conidia of *Aspergillus flavus* appear yellowish green when young but the spores turn a darker green with age. In nature, aspergillus flavus is capable of growing on many nutrient sources. It is predominately a saprophyte and grows on dead plant and animal tissue in the soil, therefore the

significance of this fungus in nutrient recycling. Growth of the fungus on a food source often leads to contamination with aflatoxin, a toxic and carcinogenic compound. Aspergillus flavus is also the second leading cause of aspergillosis in humans. Commonly aspergillus flavus grows on food items such as seeds of corn, peanuts, cotton, and nut trees in nature. Unlike most fungi, Aspergillus flavus is favored by hot dry conditions. The optimum temperature for growth is 37°C (98.6 F), but the fungus readily grows between the temperatures of 25°C-42 °C (77-108 F), and will grow at temperatures from 12-48C (54-118 F) (Ehrlich et al., 2004). Such a high temperature optimum contributes to its pathogenicity on humans.

On media like potato dextrose agar and yeast extract agar, colonies are granular, flat with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, mostly 300-400 um in diameter, later splitting to form loose columns, but having some heads with phialides borne directly on the vesicle. Conidiophores are hyaline and coarsely roughened, the roughness often being more noticeable near the vesicle. Conidia are globose to subglobose (3-6 um in diameter), pale green and conspicuously echinulate. The strain MTCC 11580 is grown on two different growth media at two temperature conditions.



MTCC 11580 (30°C) on PDA

MTCC 11580 (30°C) on YEAST EXTRACT

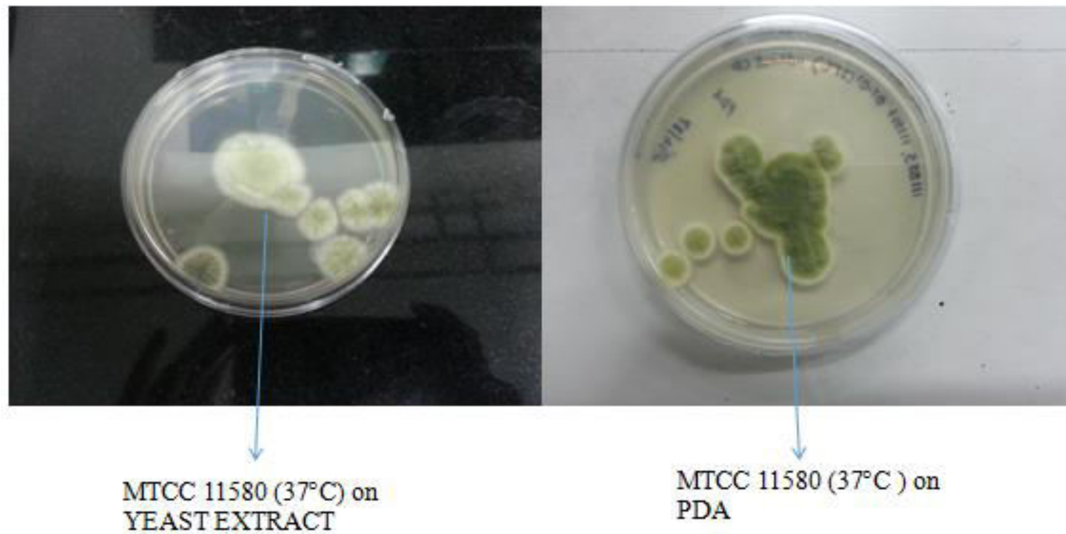


Figure 1.1: *Aspergillus flvus, MTCC 11580 grown on two different media at two different temperature.*

1.3 <u>Diseases caused by aspergillus species:</u>

The disease caused by aspergillus species is known as aspergillosis. The spores of aspergillus are present everywhere in the open environment and hence we constantly inhale it. However those spores present in an air is low in amount and hence people resist it and unable to affect our health. Various species of *Aspergillus,* which cause opportunistic infection includes *Aspergillus fumigates Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus glaucus*, *Aspergillus nidulans*, *Aspergillus Niger*, *Aspergillus oryzae*, *Aspergillus clavatus*, *Aspergillus ustus and Aspergillus versicolor.*

There are three main diseases of aspergillosis namely allergic bronchopulmonary aspergillosis, acute invasive aspergillosis and disseminated invasive aspergillosis.

1.3.1 <u>Allergic bronchopulmonary aspergillosis</u>

Allergic bronchopulmonary aspergillosis is a hypersensitivity reaction to Aspergillus species that occurs almost exclusively in patients with asthma or, less commonly, cystic fibrosis.

Immune responses to Aspergillus antigens cause airway obstruction and, if untreated, bronchiectasis and pulmonary fibrosis. Symptoms and signs are those of asthma with the addition of productive cough and, occasionally, fever and anorexia. Diagnosis is suspected based on history and imaging tests and confirmed by Aspergillus skin testing and measurement of IgE levels, circulating precipitins and aspergillus species specific antibodies. So far 10 cases have been reported approximately and those reports are mostly from regions with hot and dry climate (M. T. Hedayati et al., 2007). It is mostly caused by aspergillus fumigatus and it is rare in aspergillus flavus.

1.3.2 Chronic Pulmonary Aspergillosis and Aspergillomas

This disease is caused by *Aspergillus* infecting the body and growing in cavities in the lungs. These cavities in the lungs would usually have to be created by a previous health problem such as tuberculosis. Once the *Aspergillus* mold has infected the lungs it begins to grow into a fungal ball which then makes the person sick because of the allergens or toxin it puts out into the person's body. At first the symptoms of an aspergilloma might not be noticeable but on longer duration symptoms such as breathing problems, chronic coughing, coughing up blood, lose of weight, tiredness and fatigue will appear. Aspergillomas or the fungal balls in lungs can be detected through x-rays or performing blood tests. Drugs such as itraconazole or voriconazole can be given to the patients once aspergilloma is treated. Anti-fungal drugs may even be injected into the cavity where the aspergilloma is growing in order to fight it. Surgery is recommended in some severe cases where the size of aspergillomas gets bigger.

1.3.3 Disseminated invasive aspergillosis

Disseminated invasive aspergillosis is a variety of bronchopulmonary aspergillosis characterized by a generalized infection of the lung with Aspergillus, occurring usually in people with defective immune response. Invasive aspergillosis is a major cause of morbidity and mortality in immunocompromised patients. Immunosuppression increases the risk of dissemination of the Aspergillus to all of the solid organs via circulation or by direct tissue invasion. Invasive

aspegillosis still remains a big clinical challenge and diagnosis relies largely on histopathological evidence of mycelial growth in tissue.

1.4 Mycotoxins

A mycotoxin is toxic secondary metabolites produced by organisms of the fungi kingdom, commonly known as molds. These metabolites produced generally have toxic responses and termed as mycotoxitosis. Although all mycotoxins are of fungal origin, it is important to note not all toxic compounds produced by fungi are called mycotoxins. Most of fungi are aerobic in nature and inhabit almost everywhere due to extremely small spore size. Likewise, mycotoxins are too small for us to see with the naked eye. Mycotoxins are as small as 0.1 microns. Mold spores are between 1 and 20 microns (Chang et al, 2007). Mycotoxins can appear in the food chain as a result of fungal infection of crops, either by being eaten directly by humans or by being used as livestock feed. Mycotoxins have great resistance to digestion and chemicals, so they remain in the food chain in meat and dairy products. Even extreme temperature treatments such as cooking and freezing won't destroy some mycotoxins. Mycotoxigenic fungi involved in the human food chain belong mainly to three genera: *Aspergillus, Fusarium* and *Penicillium*. *Aspergillus* and *penicillium* are contaminants of food during drying and storage whereas *Fusarium* is Plant pathogen which produces mycotoxin before or immediately after harvesting.

There are five mycotoxins, or groups of mycotoxins, that occur quite often in food. These include deoxynivalenol/nivalenol, zearalenone, ochratoxin, fumonisins, and aflatoxins. Aflatoxins produced by *Aspergillus* species include aflatoxin B1, B2, G1, G2 and derivatives M1 and M2.

1.5 Aflatoxins

Aflatoxins are best known and most thoroughly researched and studied mycotoxins among all the mycotoxins produced by fungi. Aflatoxins have been associated with various diseases, such as aflatoxicosis, in livestock and domestic animals and aspergillosis commonly in immuno-compromised humans throughout the world. A. flavus is the second leading cause of

aspergillosis after *A. fumigatus* (Marone et al, 2001).The incidence of aspergillosis caused by Aspergilli is rising currently due to the increase of immunocompromised patients in the population due to HIV infection. The occurence of aflatoxins is influenced by certain environmental factors; hence the extent of contamination will vary with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods. Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans.

The two majorly important fungal species able to produce Aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin biosynthesis genes of *A. flavus* and *A. parasiticus* are found to be homologous and the order of the genes within the cluster are also found to be same. *Aspergillus flavus* strains produce only Aflatoxin B1 (AFB1) and B2, whereas *A. parsiticus* produce Aflatoxin B1, B2, G1 and G2. The nomenclature for aflatoxin B and G id basically derived from the fluorescent colors produced when observed under UV light. The subscript B is for blue fluorescence and G for green fluorescence. Aflatoxin AFB1 and AFB2 produces hydroxylated derivatives M1 and M2 metabolized from aflatoxin B1 by cows and secreted in milk (Watson et al., 1999)

The production of aflatoxin is influenced by physical and biological factors. They are reported to be produced between 25°C - 35°C optimum temperature and acidic pH (Tarun et al., 2014) Relative humidity between 83%-88% and appropriate level of CO2 & O2 has also been reported to influence the mold growth and aflatoxin production. As biological factors, the preferred carbon sources for aflatoxin production are sucrose, glucose and fructose. Traces of ions like zinc and manganese are also essential for aflatoxin production as it enhances the mold growth.

Aflatoxins are known to cause severe diseases in animals, humans and food crops. It poses both hepatotoxic and carcinogenic properties depending on the duration of exposure. Common symptoms in humans include vomiting, abdominal pain, limb and pulmonary edema, convulsions, progressive jaundice, high fever, swelling of liver, coma and finally it can be fatal (Jiujiang Yu et al., 2011) Among the four major types of aflatoxins, aflatoxin B1 is the most toxic and the most potent carcinogen in humans and animals including nonhuman primates, birds, fish, and rodents. Exposure to B1 aflatoxin can result in suppressed immune response,

malnutrition bile duct proliferation necrosis of centilobe and fatty infiltration of the liver (Tarun Kumar Patel et al, 2014) and it is also a potential immunosuppressive agent. Even a chronic low level exposure of growing vertebrates to aflatoxins may enhance their susceptibility to infection and tumor genesis (Hadi et al., 2000)

Due to the toxic and carcinogenic properties there is huge economic significance of aflatoxins. In order to minimize the chronic exposure to aflatoxins in most of the countries around the world has set the levels of aflatoxins in food commodities. The minimum level of aflatoxins has been set to 20 ppb. U.S. Food and Drug Administration (FDA) specifically prevent the sale of commodities if contamination by aflatoxins exceeds 20 ppb total aflatoxins (Yu et al., 2011). The fungi associated with aflatoxin production are found mostly on peanut, maize, yams, cassava, and cereals. Aflatoxin contamination of agricultural crops causes annual losses of more than 100 million dollars in most of the western countries most cases being from Africa (Price et al, 2006). Nowadays many of the aflatoxin contamination in food has been associated to environmental conditions, poor processing and lack of proper storage and processing in market chain. Almost all the food commodities are supplied to open markets with improper processing which ultimately leads to consumption of such food contaminated with aflatoxins which in turn leads to various human diseases and conditions.

1.6 Biosynthetic pathway of aflatoxin

After many years of investigations regarding the biosynthetic pathway of aflatoxins researchers and scientists around the world has identified more than twenty enzymes and around 25 genes involved. Most of the aflatoxin-related genes are clustered within a 75 kb region of the genome (Yu et al., 2011; Woloshuk & Prieto, 1995). The aflR gene, which regulates these clustered genes, has been identified in *Aspergillus flavus, Aspergillus parasiticu*s, *Aspergillus sojae* and *Aspergillus oryzae*. The AflR protein contains a GAL4-type zinc-finger motif that activates most of the structural pathway genes transcriptionally. Although there is no evidence of aflatoxin production by the non-aflatoxin-producing fungi *A. oryzae* and A. sojae, some genes (nor-1, ver-1, omt-A and aflR) which are needed for aflatoxin biosynthesis are present, but not expressed, in these fungi (Watson et al., 1999). The sequence variability of a region of the aflR gene has been

studied in a few strains of A. flavus, A. parasiticus, A. oryzae and A. sojae; but those studies left with no high degree of differentiation for Aspergillus section Flavi strains (Chang et al., 1995).

The aflatoxin biosynthetic pathway is similar to fatty acid synthesis, consists of at least 20 multi-enzymatic conversion reactions initiated by polypeptide synthesis from acetate. The first stable intermediate appear in its synthesis is Norsolorinic acid (NOR). The fatty acid synthases (fas-1, fas-2) and polyketide synthase (pksA), are two enzymes involved in the conversion steps between initial acetate units to the synthesis of polyketide. The nor-1 gene encodes a reductase for the conversion of norsolorinic acid (NOR) to averantin (AVN). The avnA gene encodes a cytochrome P450 mono-oxygenase involved in the conversion of AVN to averufin (AVF). The avfA gene encodes an oxidase involved in the conversion of AVF to versiconal hemiacetal acetate (VHA). The ver-1 and ver-2 genes encode dehydrogenase for the conversion of VER A to demethylsterigmatocystin (DMST). The omtA gene encodes an O-methyltransferase for the conversion of sterigmatocystin (ST) to O-methylsterigmatocystin (OMST) and dihydrosterigmatocystin (DHST) to dihydrodemethylsterigmatocystin (DHOMST). The ordA gene encodes an oxidoreductase involved in the conversion from O-methylsterigmatocystin (OMST) to AFB1 and AFG1 and DHOMST to AFB2 and AFG2 (Yu et al. 2004).

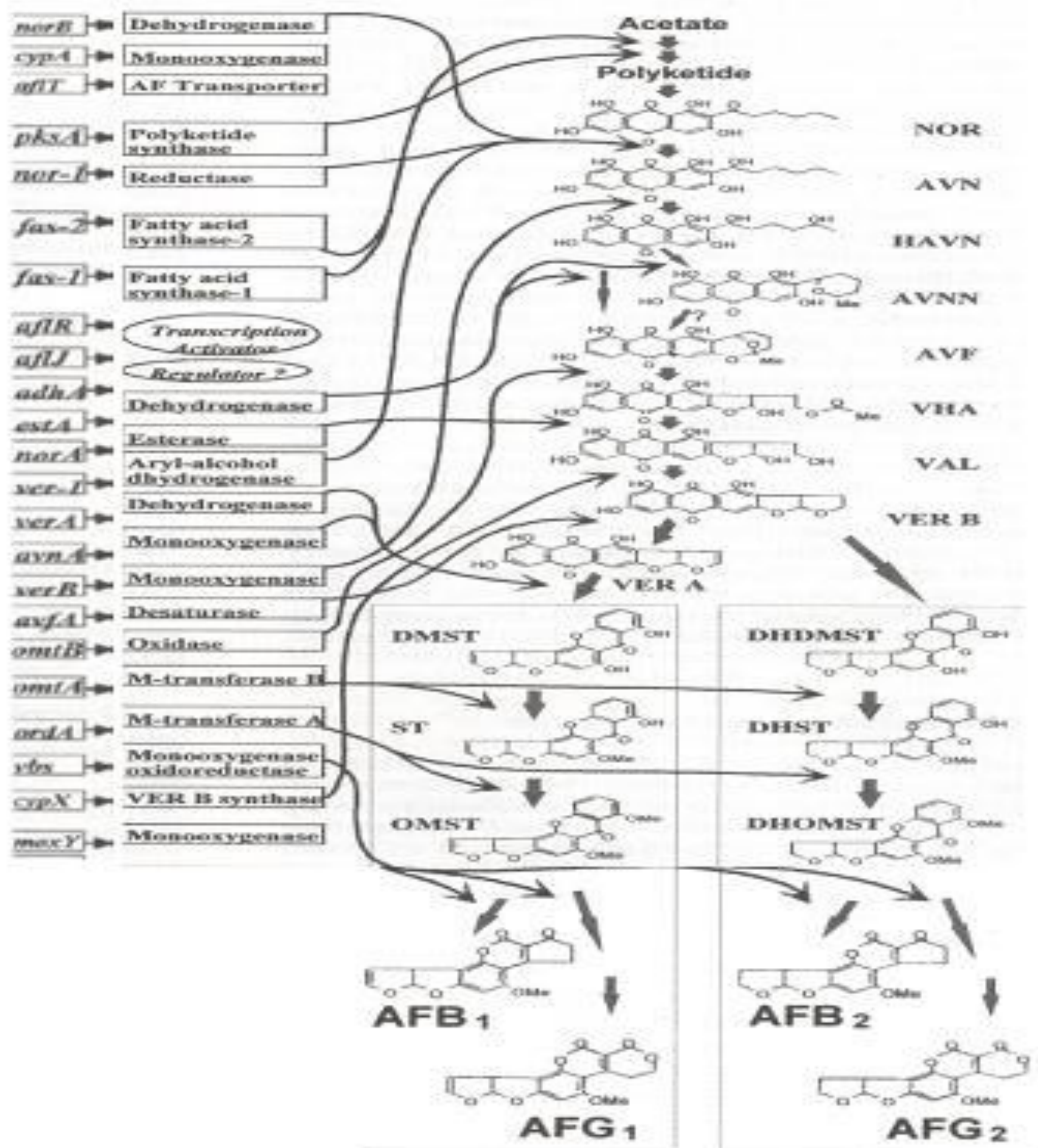


***Figure 1.2**: overall Aflatoxin biosynthetic pathway (Yu. et al., 2004)*

With reference to the literature the goal of this project study is to perform reverse transcriptase PCR for *aflR* gene to check the expression level at different temperature conditions (i.e.; 30°C and 37°C)

CHAPTER 2
MATERIALS AND METHODS

2.1 Fungal isolates used

Two strains of *Aspergillus flavus* shown in the table 1 was used as the aflatoxin is produced predominantly by AF67 and BT01 is non aflatoxin producing strains and thus comparison can be made between producing and non producing strains. Both the strains are grown at two different temperatures (30°C and 37°C) in an appropriate growth medium.

Sl. No.	Isolate name	MTCC No.	Aflatoxin production
1	BT 01	11580	No
2	AF 67	9367	Yes

Table 2.1: *Aspergillus flavus isolates.*

2.2 Media used

Fungal cultures were grown in slants containing potato dextrose agar and incubated at two different temperature conditions. PDA is common media for growing fungal and bacterial culture as it contains the essentials for bacterial and fungal species to grow. Media was autoclaved and slants were prepared. After the solidification of slants two strains of A. flavus were cultured and incubated at two different temperatures. This culture served as the mother culture throughout the experiment.

For both DNA and RNA preparation, fungi were grown in 100 ml of glucose minimal medium (1gm of ammonium phosphate monobasic, 5gms of sodium chloride, 0.2gm of magnesium sulfate, 1 gm of potassium phosphate dibasic and 5gms of glucose) in 250-ml conical flasks with shaking at 150 rpm and 25°C. Minimal Media contains the essentials for fungal species to grow. The media is often used to define if a particular microbial species is a heterotroph, namely an organism that does not have any nutritional requirements beyond core sources of carbon (sugars) and nitrogen. Auxotrophs or organisms with nutritional requirements will not be able to grow on minimal media and as fungus is heterotrophy this media is suitable.

2.3 Conidial suspension preparation

Phosphate Buffer Saline Tween-80 (PBST) was prepared and added to the petriplates. Each petriplates was shaken well so that the conidia could be washed out with the PBST and suspensions were prepared and transferred to Eppendorf. PBST acts as a washing solution, tween-80 acts as a detergent for conidia since they are of hydrophobic nature.

2.4 Microscopic analysis of isolates

Macro-morphological characteristics like colour of conidia, mycelial growth of plates were noted. Slides were prepared using Lactophenol cotton blue and observed under microscope. Lactophenol cotton blue stain is formulated with Lactophenol, which is formulated with Lactophenol, which serves as mounting fluid. Organisms suspended in the stain are killed due to the presence of phenol. High concentration of phenol deactivates the lytic cellular enzyme thus the cell do not lyses. It is an acid dye that stains chitin present in the cells of fungi.

For this analysis clean slides were taken and in the centre of it lacto phenol cotton blue dye was poured. Then the fungal cultures from the gar slants were taken and mixed it with the dye on slide. The slide was observed under microscope after teasing it well.

2.5 To check aflatoxin production by the isolates at 30°C and 37°C using Cyclodextrin

Fungal isolates were screened in YES agar medium supplemented with 3.0% β-cyclodextrin as a fluorescence enhancer and were grown at two different temperatures (30ºC and 37ºC). These YES agar plates were aseptically inoculated at the centre of culture plates with 2.0 µL of working conidial suspension, 106 conidia/ml with help of a micropipette. Plates were incubated in the dark at 27°C and 37°C for 4 days. The presence or absence of a fluorescence ring in the agar plate surrounding the mycelia, observed under UV light (365 nm), was termed as positive or negative.

2.6 Total genomic DNA isolation

The aim of the isolation processes was to obtain DNA of sufficient quality and quantity necessary for its PCR amplification and standardization of primers.

2.6.1 Isolation of gDNA from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 by SDS (freeze thawing)

Freeze thawing the fungal sample helps to break down the cell wall of fungus but this method is not effective as such. Purification of DNA from complex mixture of cellular molecules is accomplished by removal of proteins and other molecules into an organic solvent. Use of phenol chloroform leads to denaturation of proteins and phase separation in which DNA and RNA are not soluble in the organic solvents, thus remain in aqueous phases of mixture that contains solvent for Protein extraction.

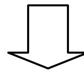
Reagents:

1) Lysis buffer: Tris acetate, EDTA, sodium acetate ,SDS
2) NaCl

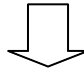
3) Chloroform
4) TE buffer: Tris Cl , EDTA

Procedure:

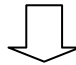
Pre chilled the motar and pestle at -80°C for 15 minutes prior to the start of the experiment



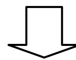
The cultures grown at two temperatures in glucose minimal media is taken and filter out the liquid and moisture content using muslin cloth under LAF hood



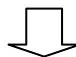
Transferred the filtered fungal cultures into chilled motar and pestle and kept at - 80°C for 20 minutes.



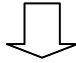
Fungal culture was then ground to fine powder with pestle and transferred into a microfuge tube and incubated it at 60°C for 15 minutes.



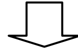
Again the tube was kept at - 80°C for 15 minutes and let it freeze. And finally thawed the powdered tissue by pouring 500 µl of lysis buffer.



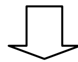
Then 165 µl of 5 M NaCl solution was added and the components were mixed by inverting the tubes several times.



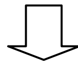
The suspension was then centrifuged at 13,000 rpm for 20 minutes at 4°C.



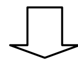
Eppendorf suspension was immediately transferred to a fresh tube and 400 µl of phenol and chloroform was added (1:1). The solution was mixed by gentle inverting the tube until their solution become milky



Centrifuged for 20 minutes at 4°C and aqueous phase was removed and extracted with equal volumes of chloroform.



DNA in the aqueous supernatant was precipitated with two volumes of 95 % chilled ethanol.



The precipitated DNA was washed 3 times with 70% ethanol at max speed for 5 minutes.

Then air dried the pellet and dissolved in 20 µl of autoclaved water and stored at -20 °C.

2.6.2 DNA isolation of *Aspergillus flavus*; strains MTCC-11580 and MTCC - AF9367 by SDS (liquid nitrogen crushing method)

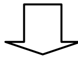
Liquid nitrogen breaks down the thick fungal cell wall and exposes the fungal chromosomes containing genetic material outside. liquid nitrogen (-180 C) which it freeze the tissue to become fragile to be a fine powder which increase the surface area of extraction, and the very low temperature prevent DNase activation.

Reagents:

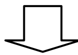
1) Lysis buffer: Tris acetate, EDTA, sodium acetate ,SDS
2) NaCl
3) Chloroform
4) TE buffer: Tris Cl , EDTA,

Procedure:

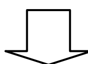
30 mg of freeze dried fungal balls was ground to a fine powder in liquid nitrogen using mortar and pestle.



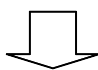
The grounded fungal balls were resuspended and lysed in 500 µl of the lysis buffer by pipetting with pipette until the viscosity of the suspension was significantly reduced and the formation of froth indicated the detachment of DNA polysaccharides.



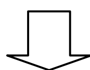
Then 165 µl of 5 M NaCl solution was added and the components were mixed by inverting the tubes several times



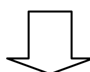
The suspension was then centrifuged at 13,000 rpm for 20 minutes at 4°C.



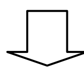
Eppendorf suspension was immediately transferred to a fresh tube and 400 µl of phenol and chloroform was added (1:1). The solution was mixed by gentle inverting the tube until their solution become milky.



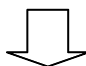
Centrifuge for 20 minutes at 4°C and aqueous phase was removed and extracted with equal volumes of chloroform.



DNA in the aqueous supernatant was precipitated with two volumes of 95 % chilled ethanol.



The precipitated DNA was washed 3 times with 70% ethanol at max speed for 5 minutes.



Then air dry the pellet and dissolve in 20 µl of autoclaved water and stored at -20 °C.

2.7 Total RNA isolation

Efficient RNA isolation is a prerequisite for gene expression studies and it has an increasingly important role in the study of fugal pathogens in plants especially food crops. But RNA isolation is difficult in filamentous fungi. These organisms are known for their rigid cell walls and the presence of high levels of carbohydrates, excreted from the fungal cells during submerged

growth, which interferes with the extraction procedures. Many commercial kits are available but at the same time user developed procedures are also used.

2.7.1 Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured 37°C using CTAB

In this method CTAB was used to isolate total RNA. It's a detergent which simultaneously solubilizes the fungal cell wall and lipid membranes of internal organelles and denatures protein and enzymes. Hence the RNA won't get hydrolyzed during the isolation process.

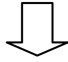
Buffer and solutions:

1. CTAB: cetyl trimethyl ammonium bromide
2. NaCl
3. EDTA (pH 8)
4. 1M Tris
5. Isopropanol
6. 75% ethanol
7. Water

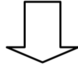
Procedure:

About 200 mg of each sample tissue were ground to the fine powder using liquid nitrogen and transferred into a 2ml centrifuge tubes. 900 µl of the CTAB buffer and 100 µl of beta mercaptoethanol were added.

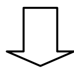
The mixture was shaken for 30 seconds and then incubated at 65°C for 10 minutes, inverting the tube 3-4 times every now and then during incubation.



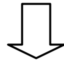
Then 800 µl of chloroform were added. The mixture was shaken for 30 seconds. Later the mixture was centrifuged at 10,000 RPM for 10 minutes at 4°C. The supernatant was transferred to a new tube.



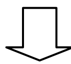
Next 800 µl of phenol/chloroform (1:1) were added and shaken for 30 seconds. Later the mixture was centrifuged at 10000 RPM for 10 minutes at 4°C.



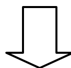
The supernatant was transferred to a new tube and an equal volume of chloroform /isoamyl alcohol was added. Samples were shaken for 30 seconds and centrifuged at 10000 RPM for 10 minutes at 4°C.



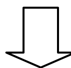
Added equal amounts of isopropanol and mixed well.



Then RNA pellet was washed with 75% ethanol and centrifuged tubes at 7500 RPM for 5 minutes at 4°C. Repeated this step twice.



Discarded supernatant and RNA pellet at bottom was obtained.



Dried pellet at room temperature for 20 minutes and finally dissolved in DEPC treated water.

2.7.2 Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured at different temperatures 30°C and 37°C by Trizol reagent

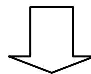
TRIzol Reagent is a ready-to-use reagent used for RNA isolation from cells and tissues. Firstly it consists of guanidinium isothiocyanate, a powerful protein denaturant and helps inactivation of RNases and secondly it has acidic phenol/chloroform which has a role in partitioning of RNA into aqueous supernatant for separation. TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase. Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA

Reagents:

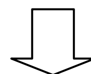
1) DEPC (diethyl pyro carbonate)
2) Trizol
3) Chloroform
4) Isopropanol
5) 75% ethanol in DEPC water

Procedure:

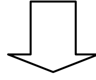
Taken 100 mg of fungal culture (both strains at two different temperature conditions)



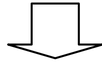
Fungal sample was frozen in liquid nitrogen



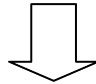
Homogenized with motar pestle



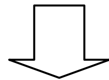
Added trizol reagent (1ml reagent for 100 mg culture)



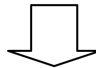
Incubated for 5 minutes at room temperature to permit complete dissociation of nucleo protein complex



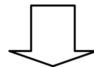
Added .2 ml of chloroform per ml f trizol reagent under the hood and closed the tube



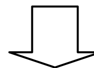
Shaken tube vigorously by hand for 15 seconds and incubated them at room temperature for 2-3 minutes



Centrifuged the sample at 12000 RPM for 15 minutes at 4°C and transferred the supernatant or aqueous phase to the fresh tube.

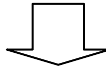


Precipitated RNA from aqueous phase by mixing with isopropanol (.5ml per 1 ml of trizol)

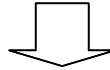


Incubated the sample at room temperature for 10 minutes and centrifuged at 12000 RPM for 10 minutes at 4°C. Discarded the supernatant.

Then RNA pellet was washed with 75% ethanol and mixed well.



Centrifuged tubes at 7500 RPM for 5 minutes at 4°C. Repeated this step twice. Discarded supernatant and RNA pellet at bottom was obtained.



Dried pellet at room temperature for 20 minutes and finally dissolved in DEPC treated water.

3.1 cDNA synthesis and PCR using housekeeping genes

Thermo scientific verso cDNA synthesis kit was used to synthesized cDNA

Components:

1) Verso enzyme mix: It includes verso reverse transcriptase which is active at high temperatures, is highly sensitive and can generate long cDNA strands. This mix also contains RNase inhibitor to protect RNA templates from degradation.
2) 5X cDNA synthesis buffer: a proprietary reaction buffer which has been optimized to improve reverse transcription across a wide range of templates.
3) Anchored oligo dT primers and random hexamers: provide flexible RNA priming methods for cDNA synthesis
4) RT enhancer: It is included to remove contaminating DNA, eliminating the need for DNase I treatment. It degrades double stranded DNA during transcription of RNA and inactivated after 2 minutes at 95 °C.

Verso is RNA dependent DNA polymerase with the significantly attenuated RNase H activity. Verso can synthesize long cDNA strands up to 11kb at temperature range of 42 °C to 57 °C. The

recommended amount of total RNA to use is between 1 pg to 1 µg. It should be stored at –20 °C until ready for use and repeated freeze thawing should be avoided.

Reaction mix:	Reaction volume:
5X cDNA synthesis buffer	4 µl
dNTP mix	2 µl
RNA primer	1 µl
RT enhancer	1 µl
Verso enzyme mix	1 µl
Template (RNA)	1-1.5 µl
Water	up to 20 µl
Total	20 µl

Table 2.2: *Reaction mixture for PCR reaction.*

Conditions	**Temperature**	**Time**	**No. of cycles**
cDNA synthesis	42°C	30 minutes	1

Table 2.3: *PCR conditions*

Housekeeping gene, beta Tubulin gene (size: 122 bp) was used and then performed PCR to check cDNA

Housekeeping genes are involved in basic cell maintenance and, therefore, are expected to maintain constant expression levels in all cells and conditions (both include HSP6 (size 121bp), HSP70 (size 141bp), HSP 989 (size 102bp) and GAPDH (size 119bp). Following PCR reactions and conditions were shown in following tables.

Reaction mixture	Volume
Forward primer	.5 µl
Reverse primer	.5 µl
DNA	1 µl
PCR mix	6 µl
DH2O	4 µl
Total	12 µl

Table 2.4*: PCR reactions for amplification of housekeeping genes.*

3.1.1 *aflR* gene amplification

aflR is the aflatoxin biosynthetic pathway regulatory gene which encodes putative 47kDa protein containing a zinc clusters DNA binding motif. It is required for the transcription of all the characterized aflatoxin pathway genes in *Aspergillus flavus*. An equal concentration of cDNA was taken for both isolates at all the temperatures. Following PCR reactions and conditions were being used for amplification.

Reaction mixture	volume
aflR forward primer	0.30µl
AflR reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
water	4.63 µl
Total	12.5 µl

Table 2.5: *PCR reaction mixtures for the amplification of aflR gene.*

Temperature	Time
95°C	5 mins
95°C	45 secs
53.7°C	30 secs
72°C	45 secs
72°C	7 mins

Table 2.6*: PCR reaction conditions for amplification of aflR gene*

3.1.2 Semi quantitative reverse transcriptase (RT PCR)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a highly sensitive and specific method useful for the detection of transcripts or for the analysis of very little amount of sample. The cases in which when RNA analysis is required, a qualitative study is not sufficient to deliver a sufficient results. For the detection of expression levels under different experimental conditions can be done effectively with quantification of RNA transcripts. After PCR, same volumes of reaction products are electrophoresed on an agarose gel.

For semi quantitative RT-PCR analysis we extracted total RNA from fungal cells with TRIZOL reagent, according to the manufacturer's instructions. RT-PCR experiments were carried out with cDNAs generated from of total RNA using a verso cDNA synthesis kit. The RT-PCR was carried out for 35 cycles to allow semiquantitative comparisons of cDNAs of two different isolates. The primers and PCR conditions are shown in supplementary Tables 7 and 8, respectively.

Reaction mixtures	Volumes
aflR forward primer	0.30µl
AflR reverse primer	0.30 µl
Master mix	6.25 µl
cDNA	1.00 µl
Water	4.63 µl
total	12.5 µl

Table 2.7: *PCR reaction mixtures for RT PCR*

Temperature	Time
95°C	5mins
95°C	45 secs
53.7°C	30 secs
72°C	45 secs
72°C	7 mins

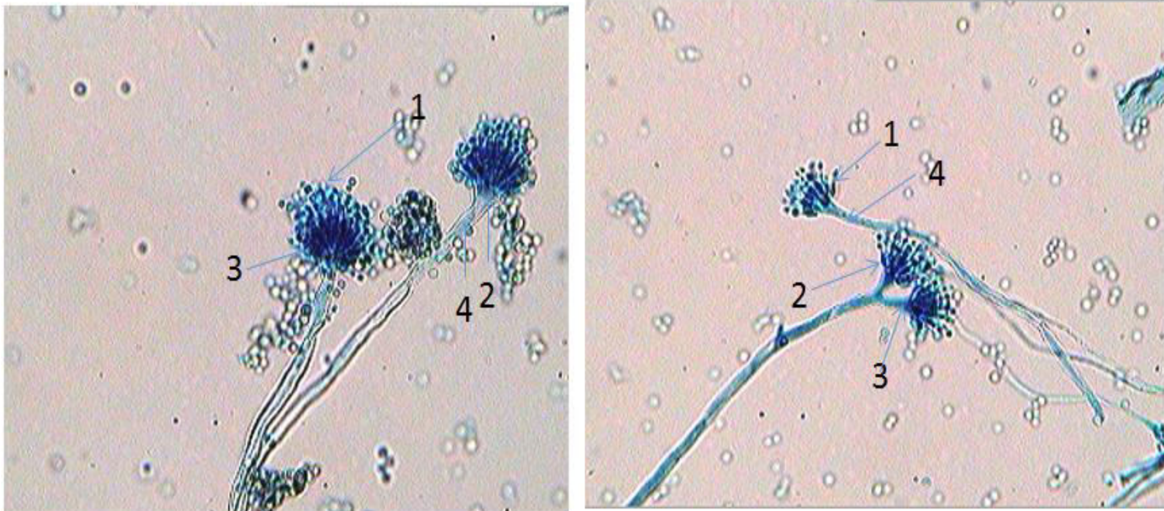
Table 2.8: *PCR conditions for RT PCR*

CHAPTER 3

RESULT AND DISCUSSION

3.1 Microscopic analysis of isolates

For the identification of conidia and conidiophores of *Aspergillus flavus* in laboratory, lacto phenol cotton blue staining is the simplest method.



Aspergillus flavus(MTCC AF9367) and MTCC 11580 when viewed under 100 x

Figure 3.1: *staining of aspergillus flavus strain MTCC AF9367 and MTCC 11580.*

Following features were observed.

1) Conidia
2) Phialides
3) Vesicles
4) Conidiophore

3.2 To check aflatoxin production by the isolates at 30°C and 37°C using Cyclodextrin

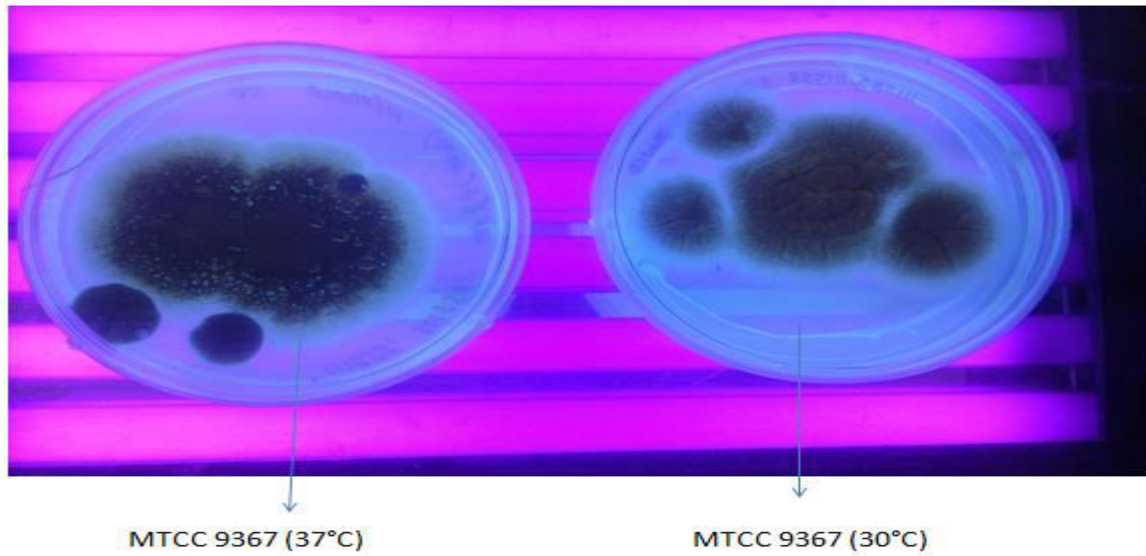


Figure 3.2: *Plates containing cultures of MTCC AF9367 isolate was viewed under UV light after incubating it for 4 days.*

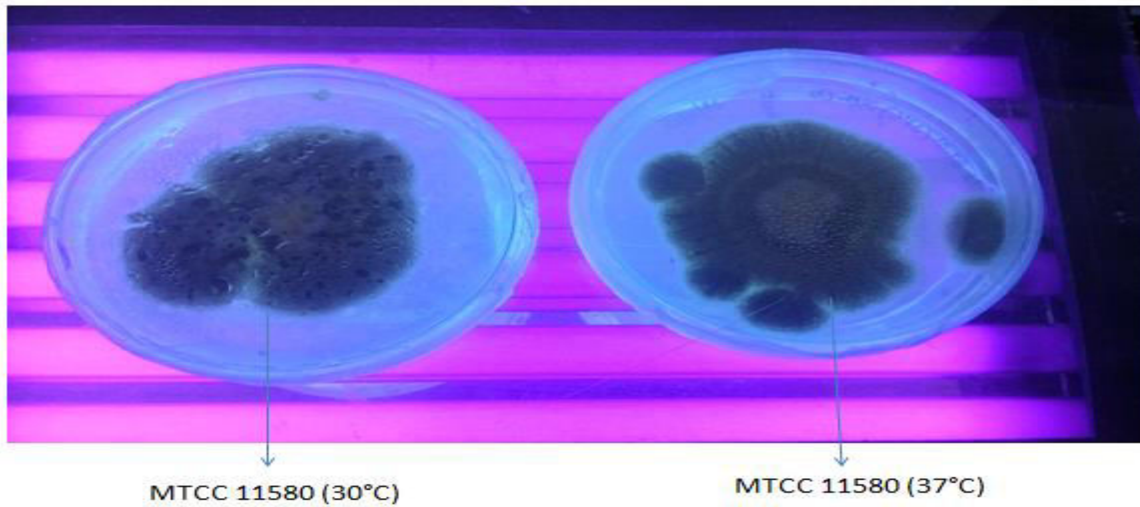


Figure 3.3: *Plates containing cultures of MTCC 11580 isolate was viewed under UV light after incubating it for 4 days.*

Discussion: The presence of fluorescence ring around the conidia indicates the presence of Aflatoxin production. Presence of fluorescence ring (white beiges) in AF67 at 30°C and 37°C indicates the Aflatoxin production whereas BT-01 at both these temperatures produces very little or no Aflatoxin because the fluorescence ring is not as prominent as the first one. Therefore the isolate MTCC 11580 I atoxigenic whereas the strain MTCC AF9367 is toxigenic.

3.3 Isolation of gDNA from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 by SDS (freeze thawing)

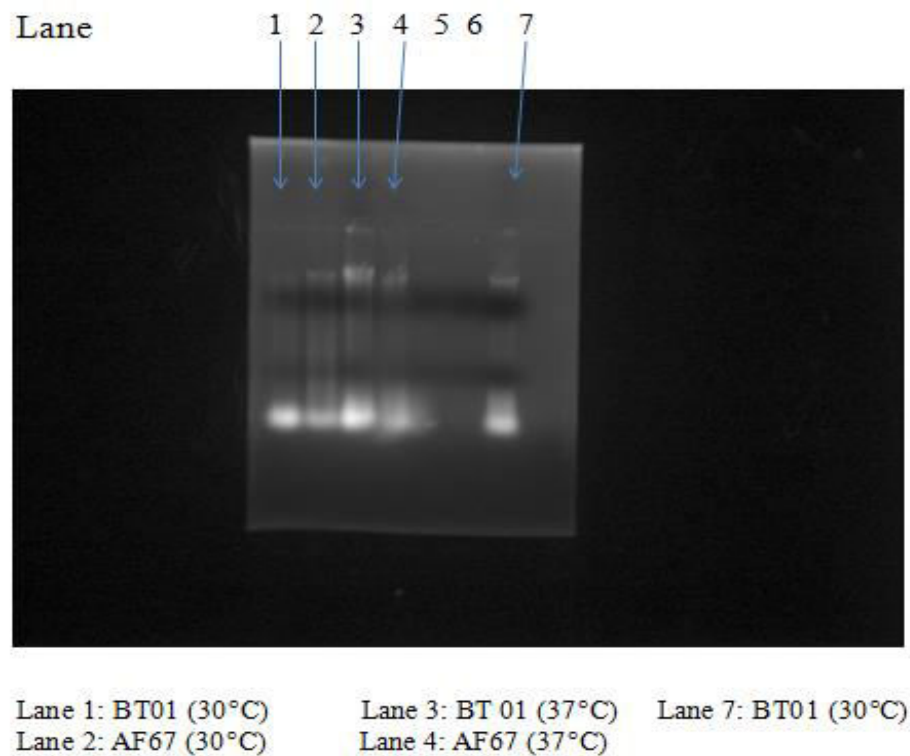


Figure 3.4: *DNA bands obtained using SDS method.*

Discussion: The DNA isolation without liquid nitrogen was not efficient. Alternative method like freeze thawing is tedious and doesn't give satisfactory results. One of the reasons behind could be due the improper lysis of fungal cell wall. Fungal cell wall is composed of chitin and it's very thick, thus liquid nitrogen is required to break down this cell wall. Therefore DNA bands are not clear in both isolates, MTCC AF9367 and MTCC 11580 at both temperatures.

3.4 DNA isolation of *Aspergillus flavus*; strains MTCC-11580 and MTCC -AF9367 by SDS (liquid nitrogen crushing method)

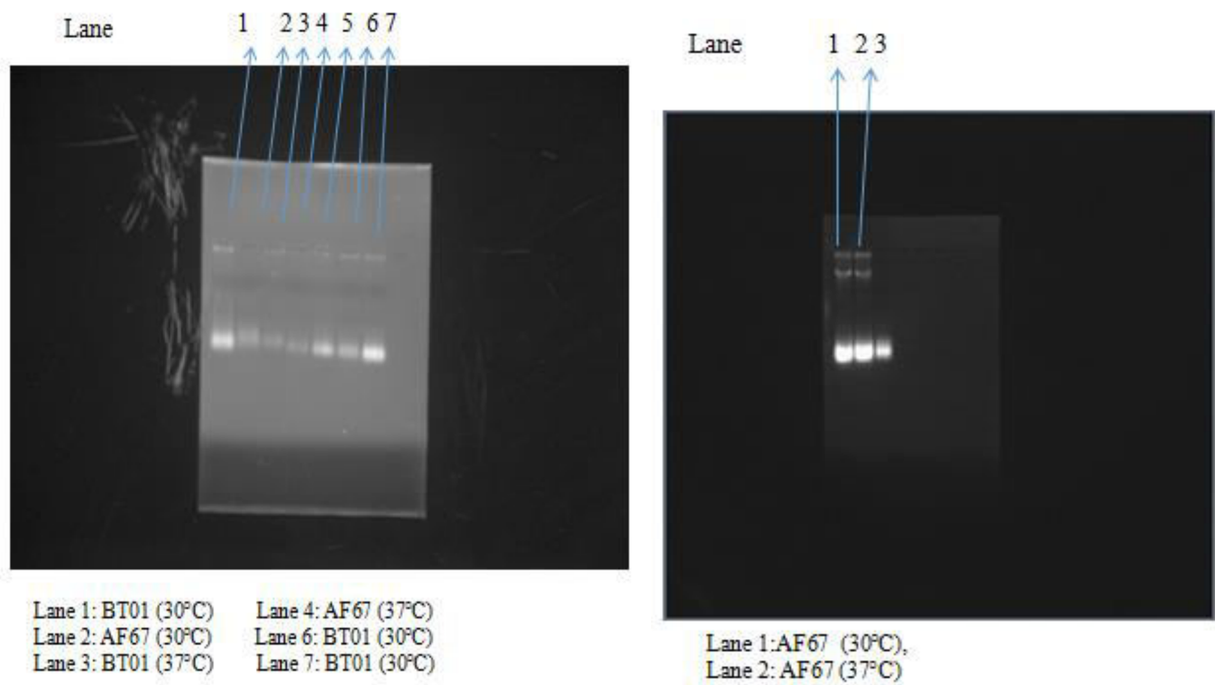
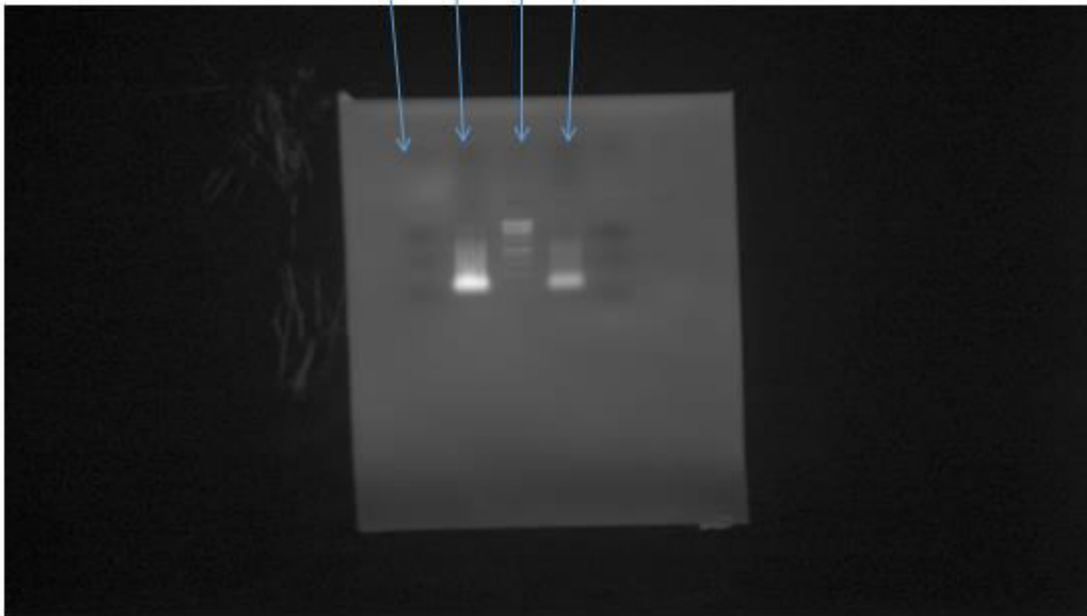


Figure 3.5: *DNA bands obtained using SDS method by liquid crushing the samples*

Discussion: DNA bands can be seen in both the strains. Along with bands degraded RNA and contaminations are also detected. Even though we used same reagents in both methods, we obtained clear DNA bands in this case. The only difference was we used liquid nitrogen in this method. So we can conclude that proper break down of fungal cell wall has taken place in this case thus exposing fungal nucleic materials outside.

3.5 Total RNA isolation from *Aspergillus flavus* MTCC-Af9367 and MTCC-11580 cultured 37°C using CTAB



Well 2: MTCC- AF9367, well 3: ladder, well 4: MTCC 11580 (at 37°C)

Figure 3.6: *RNA bands obtained using CTAB method.*

Discussion: The total RNA isolation by CTAB was not efficient as compare to isolation using TRIzol reagent. One of the reasons behind could be due the improper lysis of fungal cell wall. Fungal cell wall is composed of chitin and it's very thick, it is difficult to lyse using CTAB. Therefore DNA bands are not clear in both isolates, MTCC AF9367 and MTCC 11580 at 37°C. When it was run 1.5% gel the degraded bands were being observed. Certainly this method is not preferred for isolation of fungal RNA in most of cases.

3.6 Total RNA isolation from Aspergillus flavus MTCC-Af9367 and MTCC-11580 cultured at different temperatures 30°C and 37°C by trizol reagent

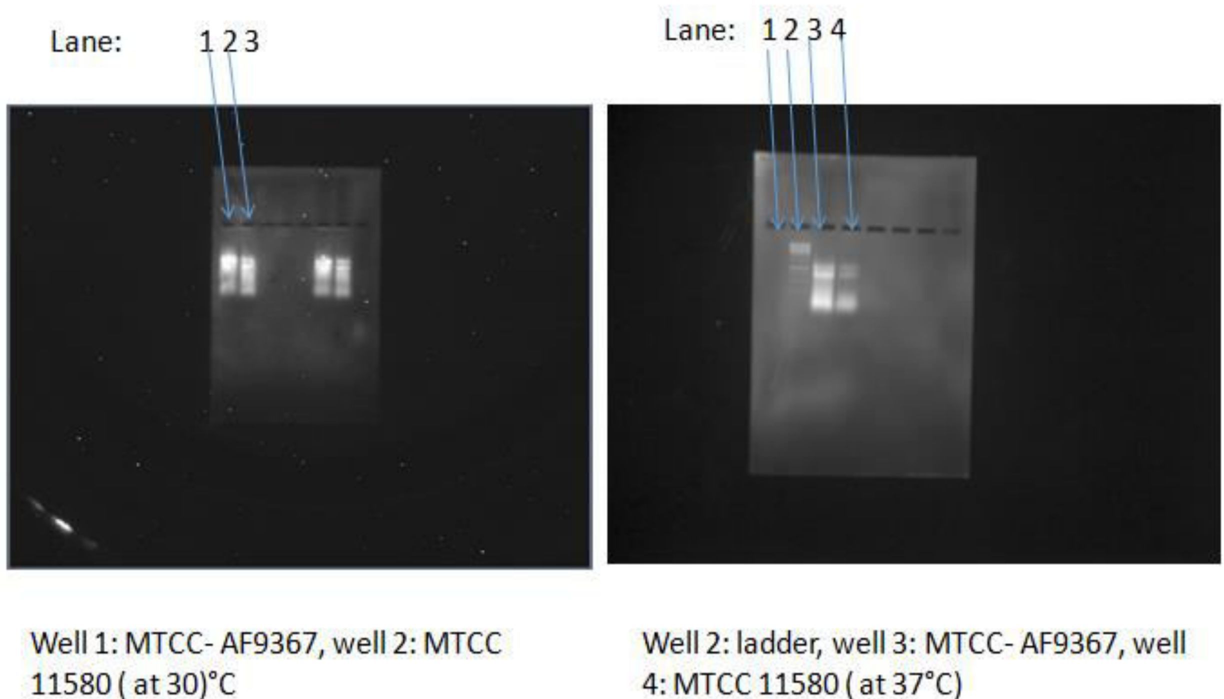


Figure 3.7: *RNA bands obtained using Trizol reagent*

Discussion: The success of total RNA extraction from fungal isolates depended on the thorough grinding of the fungal tissues. Liquid nitrogen was added to prevent the sample to dissolve and for efficient cell lysis. This method was found better among the two methods we have used for RNA isolation. 1.5% agarose gel electrophoresis showed that 28S and 18S bands were intact and visible. Although the 28S bands with apparent degradation were observed, it's much better than fist method.

Total RNA extracted by this method was suitable for downstream applications such as reverse transcription and gene amplification.

3.7 cDNA synthesis and PCR using housekeeping genes

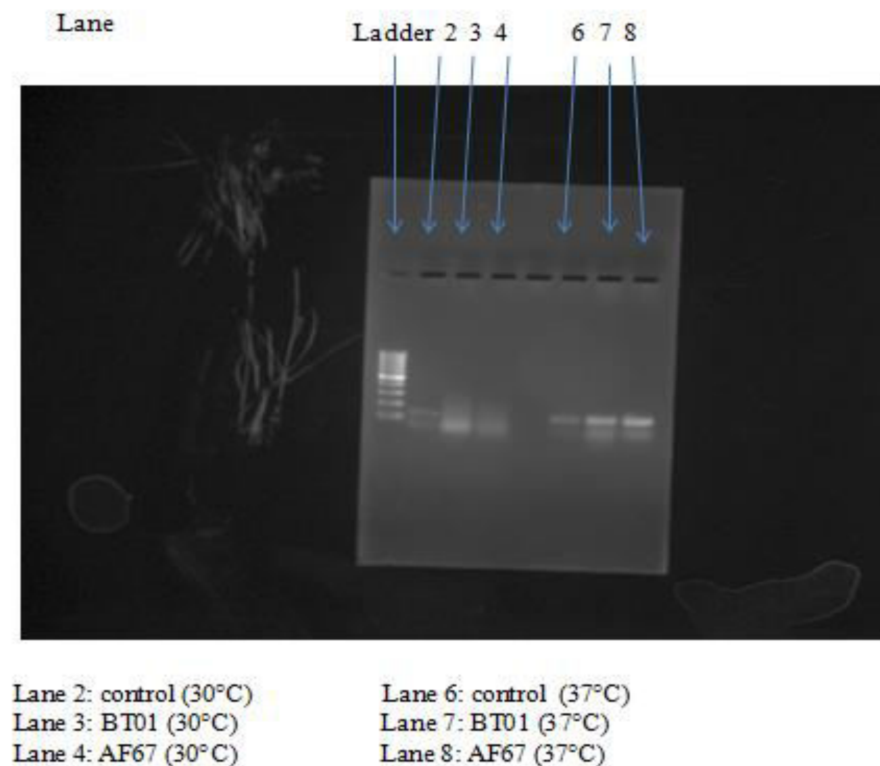


Figure 3.8: *Amplified products of housekeeping gene*

The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). Reverse transcriptases (RTs) use an RNA template and a primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR).The beta Tubulin gene was in this case in order to check whether the gene has been amplified or not. Housekeeping genes encode proteins that are usually essential for the maintenance of cellular function in almost all organisms. Their expression remains constant under most experimental conditions. The expression of housekeeping genes can be evaluated by performing PCR. The sample was then run on 1.5% gel and the above bands were being observed. It’s clear that the gene has been amplified.

3.8 *aflR* gene amplification

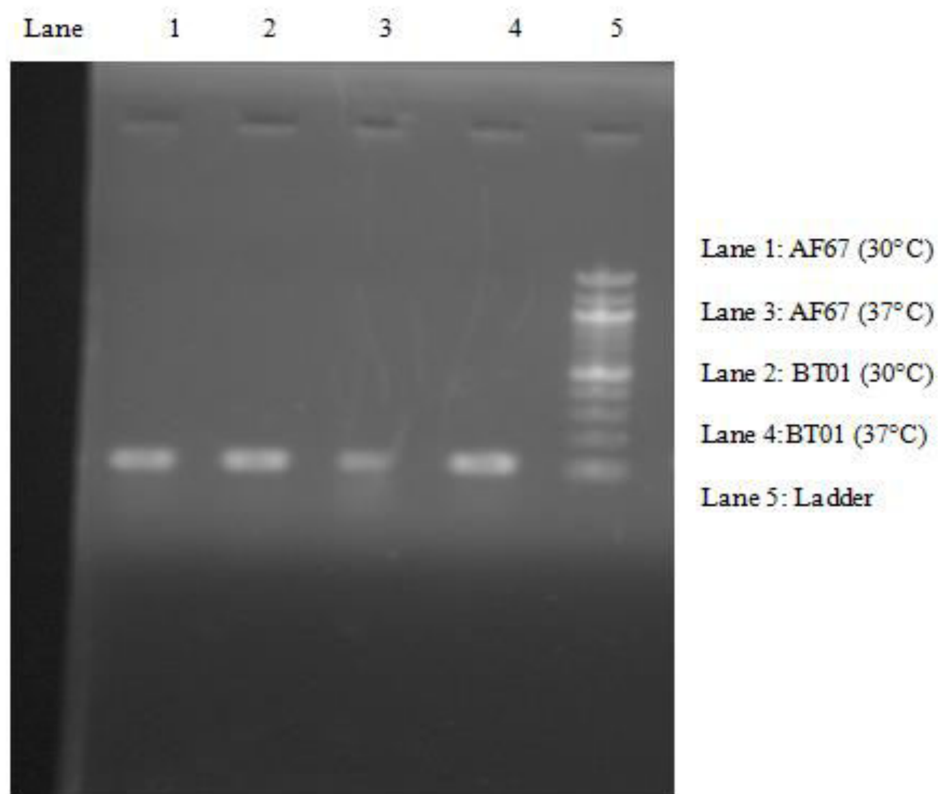


Figure 3.9: Amplified products of aflR gene

Discussion: The above picture shows the amplified *aflR* gene. The sharp bands are formed for MTCC AF9367 at both the temperatures and slightly lighter bands are being formed in case of MTCC 11580 at both temperature conditions.

3.9 Semi quantitative reverse transcriptase (RT PCR)

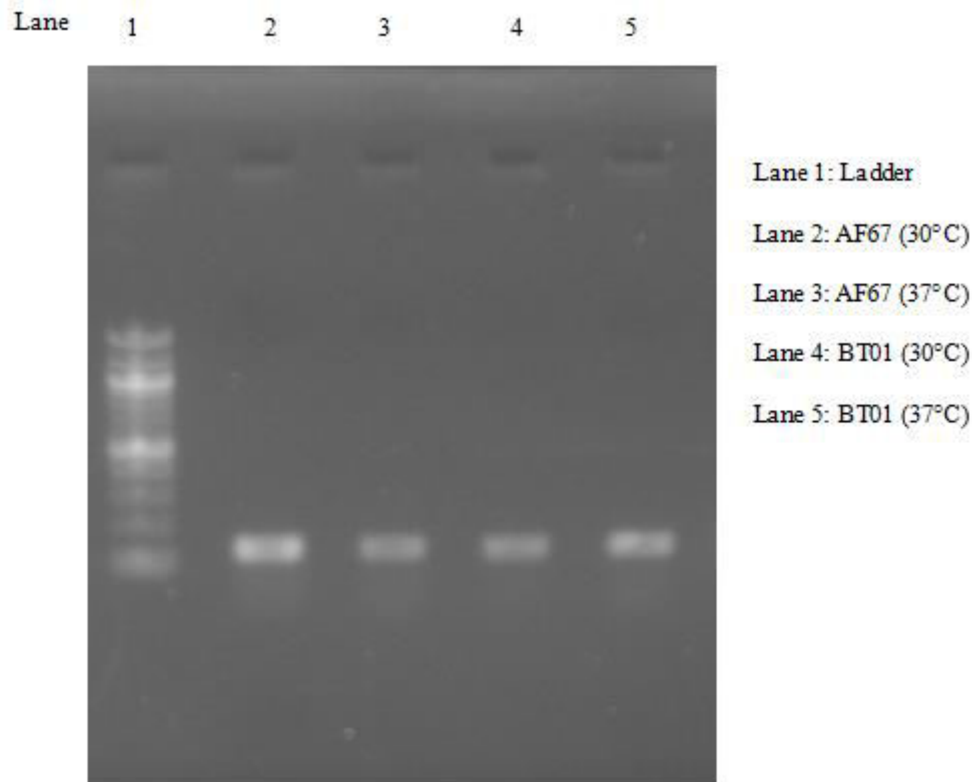


Figure 3.2.1: *Bands showing the expression level of the aflR gene*

Discussion: The expression of gene was checked by amplification of the cDNA with primers specific to the gene. The PCR product was then run on an Agarose Gel electrophoresis of 1.5% along with a ladder of 100bp to detect its size as well. The results obtained after viewing the gel under Gel Documentation System were as follows. When RT-PCR was performed, the

expression level of the aflR gene for different isolates at different temperature can be seen. MTCC AF9367 at 30°C has the sharpest band which means it was highly expressed comparing to others. MTCC 111580 was also expressed at both the temperatures but the expression level was quite low as compared to MTCC AF 9367 as the bands are slightly faint.

CHAPTER 4

CONCLUSION

Aflatoxins are highly toxic and carcinogenic secondary metabolites produced by Aspergillus flavus and Aspergillus parasiticus. This toxin is known to contaminate pre harvest crops and post harvest during storage. Many scientists have researched on the biosynthesis of Aflatoxins and were able to devise the whole biosynthetic pathway. In this pathway there are more than 20 genes identified and sequenced. The role of *aflR* gene in this pathway has also been shown in many studies. *aflR* is aflatoxin pathway regulatory gene which codes for proteins within 75kDa containing zinc finger binding motif. This gene is required for the regulation of almost all the genes which are present in this pathway.

In this project study the expression level of *aflR* gene in two different strains of Aspergillus flavus were checked by performing semi quantitative RT-PCR and the results were compared. Many biological and physical factors affect the growth of *Aspergillus flavus* and thus production of aflatoxin. Temperature is one of the physical factors which play a significant role in the production of aflatoxin. It is being produced at 30°C and inhibited at higher temperature. Two isolates of *Aspergillus flavus* were grown at two different temperatures and their results were compared. The result shows that the aflatoxin producing isolate grown at 30°C is highly expressed while non aflatoxin producing strain grown at higher temperature (37°C) was not well expressed.

Aflatoxins are highly toxic, mutagenic, and carcinogenic to plants, animals and humans. It has been found that they may also be involved, to some degree, in primary liver cancer in humans.
The Aflatoxins have been implicated in hepato cellular carcinoma, acute hepatitis, Reye's syndrome and cirrhosis in malnourished children. Many cases of aflatoxicosis in humans have been reported in many countries including Southeast Asia and Africa. Moreover aflatoxin contamination of agricultural commodities, such as maize, peanuts and cottonseed, is a serious risk to human and animal health, and has a significant economic impact. Therefore it is important to study the mechanisms of gene expression in these aflatoxigenic fungi such as Aspergillus flavus and Aspergillus parasiticus. If we are able to study the genes involved then it can be used

further to elucidate the aflatoxin biosynthetic pathway. With this we can devise strategies to control aflatoxin contamination of pre-harvest agricultural crops and post-harvest grains during storage.

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APPENDICES

Potato dextrose agar composition:

Sl.No.	COMPOUND	AMOUNT(gm/l)
1.	Potatoes, infusion form	200
2.	Dextrose	20
3.	Agar	15

Phosphate buffer saline Tween 80:

Sl. No.	COMPOUND	AMOUNT(1L)
1.	Sodium chloride	8.0 g
2.	Potassium chloride	0.2 g
3.	Disodium hydrogen phosphate	1.44 g
4.	Tween-80	0.05 % (v/v)

Glucose minimal media composition:

SL. NO.	COMPONENTS	AMOUNT (gms/100ml)
1	Ammonium phosphate monobasic	1.0 gm
2	Glucose	5.0 gm
3	Sodium chloride	5.0 gm
4	Magnesium sulfate	0.2 gm
5	Potassium phosphate dibasic	1.0 gm

Lacto phenol cotton blue stain:

Sl. No.	COMPONENTS	AMOUNT
1	Phenol crystals	20 gms
2	Cotton blue	0.05 gms
3	Lactic acid	20 ml
4	Glycerol	20 ml
5	Distilled water	20 ml